MASARYKOVA UNIVERZITA
PŘÍRODOVĚDECKÁ FAKULTA
Ústav experimentální biologie

Emergentní zoonózy přenášené hematofágními členovci – nové hrozby i výzvy

Habilitační práce

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Souhrn:

Předkládaná habilitační práce je koncipována jako soubor 42 vybraných prací (39 prací s impakt faktorem), 1 neimpaktované práce, 1 anglické monografie a 1 kapitoly v anglické monografii), které vznikaly jako součást dlouhodobého základního výzkumu zoonóz na valtickém pracovišti Ústavu biologie obratlovců AVČR, v.v.i. a v těsné spolupráci s Ústavem experimentální biologie Přírodovědecké fakulty Masarykovy univerzity. Jde vesměs o práce povahy eko-epidemiologické, tedy spojujících složku ekologickou (ekologie vektorů a patogenů) a epidemiologickou (entomologická a epidemiologická surveillance). Komentované části jsou rozděleny do dvou hlavních kapitol: infekce přenášené klíšťaty a infekce přenášené komáry, s hlavním důrazem zejména na emergenci patogenů potažmo vektorů a zdravotní riziko pro obyvatele.

Summary:

Present thesis is a collection of selected 42 scientific publications (39 of them published in peer reviewed journal indexed by impact factor, 1 paper in peer reviewed journal, 1 monograph in English and 1 chapter in monograph in English), which originated as a part of long-term basic research in Laboratory of Medical Zoology of the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic and in close collaboration with Department of Experimental Biology of Faculty of Science, Masaryk University). There are mostly eco-epidemiological studies linking up ecological (ecology of disease vectors and pathogens) as well as epidemiological (entomological and epidemiological surveillance) approach. Annotated parts are divided into two chapters: tick-borne diseases and mosquito-borne diseases, both with main emphasis laid out on pathogen/vector emergence and public health risk.

Prohlášení: Prohlašuji, že jsem habilitační práci vypracoval sám za použití literárních zdrojů, které jsou v práci řádně ocitovány.

V Brně dne 11.8. 2016 2016

Ivo Rudolf

Podpis autora

věnováno

Silvii, Sofii a Davidovi

Mé poděkování patří rodině za nezměrnou podporu a toleranci. Bez těchto prerekvizit bych se nemohl věnovat vědě na plný úvazek. Rád bych také poděkoval všem svým současným i bývalým spolupracovníkům a studentům, kteří se mnou podíleli a podílejí na výzkumu zoonotických mikroorganizmů a bez kterých by tato práce nemohla vzniknout. V neposlední řadě děkuji domácím grantovým agenturám a Evropské komisi za finanční podporu našeho výzkumu, zejména v rámci interdisciplinárních projektů EDEN (*Emerging diseases in changing european environment*) a EDENext (*Biology of vector-borne infections in Europe*), které v posledních deseti letech extenzivně podpořily naše výzkumné snažení.

Motto:

Ve vědě existuje hlavní proud, v němž vědci na slavných univerzitách pracují na velkých tématech jako rakovina, AIDS, globální oteplování nebo cokoliv s předponou nano na začátku. Vedle toho jsou však i klidnější zátočiny, více či méně vzdálené od hlavního proudu, v nichž se řeší témata jako „ Květena Jindřichohradecka se zvláštním zřetelem ke Kardašově Řečici, „Rozvoj českého rybářství v době Ludvíka Jagellonského, případně „Srovnávací genomika koronavirů drobných savců“. Nejzajímavější okamžiky v dějinách vědy nastávají tehdy, když se najednou takové poklidné zátočinky zmocní dravý hlavní proud. Z trpělivého srovnávání sekvencí koronavirů u koček a cibetek, jehož výsledky může učenec po letech vložit do nějaké monografie, případně po částech publikovat ve velmi specializovaných časopisech (kde si je přečte všech osm jeho kolegů, které koronoviry u cibetek také zajímají), se náhle stane „velká věda“, jejíž výsledky se přednášejí na konferencích od San Franciska po Šanghaj, publikují v prestižních časopisech jako Nature nebo Science a zmatení redaktoři zpravodajských deníků se je pracně pokoušejí přežvýkat pro svoje čtenáře. Můžeme se tomu pošklebovat, ale tak to je, a kdo takové „protržení hráze“ klidné zátočiny na široké řece vědy někdy zažil, nikdy na to nezapomene…

(úryvek z kapitoly SARS-kapesní pandemie, knihy Viry pro 21. století autorů J. Konvalinky a M. Machaly).

Nové studie přinášejí nejen zprávy o rozšíření některých arbovirů a jejich příbuzných do míst, o nichž se dosud nevědělo, ale ukazují, že je nutno počítat i se vznikem kombinací virů s novými vlastnostmi, které mohou kdykoli přinést velká překvapení. Viry, které jsou dnes málo významné, se mohou stát velkými patogeny, mohou měnit svá působiště, hostitele i přenašeče. Je na místě skromnost a zapotřebí smířit se s tím, že všechny vědecké poznatky mohou platit jen dočasně, protože příroda a přírodní ohniska se vyvíjejí a mění dál, i když velmi pomalu…

(úryvek z knihy Přírodní ohniska nákaz autora L. Daneše).

Arbovirózy patří mezi nejdůležitější emergentní nákazy, se kterými se budeme potýkat v příštích 10-20 letech.

(D. Gubler, bývalý ředitel Divize nákaz přenášených vektory, Fort Collins, CDC, Colorado, USA)

OBSAH

1. Předmluva autora

Už na střední škole mne udivoval svět mikrobů a před maturitou jsem tušil, že se v budoucnu mikrobiologii budu věnovat profesionálně. Stále mne fascinuje, jak tak miniaturní organizmus (virus, baktérie či prvok) dokáže ovlivnit širokou škálu pochodů zde na Zemi včetně jeho nezastupitelné role v přenosu infekčních chorob. Na univerzitě jsem od počátku věděl, že moje vysněná meta je infekční mikrobiologie a když jsem se dostal k jejímu studiu, byť zpočátku jen zprostředkovaně skrze lékařskou mikrobiologii či taxonomii baktérií, byl jsem nevýslovně šťasten. Hlavně témata zabývající se roli mikrobů v přenosu nebezpečných nebo exotických infekcí si mne podmanily a přes malou odbočku v diplomové práci, která se zabývala magnetickými nosiči a jejich využitím při izolaci DNA, mé další kroky směřovaly již jen za opravdovými patogeny.

Úplně náhodou, ale tak to často ve vědě bývá, mne osud (teď už vím, že správně) nasměroval na valtické pracoviště Akademie věd, kde se mým školitelem (tutorem a posléze i kolegou stal prof. Zdeněk Hubálek). V podstatě lepšího školitele v této oblasti jsem si nemohl přát. Zdeněk zoonotickým onemocněním věnoval velkou část své profesní dráhy a přes svůj až renesanční záběr mě mohl zasvěcovat do tajů 'infekční mikrobiologie' napříč různými skupinami patogenních agens. Stejně jako jeho i mne naplňuje propojení terénního výzkumu (odebírání vzorků, vlajkování klíšťat, odchyt komárů do speciálních pastí) s výzkumem laboratorním (izolace a detekce pro vědu nových mikroorganizmů, jejich identifikace a zkoumání možného patogenního potenciálu) a to vše s přesahem do epidemiologie. Opravdu není pro mne nic více vzrušujícího než objevovat, izolovat a posléze charakterizovat nové mikrorganizmy nebo jejich varianty, se kterými lze dále aplikovat v mnoha disciplínách (diagnostika, léčba či biotechnologie).

V devadesátých letech byla 'naše' discíplína tzv. microbe hunting odsunuta na vedlejší kolej, ale v posledním desetiletí zvláště při objevech nových převážně zoonotických nákaz (koronaviry SARS, MERS, ptačí chřipka, nové infekce přnášené hematofágy) zjišťujeme, že nové patogenní mikroby nebo jejich varianty nás dokáží překvapovat i v 21. století. Náš dlouholetý výzkum viru West Nile, původce západonilské horečky, je toho pravým důkazem. Původně relativně exotické onemocnění s endemickým výskytem v Africe expandovalo až do Střední Evropy, kde se stává nyní pro nás opravdovou zdravotní hrozbou. V roce 1997, kdy bylo u nás poprvé naší laboratoří diagnostikováno na jižní Moravě, o něm vědělo jen pár zasvěcených odborníků, ale po jeho introdukci do Spojených států amerických v roce 1999, kdy se virus lavinovitě rozšířil během několika následující let po celé USA, se náhle stal

středobodem pozornosti světových virologů. V současnosti je výskyt tohoto viru takřka kosmopolitní. Stejně tak po nedávných epidemiích západonilské horečky v Evropě (Itálie-2008-2010, Maďarsko-2008, Řecko-2010, Srbsko-2012) již Evropské centrum pro prevenci a kontrolu nemocí (ECDC) velmi bedlivě sleduje vývoj kolem tohoto patogenního viru. Díky globalizaci infekčních chorob se tak výzkum tzv. emergentních zoonotických nákaz dostává do popředí, protože v sobě kromě rizika infekcí ukrývá spoustu nových vzrušujících výzkumných témat jako je jejich léčba, výzkum patogeneze či vývoj nových vakcín. Emergentní zoonózy se tak pro obyvatele Evropy stávají hrozbou, ale pro nás vědce hlavně netušenou výzvou. Tzv. One health koncept, který naše laboratoř jako jedna z mála u nás propaguje, tj. komplexní a interdisciplinární pohled na tato onemocnění, kombinující pohled zoologů, veterinářů, mikrobiologů, infekcionistů, matematiků či epidemiologů, je do budoucna jedinou možnou alternativou, jak účinně čelit přicházejícím hrozbám nových nebo se znovu objevujících infekčních chorob jako je nyní epidemie horečky Zika v Pacifiku. Jsem šťasten, že u tohoto nikdy nekončícího boje mezi člověkem a mikrobiálními patogeny mohu být.

Autor

Ve Valticích dne 11.8. 2016

2. Struktura a zaměření habilitační práce

Předkládaná habilitační práce je koncipována jako soubor komentovaných prací, které vznikaly jako součást dlouhodobého výzkumu zoonóz na valtickém pracovišti Ústavu biologie obratlovců AVČR, v.v.i. a v těsné spolupráci s Ústavem experimentální biologie Přírodovědecké fakulty Masarykovy univerzity. Jde vesměs o práce povahy eko-epidemiologické, tedy spojujících složku ekologickou (ekologie vektorů a patogenů) a epidemiologickou (zahrnující hodnocení zdravotní rizik daných zoonotických nákaz s důrazem na možnou emergenci vektorů potažmo patogenů).

Habilitační práce je komentovaným souborem 42 recenzovaných prací (39 s impakt faktorem), 1 neimpaktované práce, 1 anglické monografie a 1 kapitoly v anglické monografii). Komentované části práce předchází stručný úvod do studované problematiky (kapitola 3.). Samotný komentář (kapitola 4.) je členěn na 3 hlavní podkapitoly, které 'neorganicky' dělí naše studie na projekty zabývající se patogenními mikroorganizmy přenášenými klíšťaty (kapitola 4.1.), komáry (kapitola 4.2.), vše doplněné kapitolou zahrnující souborné publikace typu review, 1 knihy a 1 kapitoly v knize (kapitola 4.3.). Habilitační práci doplňuje Závěr (kapitola 5.), Literatura (kapitola 6.) a Přílohy-tištěné publikace (kapitola 7.).

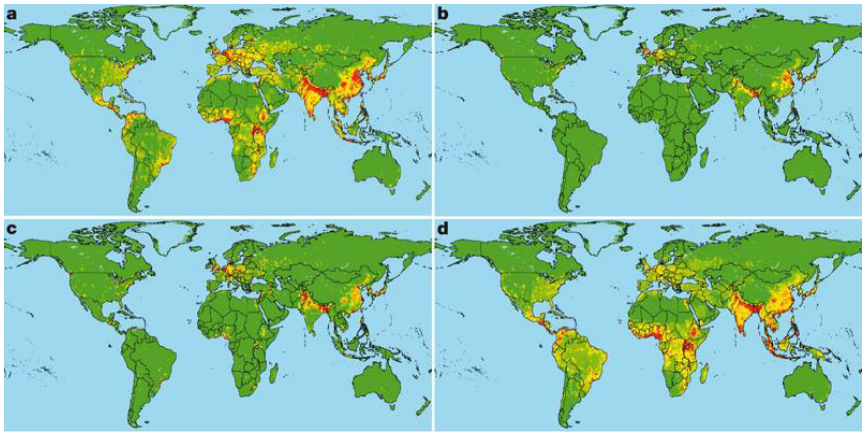
3. Obecný vhled do studované problematiky

3.1. Emergentní zoonózy

Zoonózy jsou nemoci přenosné z živočichů na člověka. Termín vytvořil Rudolf Virchow roku 1855 při studiu trichinelózy. Pravé zoonózy zpravidla nejsou přenosné kontaktem z jednoho člověka na druhého (výjimky: hemoragické horečky Lassa, Ebola, Marburg, hantavirový plicní syndrom, krymsko-konžská hemoragická horečka, mor aj.). Dříve byl pro choroby přenosné ze zvířat na člověka používán termín "antropozoonózy". Analogicky byl zaveden pojem "zooantroponózy" pro nemoci přenosné naopak z člověka na zvířata; počet takových chorob je malý (např. chřipka, tuberkulóza). Bohužel mnozí epidemiologové používali tyto termíny v opačném smyslu (zooantroponózy jako nemoci přenosné ze zvířete na člověka), anebo promiskue (Hubálek a Rudolf, 2011). Na doporučení WHO se proto oficiálním termínem stalo označení "zoonózy", a další dva uvedené termíny se používat nadále nemají. Podle společné komise expertů WHO/FAO zní definice v originále: "*Zoonoses are diseases and infections which are naturally transmitted between vertebrate animals and man*" (WHO Tech. Rep. Ser. 169, 1959). Tato definice byla potvrzena 3. i 4. zprávou této komise (WHO Tech. Rep. Ser. 378, 1967; WHO Tech. Rep. Ser. 682, 1982). Počet známých zoonóz neustále roste a v současnosti přesahuje 250, z toho přibližně 80 je běžných. Ze zoonóz nověji prokázaných lze uvést např. lymskou borreliózu, anaplazmózu, hantavirový plicní syndrom, koronavirózy SARS a MERS nebo horečku způsobenou paramyxoviry Nipah a Hendra. Jen malý počet zoonotických agens však vyvolává rozsáhlé epidemie – k nim patří např. salmonelóza, kampylobakteróza, Q horečka, žlutá zimnice, dengue, japonská encefalitida, západonilská horečka, horečka údolí Rift anebo americké koňské encefalomyelitidy (Bisen a Raguvanshi, 2013; Singh 2014). Lokálním pohledem se na jižní Moravě objevila velká epidemie tularémie před 2. světovou válkou, na Slovensku potom rožňavská epidemie klíšťové encefalitidy v roce 1951. Jiné zoonózy ovšem přitahují pozornost veřejnosti (a médií) pro svou vysokou letalitu, někdy spojenou s velkou nakažlivostí pro ošetřující personál (např. rozsáhlá epidemie hemoragické horečky Ebola, která propukla v roce 2013 v západní Africe s letalitou dosahující téměř 40%).

Mezi zoonózami se kupodivu i v dnešní době stále objevují závažné nemoci zcela nové (např. SARS, virózy Hendra a Nipah, hantavirový plicní syndrom), nově poznané (lymská borrelióza, ehrlichióza a anaplasmóza), vracející se (západonilská horečka v Evropě), se vzrůstající incidencí (salmonelóza po r. 1988, kampylobakteróza), geograficky expandující (západonilská horečka v Americe nebo Evropě), s měnícím se okruhem hostitelů či přenašečů

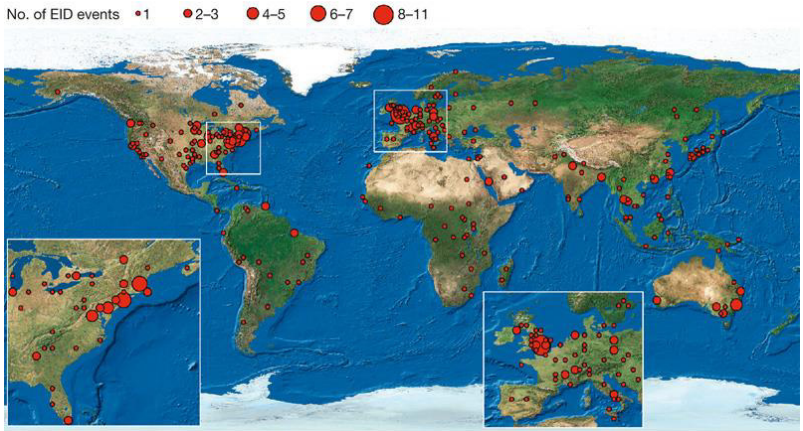
(*Ae. albopictus* a horečka chikungunya), anebo nově se klinicky manifestující (malformace plodu způsobené virem Zika), pro něž se v angličtině používá souhrnného termínu "emerging/re-emerging diseases", a v češtině je lze označit jako „**nákazy (re-)emergentní**“ (Obrázek 1) (Hubálek, 2002; Hubálek, 2003; Hubálek a Kříž, 2003). Řada těchto onemocnění je vyvolána schopností některých patogenů překonat mezidruhovou bariéru hostitelů, což dokazují např. ptačí a prasečí chřipka, SARS nebo AIDS (Singh, 2014).



Obrázek 1. Globální pohled znázorňující relativní risk výskytu emergentních nákaz: a – zoonotické patogeny (wildlife); b – zoonotické patogeny (non-wildlife); c – rezistentní patogeny; d – patogeny přenášené vektory (převzato z Jones a kol., 2008).

Bylo zjištěno, že z celkového počtu 177 (re)emergentních nákaz představují 73-75% právě zoonózy (Taylor a kol. 2001, Woolhouse a Gowtage-Sequeria 2005). Řada zoonóz, především nákaz přenášených bodavým hmyzem (např. malárie, dengue, filariáza, trypanosomóza, leishmaniáza), každoročně ohrozí na životech milióny lidí na celé planetě.

Počet známých zoonotických patogenů člověka je značný, stále rostoucí, odrážející se i v nárůstu epidemických událostí (Obrázek 2) (Woolhouse a Gowtage-Sequeria 2005).



Obrázek 2. Mapa znázorňující geografický původ nejvýznamnějších epidemických údalostí způsobených emergentními patogeny v letech 1940-2004 (převzato z Jones a kol., 2008).

3.2. Emergentní zoonózy přenášené hematofágními členovci

Emergentní nákazy přenášené hematofágními členovci patří mezi nejdůležitější nemoci, kterým čelí lidstvo na začátku třetího tisíciletí, a stávají se (vedle malnutrice a helmintóz) největším zdravotnickým problémem zejména v zemích třetího světa. Důsledný monitoring emergentních zoonotických nákaz může napomoci jejich lepší prevenci a případně kontrole.

V této kapitole jsem se snažil stručně vymezit nejrizikovější agens přenášená hematofágy, se kterými se bude lidstvo potýkat v blízké budoucnosti.

3.2.1. Přehled nejvýznamnějších mikrobiálních agens přenášených klíšťaty a komáry

Následující přehled shrnuje nejdůležitější patogeny člověka biologicky přenášené hematofágními členovci (konkrétně klíšťaty a komáry) (Hubálek a Halouzka, 1996; Marquardt, 2006; Service, 2012; Hubálek a Rudolf, 2011; Vasilakis a Gubler, 2014). Ojedinělý anebo mechanický přenos je zde opomenut. Tučně jsou zvýrazněny patogeny s charakterem emergence.

Čeleď **Klíšťatovití (*Ixodidae*)**

arboviry: **flaviviry středoevropské klíšťové encefalitidy**, Louping ill, ruské jaro-letní encefalitidy, Powassan, Omské hemoragické horečky, horečky Kyasanurského pralesa, *orbiviry* Kemerovo, Tribeč, reovirus Koloradské klíšťové horečky, flebovirus Bhandža, **nairovirus krymsko-konžské hemoragické horečky**, Dugbe, orthomyxoviry Dhori, Thogoto;

rickettsie: *Rickettsia rickettsii*, *R. sibirica*, ***R. slovaca***, ***R. monacensis***, ***R. helvetica***, *R. japonica*, *R. australis*, *R. conorii*, ***R. africae***, *Ehrlichia chaffeensis*, *E. ewingii*, ***Anaplasma phagocytophilum*** s.l., **'*Candidatus* Neoehrlichia mikurensis'**

jiné bakterie: ***Borrelia burgdorferi*** s.l. (řada patogenních genomických druhů), ***B. miyamotoi***, *Francisella tularensis*, ***Coxiella burnetii***;

prvoci: ***Babesia microti***, ***B. venatorum***, ***B. canis***, *B. divergens*, *B. bovis*, *B. equi*, ***B. gibsoni***.

Čeleď **Komárovití (*Culicidae*)**

arboviry: **togaviry východoamerické koňské encefalomyelitidy, západoamerické koňské encefalomyelitidy, venezuelské koňské encefalomyelitidy,** Sindbis, **Chikungunya**, Onyon nyong, Ross River, Barmah Forest, Mayaro, flaviviry japonské encefalitidy, **West Nile**, encefalitidy St Louis, **žluté zimnice**, **dengue**, encefalitidy Murray Valley, **Zika**, Rocio, Bunyamwera, Bwamba, Pongola, skupina California - např. Ťahyňa a LaCrosse, Oropouche, horečky údolí Rift, Keterah, Vesikulární stomatitidy

prvoci: ***Plasmodium*** spp.

3.3. Eko-epidemiologie emergentních zoonóz

3.3.1. Epidemiologická surveillance a koncept One-Health

Termínu **surveillance** (česky nepříliš přesně přeložitelné jako dozor, dohled nad, bdělost) bylo v epidemiologii poprvé užito v roce 1950 v souvislosti s programy kontroly malárie, neštovic či urbánní formy žluté zimnice. Koncepci surveillance přenosných nemocí doporučila WHO v letech 1968-69 všem členským státům jako moderní strategii v boji s infekcemi. Profesor K. Raška ji definoval jako "epidemiologické studium nemoci jako dynamického procesu, včetně ekologie původce nákazy, hostitele, rezervoárů a vektorů nákazy, jakož i studium zevních podmínek prostředí a všech mechanismů, které se uplatňují v procesu šíření nákazy v rozsahu, ve kterém se daná nákaza vyskytuje". Je to tedy monitorování nákazy a všech vnějších podmínek, které mohou mít význam pro její dynamiku; získávání všech dostupných informací, jejich ukládání do databáze a průběžné vyhodnocování. Souběžně se pokusil o totéž i Dr. Alexander Langmuir ze CDC, pozdější představený prof. K. Rašky v ústředí Divize infekčních onemocnění WHO v Ženevě. Konečným cílem epidemiologické surveillance je kontrola (potlačení) dané infekce na základě vyhodnocení, poznání a ovlivnění faktorů determinujících či modifikujících její epizootický a epidemický proces.

Podle WHO (Tech. Rep. Ser. 682, 1982) je náplní surveillance:

1) přesná a rychlá diagnostika nákazy (klinicko-patologická a laboratorní, včetně izolace a identifikace původce ze vzorků lidských, zvířecích a vektorů, a sérologické diagnostiky);

2) racionální použití dostupných prostředků k potlačení zoonózy v živočišném rezervoáru (př.: deratizace, dezinsekce, tj. hubení přenašečů – členovců, a dezinfekce prostředí).

Komplexněji, "*epidemiological surveillance is the process of collection*, *interpretation*, *and distribution of information on rates of occurrence of a particular disease to estimate the variation of incidence and prevalence in order to take appropriate action for the control or eradication of the disease*". Schéma surveillance lze tedy vyjádřit v angličtině jako "collection → interpretation → distribution → action." Distribucí se míní zpětná informace pro pracovníky v terénu.

Až později se vžil moderní termín **public health surveillance**, který navrhli Stephen Thacker a Ruth Berkelman ze CDC. Definice zní následovně: *"Public health surveillance is the ongoing, systematic analysis, interpretation, and dissemination of data regarding a health*

related event for use in public health action to reduce morbidity and mortality and to improve health."

Velmi podobná definice je využívána i WHO: *"Public health surveillance is the continuous, systematic collection, analysis and interpretation of health-related data needed for the planning, implementation, and evaluation of public health practice"* (http://www.who.int/topics/public_health_surveillance/en).

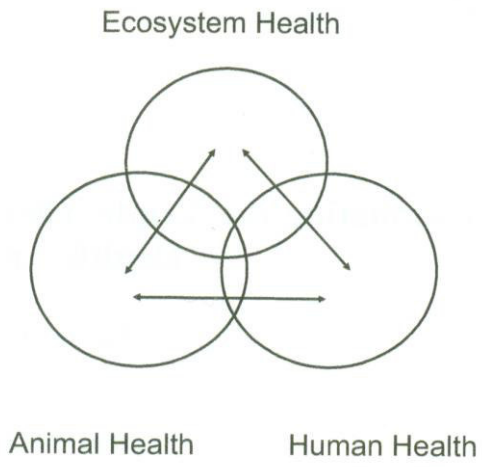
Indikace zoonózy v určité oblasti může být založena na klinických pozorováních zvířat (vzteklina, virové encefalitidy, hantavirózy, virus západonilské horečky), jejich vyšetření autoptickém a při inspekci masa (např. tuberkulóza, antrax), s využitím sérologické surveillance (japonská encefalitida u selat, brucelóza u skotu) či alergických kožních testů (bovinní tuberkulóza), s pomocí monitorovacích izolačních vyšetření vektorů (např. komárů u amerických koňských encefalomyelitid) a potravin živočišného původu. Vhodně lze využít ekologických údajů o vysokých až kritických populačních hustotách vektorů a hostitelů (např. lišky u vztekliny, hlodavci u tularémie nebo hemoragické horečky s renálním selháním). Velmi významná je rychlá mezinárodní výměna informací o všech těchto kritických veličinách a incidenci zoonóz v jednotlivých oblastech světa na bázi WHO a FAO. K tomu účelu je mj. pro standardizaci hlášení vypracována mezinárodní klasifikace nemocí (včetně zoonóz), a seznam nemocí podléhajících hlášení ('notifiable diseases'). V ČR existuje databáze EPIDAT v rámci SZÚ, která monitoruje nemocnost infekčními chorobami na celostátní úrovni od roku 1982, ale u některých nákaz zasahuje i dále do minulosti (např. u tularémie nebo klíšťové encefalitidy až do 50. let 20. století). Dalšími participujícími institucemi ve světě jsou Center for Disease Control and Prevention (CDC) v Atlantě, European Centre for Disease Prevention and Control (ECDC) ve Stockholmu, které disponují zázemím expertních týmů a referenčních laboratoří. Revoluční formou je šíření informací o nakažlivých nemocech včetně zoonóz pomocí Internetu (např. ProMED mail). Jsou zaváděny pojmy jako včasné varování ("early warning") a rychlá reakce ("rapid response").

Na epidemiologické surveillanci u nás se podílejí mikrobiologické laboratoře regionálních Zdravotních ústavů, laboratoře Státního zdravotního ústavu včetně referenčních laboratoří, Státní veterinární správa, Česká zemědělská a potravinářská inspekce a Česká obchodní inspekce.

Konkrétně u zoonotických nákaz přenášených hematofágy mezi metody surveillance řadíme především periodické vyšetřování vektorů v endemické oblasti výskytu viru (s jejich následným hubením v případě přemnožení), sérologické přehledy hostitelů (hlodavci, volně

žijící zvěř, stálí i stěhovaví ptáci), monitoring domácích sentinelů (např. slepic a kachen) na specifickou sérokonverzi, vyšetřování lokální lidské populace na protilátky k virům přenosným hematofágními členovci (zvláštní pozornost by měla být soustředěna při zjišťování etiologie letních chřipkovitých stavů, spalničkového exantému, aseptických meningitid nebo memingoencefalitid nejasného původu) a důsledný monitoring importovaných nákaz. V poslední době je systém surveillance doplňován o kontrolu krevních derivátů na vybrané agens, např. viry West Nile a Zika (Kolodziejek a kol., 2015).

Při šetření v přírodním ohnisku nákazy v epidemickém období je pak více než žádoucí spolupráce širokého týmu odborníků z řad epidemiologů, medicinských akaroentomologů, zoologů, parazitologů, ekologů, veterinářů, terénních i klinických mikrobiologů a infektologů včetně matematických modelistů a informatiků, tedy tzv. koncept "One Health" (Atlas a Maloy, 2014). Jde o mezinárodní interdisciplinární spolupráci propojující zdraví člověka, zvířat i všech složek prostředí (Obrázek 3).



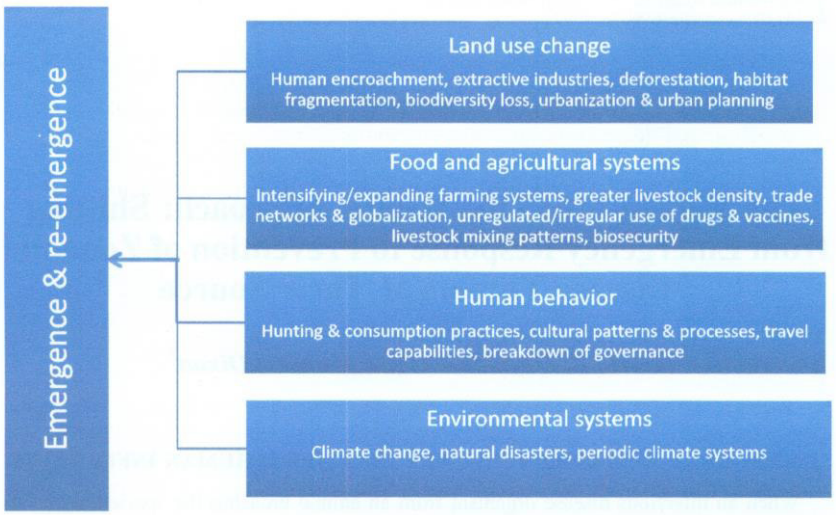
Obrázek 3. Schématické znázornění jednotlivých složek konceptu One Health (převzato z Atlas a Maloy, 2014).

Dle mezinárodní iniciativy zaštitěné uznávanými kapacitami v oboru jako jsou Laura H. Kahn, Bruce Kaplan, Thomas P. Monath, Jack Woodall a Lisa A. Conti, je tento koncept definován následovně: „*The One Health concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment*“ (http://www.onehealthinitiative.com). Dle definice CDC: „*The One Health concept recognizes that the health of humans is connected to the health of animals and the environment“* (http://www.cdc.gov/onehealth.html) a nahrazuje tak již zastaralé paradigma zahrnující pouze surveillance daného onemocnění (Obrázek 4).

Obrázek 4. Dřívější a současné paradigma znázorňující odlišné pojetí surveillance infekčních onemocnění.

3.3.2. Faktory určující emergenci zoonotických nákaz

Tyto činitele lze dělit na sociální/antropogenní (působící na infekční proces v rámci lidské společnosti) a přírodní (tj. fyzikálně-geografické a biotické) (Obrázek 5). Obě tyto skupiny faktorů se uplatňují zvláště výrazně u emergentních nákaz (Morse, 1995; Karesh a kol., 2005; Jones a kol., 2008; Taylor a kol., 2001; Hubálek a Rudolf, 2011; Bisen a Raghuvanshi, 2013).



Obrázek 5. Příklady vybraných činitelů (sociálních i přírodních) určujících emergenci zoonotických onemocnění (převzato z Atlas a Maloy, 2014).

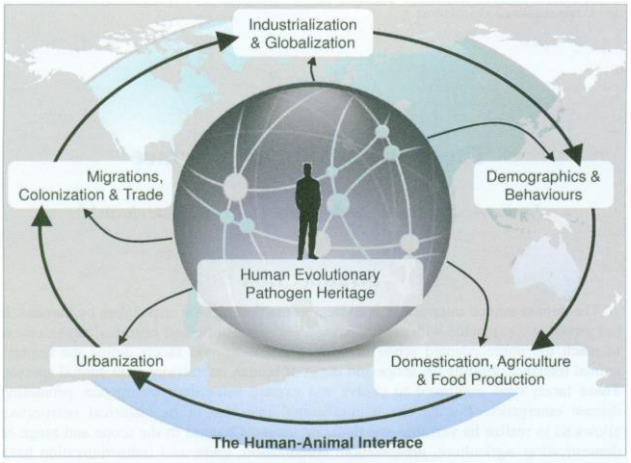
3.3.2.1. Faktory sociální

Sociální faktory, označované mnohdy také jako faktory socio-ekonomické nebo antropogenní, hrají samozřejmě zásadní roli u antroponóz, ale jsou velmi důležité také u zoonóz (Morse, 1995; Karesh a kol., 2005; Hubálek a Rudolf, 2011). K socio-ekonomickým činitelům, kteří mohou do značné míry ovlivnit prevalenci nákaz, patří zejména:

- hustota lidské populace v dané oblasti;
- sociální a hygienické poměry (životní styl a úroveň);
- úzký kontakt lidí s hospodářskými zvířaty;
- kolektivní způsob života: společné stravování, charakter jídelen ('fast-food' bufety) a způsob ubytování;
- stupeň urbanizace krajiny (expanze měst, zvláště v tropických oblastech: 'slums');
- 'suburbanizace', tj. výstavba rodinných domků v zalesněných příměstských oblastech;
- osvojování nových území (kolonizace, 'pionýři') a návazné antropogenní zásahy do ekosystémů budováním sídel, většími terénními úpravami, odlesňováním – deforestací ale i zalesňováním – reforestací, zřizováním vodních nádrží, zavlažováním a odvodňováním;
- rychlý mezinárodní transport a rozvoj mezinárodního obchodu: zavlékání agens, vektorů a rezervoárů nákaz;
- zvýšená mobilita a migrabilita lidské populace: cesty za obchodem a na trhy;
- migrace pracovníků ze zahraničí, migrace lidí v důsledku válečných konfliktů;
- turistika a rekreace: tuzemská rekreace spojená se vstupem do PON; cesty do cizích zemí a náboženské poutě, spojené se vstupy do lokálních ohnisek nákaz, 'adrenalinová turistika';
- některé aktivity v rámci volného času (lovectví, sběr hub a lesních plodin), vedoucí ke zvýšenému kontaktu s vektory nákaz;
- zvýšený kontakt se zvířaty pro potěchu (angl. "pets" nebo "companion animals");
- expanze a intenzifikace (koncentrace, specializace) zemědělství;
- zpracování a konzum zvířecích produktů (např. "bushmeat") a odpadků;
- přesuny stád dobytka na nové pastviny, případně nomadizmus;
- import a export domácích zvířat, jejich produktů a živočišných potravin;
- domestikace zvířat (zejména v minulosti), farmový chov původně divokých zvířat;
- import a chov exotických živočichů pro ZOO, safari, privátní potřeby a výzkum;
- profesionalita; u synantropních zoonóz jsou riziku nákazy zvláště vystavena povolání: chovatel a ošetřovatel zvířat, veterinární lékař, řezník, lesní dělníci, kopáči, pracovníci specializovaných diagnostických laboratoří;
- nozokomiální/iatrogenní zoonotické infekce (transfuze krve a transplantace orgánů);
- xenotransplantace (kontaminované orgány zvířat transplantované lidem), živočišné buněčné kultury užité např. pro přípravu vakcín;
- kosmetické zásahy ("piercing", tetování), injekční aplikace drog;
- zavádění dokonalejších diagnostických a epidemiologických technik;
- nedostatečnost či absence zdravotnické prevence (včetně osvěty) a zásahů, nečinnost nebo rozklad infrastruktury zdravotnického systému;
- sociální katastrofy nebo stresy (války, uprchlické tábory, hladomor).

S jistou nadsázkou lze říci, že společným jmenovatelem většiny sociálních faktorů u zoonóz je globalizace: ekonomiky, transportu zboží, zvířat a osob, turistiky a rekreace,

živočišné i rostlinné produkce, životního stylu, osvojování ekosystémů atp. Mluvíme tedy o globalizaci infekčních onemocnění (Obrázek 6) (Hubálek a Rudolf, 2011).



Obrázek 6. Vybrané socio-ekonomické činitele určující emergenci zoonóz (převzato z Atlas a Maloy, 2014).

3.3.2.2. Faktory přírodní

Přírodní činitelé, nesouvisející s lidskou činností, jsou v zásadě faktory ekologické, určující nebo modifikující cirkulaci všech patogenních agens. Patří k nim proměnné (a) abiotické (geomorfologické, geologické, hydrologické, pedologické, klimatické, aktuální podmínky meteorologické); (b) biotické (vegetace, fauna). Komplex všech těchto faktorů např. rozhoduje o zeměpisném rozšíření původců jednotlivých zoonóz (ve vazbě na distribuci rezervoárů, hostitelů, a případně přenašečů).

Z abiotických činitelů je obecně nejdůležitějším klima (teplota, srážky), dále zeměpisná šířka, nadmořská výška a členění reliéfu (geomorfologie ovlivňuje např. mikro- a mezoklima). Byla např. zjištěna průkazná korelace mezi aktivitou tzv. Jižní Oscilace El-Niño (ENSO) v Pacifiku, ovlivňující globální atmosférickou cirkulaci, a zvýšenou incidencí některých nákaz v rozsáhlých oblastech v letech 1991-93 (cholery, malárie a hantavirového plicního syndromu). Existují opodstatněné obavy, že probíhající globální oteplování klimatu by mohlo výrazným způsobem ovlivnit rozšíření především nemocí přenosných hematofágním hmyzem (např. malárie, dengue, leishmanióza). U analogického klimatického systému Severoatlantické Oscilace (NAO), který výrazně ovlivňuje podnebí v Evropě, nebyla doposud jasná korelace s incidencí zoonóz prokázána (Hubálek, 2005; Hubálek a Rudolf, 2011).

Abiotické přírodní podmínky se podílejí také na sezonnosti mnohých nákaz, zvláště transmisivních zoonóz (např. klíšťové encefalitidy) - to je dáno sezónní distribucí (fenologií) jejich vektorů. Pro některé přenašeče (např. komáry) je nezbytná přítomnost vodních ploch nebo mokřadů. Teplota prostředí může zase velmi ovlivnit vývoj patogenních agens (např. arbovirů) v přenašečích (Vasilakis a Gubler, 2016).

K abiotickým činitelům patří také přírodní katastrofy, které mohou být meteorologické (vichřice, tornáda), hydrologické (povodně) nebo geologické (zemětřesení, rozsáhlé sesuvy půdy, cunami). Např. po zemětřesení poblíž Los Angeles v r. 1994 došlo ke zvýšení incidence mykóz, jejichž agens sporulují v půdě: popsáno bylo 170 případů kokcidioidomykózy a histoplazmózy (Guevara a kol., 2015). Po povodních se obvykle zvyšuje populační hustota krevsajícího hmyzu, především komárů, a může docházet ke zvýšení incidence virových nákaz (Hubálek a kol., 2004) a malárie jimi přenášených, a rovněž k nárůstu některých dalších zoonóz v důsledku narušení zásobování obyvatelstva pitnou vodou nebo kontaminace znečištěnou vodou: např. kolibacilóza (enteropatogenní *Escherichia coli*), salmonelóza, melioidóza (v tropech), leptospiróza, tularémie, giardióza, kryptosporidióza (Hubálek a Rudolf, 2011).

Biotické faktory, ovlivňující epidemický proces, jsou zejména:

- velikost, hustota a vývoj populace (populační dynamika) jak obratlovčích hostitelů (rezervoárů) nákaz, tak bezobratlých přenašečů ('gradace');
- jejich bionomie a etologie (způsob života a chování: např. existence kolonií, společných shromaždišť a nocovišť, synantropie), fenologie (sezonnost);
- mobilita (velikost teritoriálních okrsků - "home range") a migrabilita (tah ptáků, invaze, potulky) hostitelů i bezobratlých přenašečů (invazivní druhy komárů);
- imunita populací obratlovců;
- přítomnost stresových faktorů (malnutrice, přemnožení aj.) v populaci;
- charakter a typ vegetace;
- změny patogenů samotných, druhového okruhu jejich hostitelů, vektorů.

Velmi významným ekologickým faktorem v šíření některých zoonóz je populační dynamika hostitelů patogenních agens, a nejzávažnější je přemnožení volně žijících obratlovců, které může vést k jejich přibližování lidským sídlům. Také tuhá zima nutí některé exoantropní savce a ptáky k přechodně synantropnímu způsobu života. U transmisivních nákaz je neméně důležitá populační dynamika jejich vektorů, především mimořádné přemnožení hematofágních členovců (Hubálek a kol., 2004).

Při disperzi patogenů může hrát roli i tah ptáků, tj. přenos agens táhnoucími ptáky jako jejich hostiteli anebo přenos jejich infikovaných ektoparazitů - vektorů. Podle odhadů migruje

každoročně z Evropy do Afriky a zpět asi půl miliardy ptáků, z nichž určité procento bývá napadeno klíšťaty. Africká klíšťata byla prokázána na ptácích také např. v Bulharsku (*Amblyomma variegatum*, *A. hebraeum*) a v Ázerbájdžánu (*A. lepidum*), a *Hyalomma marginatum* ojediněle v ČR i ve Finsku (Hubálek 1994; Čapek a kol., 2014). Imaga afrických klíšťat *A. variegatum*, *A. hebraeum* a *A. gemma* byla zjištěna v jižní Evropě (Itálii, Francii, Bulharsku a na Krymu) na domácích zvířatech v důsledku zánosu preimaginálních stadií klíšťat ptáky. Většina těchto exotických klíšťat v Evropě nedokončí svůj vývojový cyklus, ale část z nich by mohla např. nakazit lokální obratlovce a založit tak nová přírodní ohniska nákaz. Kromě ptáků migrují na delší vzdálenosti i někteří savci - např. netopýři (*Miniopterus schreibersi*). Popsány jsou rovněž zánosy dvoukřídlých vektorů nákaz (např. pakomárci *Culicoides* infikovaní veterinárně významnými arboviry bluetongue nebo africké nemoci koní) vzdušnými proudy za specifických meteorologických situací (Sedda a kol., 2015).

U některých nákaz a jejich agens je vazba na podmínky prostředí a společenstva tak výrazná, že lze s pomocí charakteristických biogeografických indikátorů (izotermy, izohyety, určitá rostlinná společenstva nebo indikátorové druhy rostlin a živočichů) do určité míry předvídat jejich výskyt (Hubálek, 2010). Pro účel predikce je možno využít také dálkového snímkování (angl. "remote sensing") Země z družic, například při monitorování nákaz přenosných komáry, u nichž se zjišťují líhniště komářích larev (Govoetchan a kol., 2014). Z údajů poskytovaných satelity se pro tyto účely využívá nejvíce tzv. normalizovaný diferenciální vegetační index (angl. "normalized difference vegetation index", NDVI). Data získaná ze satelitů se spřahují s technikami geografického informačního systému (GIS) a vytvářejí mapu rizika výskytu nákazy pro danou oblast (Honig a kol., 2015).

4. Komentář k předloženým publikacím

4.1. Příspěvek autora v oblasti nákaz přenášených klíšťaty

Stručný komentář k předloženým pracem nemá charakter klasické diskuse, spíše se snaží reflektovat ty nejdůležitější aspekty případně přesah našich studií. Publikace zabývající se klíšťaty jako přenašeči emergentních patogenů lze rozdělit do tří odlišných celků:

(1) experimentální práce zabývající se fyziologií borrelií při kultivaci *in vitro* po přidání extraktů slinných žláz či střeva (**PRÁCE 1, 8**);

PRÁCE 1: Rudolf I., Hubálek Z.: Effect of salivary gland and midgut extract from *Ixodes ricinus* and *Dermacentor reticulatus* (Acari: *Ixodidae*) on the growth of *Borrelia garinii* in vitro. *Folia Parasitol*. (2003), 53, 159-160.

PRÁCE 8: Rudolf I., Šikutová S., Kopecký J., Hubálek Z.: Salivary gland extract from engorged *Ixodes ricinus* (Acari: *Ixodidae*) stimulates *in vitro* growth of *Borrelia burgdorferi* sensu lato. *J. Basic. Microbiol.* (2010), 50, 294-298.

(2) práce ekologické povahy zkoumající vliv antropogenních změn v krajině na prevalenci klíšťat potažmo borrelií v klíšťatech (**PRÁCE 3, 5**);

PRÁCE 3: Hubálek Z., Halouzka J., Juřicová Z., Šikutová S., Rudolf I.: Effect of forest clearing on the abundance of *Ixodes ricinus* ticks and the prevalence of *Borrelia burgdorferi* s.l. *Med. Vet. Entomol.* (2006), 20, 166-172.

PRÁCE 5: Jarošová V., Rudolf I., Halouzka J., Hubálek Z.: *Borrelia burgdorferi* s.l. v klíšťatech na ostravských haldách. *Epidem. Mikrobiol. Imunol.* (2009), 58, 90-97.

(3) molekulární a sérologická surveillance klíšťaty přenášených nákaz (**PRÁCE 2, 4, 6, 7, 9, 10, 11, 12, 13, 14**).

PRÁCE 2: Rudolf I., Golovchenko M., Šikutová S., Rudenko N., Grubhoffer L., Hubálek Z.: *Babesia microti* (Piroplasmida: *Babesiidae*) in nymphal *Ixodes ricinus* (Acari: *Ixodidae*) in the Czech Republic. *Folia Parasitol.* (2005), 52, 274-276.

PRÁCE 4: Šikutová S., Rudolf I., Golovchenko M., Rudenko N., Grubhoffer L., Hubálek Z.: Detection of *Anaplasma* DNA in *Ixodes ricinus* ticks: pitfalls. *Folia Parasitol.* (2007), 54, 310-312.

PRÁCE 6: Šikutová S., Hornok S., Hubálek Z., Doležálková I., Juřicová Z., Rudolf I.: Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. *Vet. Microbiol.* (2009), 135, 267-271.

PRÁCE 7: Rudolf I., Mendel J., Šikutová S., Švec P., Masaříková J., Nováková D., Buňková L., Sedláček I., Hubálek Z.: 16S rRNA gene-based identification of cultured bacterial flora from host-seeking *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* ticks, vectors of vertebrate pathogens. *Folia Microbiol.* (2009), 54, 419-428.

PRÁCE 9: Konvalinová J., Rudolf I., Šikutová S., Hubálek Z., Svobodová V., Svoboda M.: Likely emergence of canine babesiosis in the Czech Republic. *Acta Vet. Brno.* (2012), 81, 91-95.

PRÁCE 10: Venclíková K., Rudolf I., Mendel J., Betášová L., Hubálek Z.: Rickettsiae in questing *Ixodes ricinus* ticks in the Czech Republic. *Ticks Tick-borne Dis*. (2014), 5, 135-138.

PRÁCE 11: Venclíková K., Betášová L., Šikutová S., Jedličková P. , Hubálek Z., Rudolf I.: Human pathogenic borreliae in *Ixodes ricinus* ticks in natural and urban ecosystem (Czech Republic). *Acta Parasitol.* (2014), 59, 717-720.

PRÁCE 12: Venclíková K., Mendel J., Betášová L., Hubálek Z., Rudolf I.: First evidence of *Babesia venatorum* and *Babesia capreoli* in questing *Ixodes ricinus* ticks in the Czech Republic. *Ann. Agric. Environ. Med.* (2015), 22, 212-214.

PRÁCE 13: Venclíková K., Mendel J., Betášová L., Blažejová H., Jedličková P., Straková P., Hubálek Z., Rudolf I.: Neglected tick-borne pathogens in the Czech Republic, 2011-2014. *Ticks Tick-borne Dis.* (2016), 7: 107-112.

PRÁCE 14: Duscher G., Hodžić A., Weiler M., Vaux A.G.C., Rudolf I., Sixl W., Medlock J.M., Versteirt V., Hubálek Z. First report of *Rickettsia raoultii* in field collected *Dermacentor reticulatus* ticks from Austria Ticks and Tick-borne Dis. (2016). doi:10.1016/j.ttbdis.2016.02.022.

(1) V rámci dizertační práce jsem se soustředil na experimentální studie z oblasti eko-fyziologie borrelií. Interakce mezi klíšťaty a jimi přenášenými patogeny je klíčovým faktorem pro úspěšný biologický přenos patogena na obratlovčího hostitele (Munderloh a Kurtti, 1997; Schwann 1996). Spirochéta *Borrelia burgdorferi* sensu lato, původce lymské borreliózy, musí překonat nejméně dvě bariéry pro efektivní přenos na hostitele: slinné žlázy a střevo. V naší studii (**PRÁCE 1**) jsme zkoumali efekt extraktů slinných žláz (SGE) a střeva (MGE) u dvou druhů klíšťat: *I. ricinus* (kompetentního vektora pro přenos agens lymské borreliózy) a *D. reticulatus* (nekompetentního druhu pro přenos původce lymské borreliózy) na růst borrelií (*B. garinii*) *in vitro* a očekávali opačný efekt. Podařilo se nám vyvinout unikátní metodu *in vitro* kultivace v mikrotitračních destičkách. Pozorovali jsme statisticky významný stimulační efekt SGE z kompetentního vektora na růst borrelií, zatímco u nekompetentního druhu nedošlo překvapivě k výraznému ovlivnění růstu (stimulaci či inhibici). Práce byla ve své době ojedinělá, podobný experiment provedli pouze Američané u klíšťat *I. scapularis* a *B. burgdorferi* sensu stricto (Shih a kol., 2002). V navazující studii (**PRÁCE 8**) jsme potvrdili významnou stimulaci růstu *B. burgdorferi* (tentokrát tří patogenních genomických druhů *B. garinii*, *B. burgdorferi* s.s., *B. afzelii*) při kultivaci *in vitro* po přidání SGE z nasátých klíšťat *I. ricinus*. Práce potvrdila nezastupitelnou roli slinných žláz při multiplikaci patogena a podpořila práce týkající se role tzv. SAT faktoru ('saliva activated transmission'), který doprovází úspěšný přenos borrelií obratlovčímu hostiteli (Pechová a kol., 2002; Macháčková a kol., 2006; Horká a kol. 2009).

(2) V tomto bloku se nachází dvě studie sledující změny v abundanci klíšťat a prevalenci borrelií v klíšťatech *I. ricinus* v rámci dvou odlišných biotopů pozměněných člověkem (vymýcení lesa, haldy po těžbě uhlí), tedy v důsledku změn antropogenních. Existuje totiž omezený počet eko-epidemiologických prací zabývajících se konkrétními antropogenními vlivy na výskyt klíšťat, včetně jejich promořenosti vybranými patogeny (Gorelova a Kovalovski, 1985; Wilson, 1986; Gorelova 1987; Mather a kol., 1993; Schulze a kol.,1995; Stafford a kol., 1998).

V první studii (**PRÁCE 3**) jsme se zabývali efektem vymýcení části lesního porostu na abundanci klíšťat *I. ricinus* a jejich promořenost borreliemi ve srovnání s kontrolním nevymýceným úsekem (tj. vlivem zejména antropogenního vlivu). Na vykáceném úseku došlo po omezené období k redukci počtu klíšťat (tedy včetně klíšťat infikovaných

borreliemi) a tedy ke snížení rizika nákazy lymskou borreliózou. Promořenost klíšťat borreliemi se však významně nelišila u vymýceného transektu ve srovnání s kontrolním (nezasaženým) transektem. Již ve 3. roce studie však lze na vymýceném transektu pozorovat návrat k původnímu stavu. Další ekologická studie (**PRÁCE 5**) se zabývá frekvencí klíšťat *I. ricinus* (nymf a dospělců) a jejich promořenosti borreliemi na dvou ostravských haldách hlušinách (částečně porostlých vegetací v rámci rekultivace) vzniklých po těžbě černého uhlí a jedné kontrolní (lesní) lokalitě. Překvapivě bylo zjištěno, že ostravské haldy hlušiny, pokud jsou porostlé vegetací a navštěvovány lidmi, představují stejné potenciální riziko nákazy lymskou borreliózou jako běžné lesní biotopy. Podobné studie v evropském kontextu nebyly dosud provedeny.

(3) Molekulární detekce emergentních patogenů v klíšťatech se v posledních letech stala velmi turbulentní disciplínou. Nejen že jsou stále identifikovány nové klíšťaty přenášené patogeny (např. '*Candidatus* Neoeherlichia mikurensis', fleboviry Heartland nebo Henan, thogotovirus Bourbon, *B. myiamotoi* v klíšťatech *I. ricinus* a *I. scapularis*), musíme monitorovat patogeny již poznané i když relativně vzácné. Je třeba si uvědomit, že vedle klíšťové encefalitidy a lymské borreliózy klíšťata přenášejí i řadu jiných humánních, mnohdy opomíjených patogenů. V rámci účasti ve dvou evropských projektech (EDEN a EDENext) jsme provedli molekulární skríning zaměřený především na záchyt *Babesia* spp., *Anaplasma phagocytophilum*, *Rickettsia* spp. a '*Candidatus* Neoeherlichia mikurensis' v nenasátých klíšťatech *I. ricinus*. Již předtím se nám podařilo poprvé v České republice zachytit patogenní *Babesia microti*, původce lidské babeziózy u klíšťat *I. ricinus* (**PRÁCE 2**). Při detekci *A. phagocytophilum* u klíšťat (**PRÁCE 4**) jsme zjistili, že primery, které se běžně používaly pro detekci *A. phagocytophilum* u klíšťat nebo obratlovců amplifikují také jiné ehrlichie včetně nepatogenních druhů či variant. Naše práce byla jedna z mála, která upozornila na nutnost odlišovat patogenní a nepatogenní varianty *A. phagocytophilum* s ohledem na výskyt odlišných variant a tedy upozornila na bias prevalenčních studií týkajících se *A. phagocytophilum* v klíšťatech v Evropě.

V další studii financované Grantovou agenturou AVČR jsme se pokusili pomocí kultivačních a molekulárních metod popsat diverzitu kultivovatelných baktérií v klíšťatech (**PRÁCE 7**) s cílem izolovat nové druhy baktérií a také porovnat diverzitu mikroorganizmů mezi třemi druhy klíšťat, která se vyskytují v České republice a současně přenášejí lidské patogeny. Práce byla ve své době pionýrskou studií, která se pokoušela alespoň částečně popsat diverzitu mikroorganizmů, která může mimojiné ovlivnit např. vektorovou kompetenci klíšťat pro přenos patogenů. Dnes se díky progresivnímu vývoji molekulárních metod včetně

metody sekvenování nové generace toto pole výzkumu významně rozšířilo a poskytuje obrovský zdroj informací (Narasimhan a Fikrig, 2015). V naší studii se nám podařilo zachytit 151 bakteriálních kmenů včetně medicínsky významných druhů (např. *Stenotrophomonas maltophilia*).

Ve spolupráci s kolegy z Veterinární univerzity v Brně jsme se neúspěšně pokoušeli detegovat významného patogena psů, prvoka *Babesia canis* v klíšťatech *Dermacentor reticulatus* z oblasti jižní Moravy (**PRÁCE 9**), kde byly detegovány protilátky proti tomuto závažnému onemocnění u psů, kteří nevycestovali. Psí babezióza je dosud v České republice považována pouze za importovanou nákazu, je však nutné si uvědomit, že toto onemocnění se vyskytuje u našich slovenských a rakouských sousedů (Víchová a kol., 2016) a je tedy nutné monitorovat případnou introdukci do České republiky. V rámci molekulární surveillance klíšťat *I. ricinus* v přírodním a urbánním biotopu na Ostravsku (**PRÁCE 11**) jsme poprvé u nás detegovali *Borrelia spielmanii* v urbánním ekosystému a poukázali na riziko nákazy lymskou borreliózou při návštěvách městských parků.

Dále se nám podařilo poprvé v České republice detegovat některé patogenní rickettsie, např. '*Candidatus* Neoehrlichia mikurensis', *Rickettsia helvetica* a *R. monacensis* v nenasátých klíšťatech *I. ricinus* (**PRÁCE 10**). Ve stejném souboru klíšťat se nám podařilo detegovat patogenní *Babesia venatorum* a *Babesia capreoli* opět poprvé v České republice (**PRÁCE 12**). V rámci evropského projektu EDENext jsme během let 2011 až 2014 vyšetřili celkem 2473 klíšťat (**PRÁCE 13**) a zacílili zejména na opomíjené patogeny (*A. phagocytophilum*, '*Candidatus* Neoehrlichia mikurensis', *Rickettsia* spp., *Babesia* spp.) a to v rámci tří odlišných biotopů (přírodní, urbánní a pastvinný). Na základě získaných výsledků jsme doporučili epidemiologickou surveillance i těchto opomíjených nákaz na našem území ve všech typech biotopů. V rámci dalšího evropského projektu VECTORNET, který má za cíl mapování vektorů nákaz v Evropě jsme se zaměřili na distribuci medicínsky významného klíštěte *D. reticulatus* v Rakousku (**PRÁCE 14**), zejména v biotopech, kde zcela chybí relevantní data. Podařilo se nám najít nová místa výskytu a také místa s absencí a dále vyšetřit sbíraná klíšťata na humánní patogeny. Nejvýznamnějším výstupem je první detekce *Rickettsia raoultii*, tedy původce onemocnění provázeného lymfadenopatií (DEBONEL), v klíšťatech *D. reticulatus* v Rakousku.

Práce týkající se molekulární detekce patogenů v klíšťatech doplňuje séropřehled dalších onemocnění přenášených klíšťaty a to fleboviru Bhanja a flaviviru klíšťové encefalitidy (**PRÁCE 6**) u 400 jedinců domácích zvířat (260 krav, 100 ovcí a 40 koní) ze severovýchodního Maďarska. Zatímco protilátky k viru Bhanja nebyly detegovány, narozdíl

například od ovcí pasených na území sousedního Slovenska (Hubálek a kol., 1985), a kde byl dokonce virus již dříve izolován z klíštěte *Dermacentor marginatus* (Hubálek a kol., 1988), zjištěné protilátky k viru klíšťové encefalitidy (26,5% u krav a 7% u ovcí) odhalily ohnisko klíšťové encefalitidy v severovýchodním Maďarsku. Práce dokládá důležitost analytických epidemiologických metod jako je sérologická surveillance při monitorování ohnisek nákaz přenášených klíšťaty včetně odkrývání potenciálně nových ohnisek.

4.2. Příspěvek autora v oblasti nákaz přenášených komáry

Komentář k předloženým pracem nemá charakter klasické diskuse, spíše se snaží reflektovat ty nejdůležitější aspekty našich studií případně naznačit přesah našich výzkumů. Práce jsou v tomto komentáři členěny do pěti bloků:

(1) séropřehledy obratlovců včetně člověka sledující výskyt protilátek k viru West Nile (WNV) (**PRÁCE 15, 16, 19, 20, 27, 34**)

PRÁCE 15: Hubálek Z., Zeman P., Halouzka J., Juřicová Z., Šťovíčková E., Bálková H., Šikutová S., Rudolf I.: Protilátky k virům přenosným komáry u středočeské populace z oblasti zasažené povodní v roce 2002. *Epidem. Mikrobiol. Imunol.* (2004), 53, 112-120.

PRÁCE 16: Hubálek Z., Zeman P., Halouzka J., Juřicová Z., Šťovíčková E., Bálková H., Šikutová S., Rudolf I.: Mosquitoborne Viruses, Czech Republic, 2002. *Emerg. Infect. Dis.* (2005), 11, 116-118.

PRÁCE 19: Hubálek Z., Wegner E., Halouzka J., Tryjanowski P., Jerzak L., Šikutová S., Rudolf I., Kruszewicz A.G., Jaworski Z., Wlodarczyk R.: Serologic survey of potential vertebrate hosts for West Nile virus in Poland. *Viral Immunol*. (2008), 21, 247-253.

PRÁCE 20: Hubálek Z., Halouzka J., Juřicová Z., Šikutová S., Rudolf I., Honza M., Janková J., Chytil J., Marec F., Sitko J.: Serologic survey of birds for West Nile flavivirus in southern Moravia (Czech Republic). *Vector-borne Zoonotic Dis*. (2008), 8, 659-666.

PRÁCE 27: Hubálek Z., Ludvíková E., Jahn P., Treml F., Rudolf I., Svobodová P., Šikutová S., Betášová L., Bíreš J., Mojžíš M., Tinák M., Boldižár M., Citsoňová G., Staššíková Z.: West Nile virus equine serosurvey in the Czech and Slovak Republics. *Vector-borne Zoonotic Dis*. (2013), 13, 733-738.

PRÁCE 34: Straková P., Šikutová S., Jedličková P., Sitko J., Rudolf I., Hubálek Z.: The Common Coot as sentinel species for the presence of West Nile and Usutu flaviviruses in Central Europe. *Res. Vet. Sci.* (2015), 102, 159-161.

(2) molekulární detekce flaviviru WNV a orthobunyaviru Ťahyňa v komárech (a lidech) (**PRÁCE 17, 22, 30, 31, 32, 33, 35**)

PRÁCE 17: Bakonyi T., Hubálek Z., Rudolf I., Nowotny N.: Novel Flavivirus or New Lineage of West Nile Virus, Central Europe. *Emerg. Infect. Dis.* (2005), 11, 225-231.

PRÁCE 22: Hubálek Z., Rudolf I., Bakonyi T., Kazdová K., Halouzka J., Šebesta O., Šikutová S., Juřicová Z., Nowotny N.: Mosquito (Diptera: Culicidae) surveillance for arboviruses in an area endemic for West Nile (Lineage Rabensburg) and Tahyna viruses in Central Europe. *J. Med. Entomol.* (2010), 47, 466-472.

PRÁCE 30: Rudolf I., Bakonyi T., Šebesta O., Peško J., Venclíková K., Mendel J., Betášová L., Blažejová H., Straková P., Nowotny N., Hubálek Z.: West Nile virus lineage 2 isolated from *Culex modestus* mosquitoes in the Czech Republic, 2013: expansion of the European WNV endemic area to the North? *Euro Surveill.* (2014), 19, pii=20867.

PRÁCE 31: Hubálek Z., Šebesta O., Peško J., Betášová L., Blažejová H., Venclíková K., Rudolf I.: Isolation of Tahyna virus (California Encephalitis Group) from *Anopheles hyrcanus* (Diptera, Culicidae), a mosquito species new to, and expanding in, Central Europe. *J. Med. Entomol.* (2014), 51, 1264-1267.

PRÁCE 32: Pachler K., Lebl K., Berer D., Rudolf I., Hubálek Z., Nowotny N.: Putative New West Nile virus lineage in *Uranotaenia unguiculata* mosquitoes, Austria, 2013. *Emerg. Infect. Dis.* (2014), 20, 2119-2122.

PRÁCE 33: Kolodziejek J., Seidel B., Jungbauer C., Dimmel K., Kolodziejek M., Rudolf I., Hubalek Z., Allerberger F., Nowotny N.: West Nile virus positive blood donation and subsequent entomological investigation, Austria , 2014. *PLoS One*. (2015), 10, e0126381.

PRÁCE 35: Rudolf I., Bakonyi T., Šebesta O., Mendel J., Peško J., Betášová L., Blažejová H., Venclíková K., Straková P., Nowotny N., Hubálek Z.: Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem. *Parasites Vectors* (2015), 8:520.

(3) výskyt dvou opomíjených arbovirů ve Střední Evropě (flavivirus Usutu, orthobunyavirus Sedlec) (**PRÁCE 18, 26, 28, 35**)

PRÁCE 18: Meister T., Lussy H., Bakonyi T., Šikutová S., Rudolf I., Vogl W., Winkler H., Frey H., Hubálek Z., Nowotny N., Weissenböck H.: Serological evidence of continuing high Usutu virus (*Flaviviridae*) activity and establishment of herd immunity in wild birds in Austria. *Vet. Microbiol*. (2008), 127, 237-248.

PRÁCE 26: Bakonyi T., Kolodziejek J., Rudolf I., Bercic R.L., Nowotny N., Hubálek Z.: Partial genetic characterization of Sedlec virus (Orthobunyavirus, *Bunyaviridae*). *Infect. Genet. Evol*. (2013), 19, 244-249.

PRÁCE 28: Hubálek Z., Rudolf I., Čapek M., Bakonyi T., Betášová L., Nowotny N.: Usutu Virus in Blackbirds (*Turdus merula*), Czech Republic, 2011-2012. *Transbound. Emerg. Dis.* (2014), 61, 273-276.

PRÁCE 35: Rudolf I., Bakonyi T., Šebesta O., Mendel J., Peško J., Betášová L., Blažejová H., Venclíková K., Straková P., Nowotny N., Hubálek Z.: Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem. *Parasites Vectors* (2015), 8:520.

(4) entomologická surveillance medicínsky významných komárů (**PRÁCE 21, 23, 24, 31, 36**)

PRÁCE 21: Šebesta O., Rettich F., Minář J., Halouzka J., Hubálek Z, Juřicová Z., Rudolf I., Šikutová S., Gelbič I. and Reiter P.: Presence of the mosquito *Anopheles hyrcanus* in South Moravia, Czech Republic. *Med. Vet. Entomol.* (2009), 23, 284-286.

PRÁCE 23: Šebesta O., Halouzka J., Hubálek Z., Juřicová Z., Rudolf I., Šikutová S., Svobodová P., Reiter P.: Quantitative analysis of mosquito fauna in South Moravia (Diptera: Culicidae) fauna in an area endemic for West Nile virus. *J. Vector Ecol.* (2010), 35, 156-162.

PRÁCE 24: Šebesta O., Rudolf I., Betášova L., Peško J., Hubálek Z.: An invasive mosquito species *Aedes albopictus* found in the Czech Republic. *Euro Surveill.* (2012), 17, pii: 20301.

PRÁCE 31: Hubálek Z., Šebesta O., Peško J., Betášová L., Blažejová H., Venclíková K., Rudolf I.: Isolation of Tahyna virus (California Encephalitis Group) from *Anopheles hyrcanus* (Diptera, Culicidae), a mosquito species new to, and expanding in, Central Europe. *J. Med. Entomol.* (2014), 51, 1264-1267.

PRÁCE 36: Rudolf I., Šebesta O., Straková P., Betášová L., Blažejová H., Venclíková K., Seidel B., Tóth S., Hubálek Z., Schaffner F.: Overwintering of *Uranotaenia unguiculata* adult females in Central Europe: a possible way of persistence of the putative new lineage of West Nile virus? *J. Am. Mosquito Contr. Assoc.* (2015), 31: 364-365.

(5) Emergentní *Dirofilaria repens* v komárech v České republice a na Slovensku (**PRÁCE 25, 29**).

PRÁCE 25: Bocková E., Rudolf I., Kočišová A., Betášová L.,Venclíková K., Mendel J., Hubálek Z.: *Dirofilaria repens* Microfilariae in *Aedes vexans* Mosquitoes in Slovakia. *Parasitol. Res.* (2013), 112, 3465-3470.

PRÁCE 29: Rudolf I., Šebesta O., Mendel J., Betášová L., Bocková E., Jedličková P., Venclíková K., Blažejová H., Šikutová S., Hubálek Z.: Zoonotic *Dirofilaria repens* (Nematoda: Filarioidea) in *Aedes vexans* mosquitoes, Czech Republic. *Parasitol. Res.* (2014), 113, 4663-4667.

(1) Naše laboratoř je v rámci České republiky i Evropy jedinečná v použití sérologických metod při diagnostice nákaz způsobených arboviry, provádíme zde dlouhodobě studie s využitím neutralizačního testu. Jde o velmi specifický a citlivý test, mezi arbovirology považován za zlatý standard a právě při séropřehledech arbovirových nákaz v podstatě jediný, který z důvodu vysoké zkřížené reaktivity arbovirů poskytuje relevantní data. Sérologická surveillance patří v epidemiologii mezi základní analytické metody. I když v době rozvoje molekulárních metod může někdo tyto metody považovat za obsolentní, není tomu tak. Séropřehledy lidských onemocnění dávají epidemiologům přesné informace o stavu imunity populace a promořenosti k dané nákaze (význam např. v oblasti 'herd immunity'), hrají nezastupitelnou roli v oblasti diagnostiky arboviróz a mohou do určité míry sloužit i k odhadu rizika nákazy v budoucnu (imunně 'naivní' populace bývá vždy náchylnější k nové nákaze viz epidemie horeček Chikungunya a Zika v Pacifiku).

V roce 2002 zasáhly Čechy katastrofální povodně, které zejména v Polabí byly provázeny enormně vysokým výskytem kalamitních druhů komárů. V rámci tzv. povodňových projektů, které byly financovány v rámci GAČR, jsme zaměřili naši pozornost na vyšetření lokální lidské populace na protilátky ke komáry přenosným virům. V jedinečné studii (**PRÁCE 16**) bylo vyšetřeno 497 sér pacientů, a u 150 vzorků se nám podařilo získat párová séra nezbytná ke zjištění recentní infekce. Oblasti byly rozděleny do 4 zón (A, B, C, D), které odrážely míru rizika daného místa s ohledem na početnost komáří populace. Nejvíce nás zajímalo, zda bude podobně jako na jižní Moravě po povodních v roce 1997 prokázána sérologická odpověď proti viru West Nile. Séra byla vyšetřena proti čtyřem arbovirům, které by mohly cirkulovat v dané oblasti (orthobunyviry Ťahyňa a Batai, flavivirus West Nile a alfavirus Sindbis). Zcela očekávaně byla zjištěna vysoká prevalence protilátek k viru Ťahyňa (zcela nepřekvapivá se jeví být i pozitivní korelace s věkem), která však nedosahovala míru promořenosti vůči dané nákaze u obyvatel jižní Moravy (Hubálek a kol., 1999). Byla zjištěna pouze jedna recentní nákaza virem Ťahyňa po povodních a zajímavé jsou také zjištěné protilátky k virům Batai a Sindbis (je nutné podotknout, že byly nejprve detegovány méně

specifickým hemaglutinačně inhibičním testem a dále pouze u viru Batai doplněny neutralizačním testem). Protilátky k viru West Nile nebyly zjištěny. Práce zdůrazňuje zejména potřebu zvýšené surveillance arbovirových nákaz po povodních (významný přírodní faktor), kdy dochází k masivnímu přemnožení komárů a zvýšení rizika přenosu daných chorob. Prakticky totožné výsledky jsme publikovali poté i v rámci českého prostředí (**PRÁCE 15**), kdy mezi cílové publikum tentokrát patřili naši infekcionisté a epidemiologové.

Následující dvě studie probíhaly v rámci evropského projektu EDEN (*Emerging Diseases in a Changing European Environment*) a pracovní skupiny pro West Nile virus (WNV). V první studii (**PRÁCE 19**) jsme se zaměřili na vyšetření sér vybraných druhů ptáků z Polska na protilátky k flavivirům WNV a Usutu (USUV). Z celkového počtu 78 sér koní, 20 sér kuřat a 97 sér divokých ptáků vyšetřených neutralizačním testem na protilátky k WNV bylo pozitivních 5 mladých čápů (indikující protilátky získané přirozenou infekcí, nikoliv mateřské protilátky), 1 labuť velká (*Cygnus olor*) a jedna vrána šedá (*Corvus cornix*), tedy celková séropozitovita činila 5,2%. Zajímavostí je také detekce protilátek k viru USUV u racka chechtavého (*Larus ridibundus*). Výsledky prokázaly vcelku nízkou aktivitu WNV v Polsku v daném období korespondující s podobnými studiemi provedenými ve Francii (Lena a kol., 2006) nebo Německu (Linke a kol., 2007). V projektu EDEN jsme se v letech 2004-2006 zaměřili na studium séroprevalence WNV u ptáků na jižní a střední Moravě (**PRÁCE 20**). Celkem bylo vyšetřeno 54 domácích kachen a hus a 391 divokých tažných i domácích divokých ptáků pomocí specifického neutralizačního testu a byla zjištěna prevalence 3,3%, která odráží celkovou nízkou aktivitu WNV v dané oblasti (titry mezi 1:20 až 1:160). Byl zjištěn také titr 1:80 proti USUV u lysky černé (*Fulica atra*). Protilátky proti WNV a USUV detegované u lysek černých nás vedly k cílenému vyšetření tohoto druhu na arboviry (**PRÁCE 34**). Na základě vyšetření 146 lysek černých z oblasti střední Moravy se nám podařilo detegovat protilátky k WNV (2 jedinci), USUV (9 jedinců) a dokonce u 7 jedinců se nám pomocí neutralizačního testu nepodařilo z jistotou přiřadit konkrétní arbovirus. Snad nějaký jiný (nový) arbovirus cirkulující v dané oblasti? Každopádně jsme naznačili možnou roli lysek černých jako vhodného sentinelu pro monitoring zvýšené aktivity WNV potažmo USUV v ohnisku, podobně jako naznačily španělské studie (Figuerola a kol., 2007a,b).

Ve spolupráci s Veterinární univerzitou v Brně a zainteresovanými pracovišti na Slovensku (zejména místní Státní veterinární správou) jsme se pokusili zmapovat WNV aktivitu skríningem koní na přítomnost protilátek k WNV v České republice a na Slovensku (**PRÁCE 27**). Vyšetřili jsme celkem 395 sér koní (z toho 163 českých a 232 slovenských) v rozmezí let 2008 až 2011. Narozdíl od České republiky (nebyly detegovány specifické WNV

protilátky) jsme u 19 slovenských koní detegovali WNV protilátky (séroprevalence 8,3%) v titrech 1:40 až 1:640 (včetně autochtonních infekcí). WNV protilátky u koní byly detegovány převážně u hranic s Maďarskem, tedy země s autochotonním výskytem západonilské horečky (Bakonyi a kol., 2006; Kutasi a kol., 2011). Séroprevalenční studie koní (vhodných indikátorů pro monitoring WNV) byly v Evropě dosud prováděny jen ve Španělsku (Jimenez-Clavero a kol., 2007), Francii (Durand a kol., 2002; Leblond a kol., 2005), Rumunsku (Savuta a kol., 2007) nebo Chorvatsku (Barbic a kol., 2012).

(2) Surveillance vektorů či obratlovců včetně člověka na arboviry patří mezi naše výzkumné priority. Zvláště v důsledku zvýšené cirkulace WNV v Evropě jsme se v rámci dvou evropských projektů zaměřili na molekulární detekci WNV v přenašečích (komárech) a také u lidí. Zejména surveillance komárů na arboviry (**PRÁCE 22, 30, 31, 35**) se staly důležitým podkladem pro zjištění cirkulace arbovirů na jižní Moravě. V letech 2006 až 2008 jsme izolovali a následně identifikovali 5 kmenů orthobunyaviru Ťahyňa z komárů *Ae. vexans*, *Ae. sticticus* a *Cx. modestus* a současně jeden kmen WNV linie Rabensburg z komára *Ae. rossicus* (**PRÁCE 22**). Nález je zajímavý tím, že tento potenciální nový vektor pro virus Rabensburg preferuje při sání savce včetně člověka a naznačuje tak možný cyklus 'savec-komár' pro cirkulaci této linie v ohnisku (narozdíl od cyklu 'pták-komár', který je pro WNV charakteristický). V roce 2014 jsme publikovali v prestižním epidemiologickém časopise *Eurosurveillance* detekci WNV linie 2 z komárů *Cx. modestus* (vyšetřeno asi 30000 komárů) a to poprvé u nás (**PRÁCE 30**). Práce nabádá ke zvýšené epidemiologické surveillance tohoto onemocnění v rámci České republiky ve světle nedávno proběhlých epidemií v Maďarsku (Kutasi a kol., 2011), Řecku (Papa a kol., 2010), Itálii (Angelini a kol., 2010) nebo Srbsku (Popovič a kol., 2013).

Zajímavá izolace Ťahyňa viru z komárů *An. hyrcanus* je zmíněna v kapitole o entomologické surveillance. Molekulární detekce arbovirů v komárech uzavírá studie s prvním záchytem USUV v komárech *Cx. modestus* (**PRÁCE 35**) včetně ko-cirkulace USUV a WNV na rybnících Lednicko-valtického areálu. Práce také naznačuje možnou existenci přírodního a urbánního cyklu USUV podobně jako u jiných arboviróz (dengue, žlutá zimnice).

V rámci naší dlouholeté spolupráce s Veterinární univerzitou ve Vídni se nám podařilo popsat genomy dvou linií WNV a to WNV linie 3 - Rabensburg (**PRÁCE 17**) a WNV linie 9 (**PRÁCE 32**). Nezanedbatelná je i naše účast v popisu detekce a izolace WNV viru v krvi donora z Rakouska z roku 2014 včetně detekce WNV v komárech z místa bydliště donora (**PRÁCE 33**), naznačující autochtonní cirkulaci WNV v sousedním Rakousku. Protože v roce

2015 byl detegován lidský případ západonilské horečky nedaleko našich hranic s Rakouskem (ústní sdělení prof. Norbert Nowotny), ve spolupráci s klinikou infekčních nemocí FN Brno jsme v roce 2015 začali prošetřovat podezřelé případy aseptických meningitid a encefalitid z oblasti Břeclavska, Mikulovska a Hodonínska.

(3) Velmi zajímavý příběh se týká dalšího exotického arboviru přenášeného komáry. Jde o flavivirus Usutu (USUV), který se endemicky vyskytuje v Africe a vůbec poprvé byl izolován z komára *Cx. neavei* v jižní Africe v roce 1959 (McIntosh a Bruce, 1985). V roce 2001 však došlo v Rakousku, konkrétně ve Vídni a okolí, k masivnímu hynutí kosů obecných (*Turdus merula*). Naši kolegové z Veterinární univerzity ve Vídni virus pomocí imunohistochemiských a molekulárních metod identifikovali jako středoevropskou linii USUV (Weissenbock a kol., 2002). V následujících letech následovalo v důsledku šíření USUV hynutí i u dalších ptačích druhů (např. puštíci, *Strix nebulosa*) především ve východním Rakousku. V roce 2003-2006 jsme s vídeňskými kolegy sérologicky vyšetřili 442 sér 55 ptačích druhů (**PRÁCE 18**) a konfirmovali slábnoucí cirkulaci USUV v Rakousku (stav tzv. 'herd immunity'). Netradiční záchyt USUV se nám podařil v roce 2011 (**PRÁCE 28**), kdy nám náš kolega ornitolog Dr. Miroslav Čapek poslal uhynulého kosa, kterého našel v Brně Pisárkách. Úspěšně jsme z něj izolovali a molekulárně determinovali evropskou variantu USUV, velmi podobnou kmenům detegovaným v sousedním Rakousku (Weissenboeck a kol., 2002) nebo Maďarsku (Bakonyi a kol., 2007). V roce 2013 se nám podařilo detegovat virus i v komárech *Cx. modestus* na Břeclavsku (**PRÁCE 35**) a tím jsme naznačili možnou cirkulaci tohoto potenciálně patogenního viru na jižní Moravě. USUV byl překvapivě detegován i u případů neuroinvazivních onemocnění člověka v Chorvatsku (Vilibic-Savlek a kol., 2014) nebo u imunokompromitovaného jedince v Itálii (Pecorari a kol., 2009).

Vedle USUV se nám podařilo geneticky charakterizovat i další 'efemerní' virus pojmenovaný Sedlec (**PRÁCE 26**) a izolovaný v roce 1984 z krve rákosníka obecného (*Acrocephalus scirpaceus*). Virus byl na základě morfologických a fyzikálně-chemických vlastností přiřazen k bunyavirům. Po sekvenaci S segmentu (nukleokapsid) a L-segmentu (RNA dependentní RNA polymeráza) viru byl zařazen jako nejblíže příbuzný dvou dosud neidentifikovaných virů (1612045 a Oyo), naznačující novou séroskupinu v rámci rodu *Orthobunyavirus*, blízko antigenní skupině Simbu. Zajímavé je připomenout další dva viry ze skupiny Simbu: pro člověka patogenní virus Oropouche (Hubálek a Rudolf, 2011) a také veterinárně významný virus Schmallenberg, který byl popsán teprve nedávno v západní Evropě a který způsobuje poškození plodů zejména u domácích přežvýkavců (Hubálek a kol., 2014). Oba viry přenášejí pakomárci. Že by stejné platilo i pro virus Sedlec?

(4) Entomologická surveillance se stává velmi cennou metodou pro záchyt nebezpečných vektorů nákaz na sledovaném území. Pravidelný monitoring komárů jako přenašečů onemocnění dnes v České republice provádíme osamoceni, přesto se nám daří získávat v rámci komáří fauny medicínsky i epidemiologicky cenná data (**PRÁCE 23**). V roce 2008 se nám podařilo zachytit v rámci pravidelného monitoringu komára *Anopheles hyrcanus*, který v Asii přenáší malárii, mezi prvními na našem území (**PRÁCE 21**). K podobným výsledkům dospěla i parazitologická skupina z Karlovy univerzity (Votýpka a kol., 2008). My však tento nový druh pro Českou republiku nadále monitorujeme a zjišťujeme, že jeho početnost se rapidně zvyšuje a areál rozšiřuje. Například jsme tento druh nedávno zachytili na Hodonínsku, kde se předtím nevyskytoval (dosud nepublikovaná data). Tento druh komára k nám mohl být introdukován pravděpodobně z jižního Slovenska, odkud byl dříve popsán (Halgoš a Benková, 2004), nebo po léta odborníkům unikal v důsledku neexistence pravidelné surveillance. Navíc se nám v roce podařilo z tohoto druhu komára izolovat patogenní virus Ťahyňa a to poprvé v Evropě, jediná podobná práce pochází z Azerbajdžánu (Lvov a kol., 1972) **(PRÁCE 31)**. Ťahyňa virus se u nás endemicky vyskytuje v oblastech jižní Moravy a Polabí a je přenášen především komáry rodu *Aedes* (nejčastěji *Ae. vexans*, *Ae. cantans*). Komár *An. hyrcanus* se tak může stát dalším potenciálním přenašečem tototo viru v Evropě. Podstatné je však zjištění, že velmi záhy po introdukci tohoto komářího druhu došlo k nálezu patogenního arboviru v něm, což naznačuje možná rizika a paralely pro jiné komáří vektory a agens.

V Evropě byl dosud zaznamenán výskyt 5 invazivních druhů komárů, u kterých bylo prokázáno, že mohou být vektorem onemocnění člověka. Jedná se o druhy *Aedes aegypti*, *Ae. albopictus*, *Ae. japonicus*, *Ae. koreicus* a *Ae. triseriatus* (Medlock a kol., 2015).

Aedes albopictus (tzv. tygří komár) pochází z tropické jihovýchodní Asie, odkud se koncem minulého a v současném století rozšířil téměř do celého světa včetně Evropy, kde je nepochybně nejrozšířenějším a ze zdravotního hlediska i nejdůležitějším invazivním druhem. V Evropě byl poprvé zjištěn již v 70. létech v Albánii (Adhami a Reiter, 1998), kam byl zavlečen se zbožím dovezeným z Číny. V roce 1990 byl zaznamenán výskyt také v Itálii a tygří komár byl dosud zjištěn v 25 zemích Evropy - v Albánii, Bosně a Hercegovině, Bulharsku, Chorvatsku, Francii, Řecku, Itálii, Maltě, Monaku, Černé Hoře, Rumunsku, Rusku, San Marinu, Slovinsku, Španělsku, Švýcarsku, Turecku a Vatikánu se již trvale usídlen, v Rakousku, Belgii, Německu, Nizozemsku, Srbsku a Slovensku se pravděpodobně ještě neusadil (Medlock a kol., 2015). Úplná eradikace tohoto druhu komára je dnes v Evropě téměř nemožná. V roce 2012 jsme zaznamenali jeho výskyt v České republice (**PRÁCE 24**).

Je významným vektorem původců řady onemocnění, především virů Chikungunya, Dengue a žluté zimnice, ale i nematodů (dirofilarie). Pravděpodobně bude hrát roli i v přenosu viru Zika (Wong a kol., 2013; Di Luca a kol., 2016), což se může stát rizikovým pro možné epidemie této horečky v jižní Evropě, kde je tento komár velmi dobře etablován. Tento scénář není nereálný ve světle proběhlé epidemie horečky chikungunya v roce 2007 v italské Ravenně (Rezza a kol., 2007).

Vcelku raritní publikací (**PRÁCE 36**) je záchyt samičky komára *Uranotaenia unguiculata* v kolekci přezimujících komárů v roce 2015. Dosud totiž nebyly relevantní údaje o přezimování tohoto druhu komára ve střední Evropě, navíc jeho záchyt naznačuje možný způsob přezimování nové linie viru WNV v Evropě (Pachler a kol., 2014).

(5) Dirofilarióza patří mezi emergentní zoonózu přenášenou komáry. Může způsobovat onemocnění u kočkovitých šelem, zejména psů, a spíše vzácně onemocnění u člověka. *Dirofilaria repens* u psů způsobuje především subkutánní formu onemocnění zatímco *D. immitis* způsobují velmi nepříjemné onemocnění zasahující srdeční arterie a plíce (Genchi a kol., 2011). U člověka se nejčastěji objevují kožní či oční forma (Hrčková a kol., 2013; Antolová a kol. 2015). V rámci molekulární surveillance komárů na vybrané arboviry jsme se pokusili i o detekci patogenních dirofilárií v komárech (**PRÁCE 29**). Protože toto onemocnění bylo zjištěno u psů na jižní Moravě (Svobodová a kol., 2006), zacílili jsme právě na danou oblast. Poprvé v České republice se nám podařilo detegovat patogenní dirofilarie druhu *D. repens* v komárech *Aedes vexans*, kteří patří mezi nejpočetnější tzv. záplavové druhy a při přemnožení působí značné kalamity a mohou se tak stát nemalým rizikem právě pro přenos dirofilárii na psy potažmo člověka. Důležitost našeho výzkumu dokládá recentní práce týmu prof. Kolářové, která popisuje první čtyři lidské případy dirofilariózy z jihomoravského regionu (Matějů a kol., 2016). Na základě detekce dirofilárií v komárech, psích a lidských případů můžeme tvrdit, že dirofilarióza se stává další novou zoonózou pro Českou republiku, nejméně však pro jižní Moravu. Metodicky velmi podobnou práci jsme publikovali se slovenskými kolegy, kdy jsme rovněž detegovali dirofilarie v komárech poprvé na Slovensku (**PRÁCE 25**).

4.3. Souborné práce, kniha a kapitola v knize týkající se nákaz přenášených hematofágy

Následující kapitola obsahuje především souborné práce typu review zacílené na patogeny přenášené hematofágními členovci. Práce jsou řazeny chronologicky dle data uveřejnění.

PRÁCE 37: Rudolf. I., Hubálek Z., Šikutová S., Švec P.: Opomíjené virové infekce přenášené hematofágními členovci v České republice. *Epidem. Mikrobiol. Imunol.* (2008), 57, 80-89.

PRÁCE 38: Hubálek Z., Rudolf I.: *Microbial Zoonoses and Sapronoses*, 1st Edition., Springer, Dordrecht, 2011. ISBN: 978-90-481-9656-2.

PRÁCE 39: Hubálek Z., Rudolf I.: Tick-borne viruses in Europe. *Parasitol. Res.* (2012), 111, 9-36.

PRÁCE 40: Estrada-Pena A., Hubalek Z., Rudolf I. 2014. *Tick-transmitted viruses and climate change*. In *Viral Infections and Global Change*, First Edition. Ed. K. Singh. ISBN: 978-1-118-29787-2 Published Online: NOV 2013 DOI: 10.1002/9781118297469.ch31

PRÁCE 41: Hubálek Z., Rudolf I., Nowotny N.: Arboviruses pathogenic for domestic and wild animals. *Adv. Virus Res.* (2014), 89, 201-275.

PRÁCE 42: Rizzoli A., Silaghi C., Obiegala A., Rudolf I., Hubálek Z., Foldvari G., Plantard O., Vayssier-Taussat M., Bonnet S., Špitálská E., Kazimírová M.: *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health. *Frontiers in Public Health.* (2014), 2, 251.

Review pokrývající svým záběrem arboviry v rámci České republiky **(PRÁCE 37)** bylo sestaveno s cílem informovat zejména širokou lékařskou veřejnost včetně infekcionistů a epidemiologů o opomíjených arbovirech a onemocněních, která způsobují s cílem zdůraznit nutnost surveillance mnohdy nehlášených či jinak opomíjených onemocnění. Je třeba si uvědomit, že velké množství tzv. aseptických virových meningitid bývá v letních měsících nesprávně diagnostikováno a může být způsobeno právě těmito tzv. opomíjenými viry. Review vzniklo k 50. výročí události, kdy dva naši badatelé, Vojtech Bárdoš a Vlasta Danielová, izolovali na východním Slovensku první evropský arbovirus přenášený komáry a nazvali jej podle místa objevu Ťahyňa (Bárdoš a Danileová, 1959). V České republice se totiž kromě viru středoevropské klíšťové encefalitidy vyskytuje dalších 7 arbovirů (*Flavivirus* West Nile, *Bunyavirus* Ťahyňa, *Bunyavirus* Lednice, *Bunyavirus* Batai, *Orbivirus* Tribeč, *Uukuvirus* Uukuniemi), z nichž onemocnění člověka prokazatelně způsobují arboviry West Nile, Ťahyňa, Tribeč a pravděpodobně Batai. Navíc byly u nás detegovány protilátky k dalším dvěma pro člověka patogenním arbovirům vyskytujícím se na evropském kontinentu (*Alphavirus* Sindbis a *Coltivirus* Eyach), aniž by však tyto viry byly izolovány (Hubálek a Rudolf, 2011). V komentáři bych rád zmínil dva opomíjené viry, které jsou i odborné veřejnosti v podstatě neznámé, ovšem mohou způsobovat klinická onemocnění. Prvním je orbivirus Tribeč přenášený klíšťaty, který způsobuje horečnaté onemocnění (Grešíková a Nosek, 1981), někdy provázené aseptickou meningitidou, sérokonverze byla prokázána u pacientů v Čechách (Fraňková, 1981; Libíková a kol., 1970) i na Moravě (Hubálek a kol., 1987), kde byla navíc zjištěna akutní nákaza virem Tribeč u 14 osob na Znojemsku: převažující klinickou manifestací byla serózní meningitida. Na Moravě byly zjištěny protilátky k viru Tribeč u 16 % pacientů s diagnózou meningoencefalitidy (Libíková a kol., 1978MedlockMunderlohTatem). Druhým opomíjeným arbovirem je bunyavirus Ťahyňa,

který způsobuje valtickou horečku (Hubálek a Rudolf, 2011). Při přemnožení komáří populace, převážně po povodních nebo při jarním povodňování lužních lesů, stoupá i riziko nákazy valtickou horečkou. Určité (mnohdy nezanedbatelné) procento febrilních stavů dětí v letních měsících, stejně tak dospělých, kteří se s infekcí dosud nesetkali, může být způsobeno právě valtickou horečkou, avšak onemocnění notoricky uniká pozornosti infektologů i epidemiologů. Dalšímu závažnému onemocnění, západonilské horečce je věnován dostatečný prostor především v kapitole o surveillance WNV.

Zmíněné review je dále doplněno diagnostickým klíčem pro arbovirózy, který není triviální, zejména pokud jde o sérologické vyšetření vzorků ve světle zkřížené reaktivity mezi jednotlivými arboviry čeledi *Flaviviridae*. Review je zajímavé tím, že přesto že je psáno v českém jazyce, je díky anglickému abstraktu vyhledáváno zahraničními kolegy z oboru. Dostupných informací o stále opomíjených arbovirech je stálo málo.

Druhým souborným dílem je monografie v anglickém jazyce **(PRÁCE 38)** pokrývající dosud popsaná zoonotická a sapronotická onemocnění. Jde v podstatě o velmi podrobné kompendium informací o zoonózách a sapronózách, které je určeno především pro přírodovědce. Důležitým aspektem knihy je důraz na tzv. emergentní onemocnění. Mezi zoonózami a sapronózami se v dnešní době stále objevují závažné nemoci zcela nové (např. koronairy SARS, MERS, virózy Hendra a Nipah), nově poznané (lidská anaplazmóza), vracející se (západonilská horečka v Evropě), se vzrůstající incidencí (kampylobakteróza), geograficky expandující (západonilská horečka v Americe), s měnícím se okruhem hostitelů (bartonelózy) či přenašečů (*Ae. albopictus*), anebo nově se klinicky manifestující (horečka Zika), pro něž se v angličtině používá souhrnného termínu "emerging/re-emerging diseases", a v češtině je lze označit jako „nákazy (re)emergentní“. Řada těchto onemocnění je vyvolána schopností některých patogenů překonat mezidruhovou bariéru (angl. 'species barrier') hostitelů, což dokazují např. ptačí chřipka nebo SARS.

V celém textu je kladen větší důraz na ekologické aspekty zoonóz a sapronóz (hematofágní přenašeči nákaz a jejich bionomie; obratlovčí hostitelé zoonóz; biotopy původců a vektorů nákaz a jejich zeměpisné rozšíření; přírodní ohniskovost nákaz) než na detaily klinické nebo terapeutické, které jsou zmíněny mnohdy spíše heslovitě nebo okrajově. Hlavním přínosem práce je rovněž důraz na sapronózy, tedy nemoci přenosné na člověka z vnějšího abiotického prostředí - půdy, tlejících rostlin, exkrementů či rozkládajících se mrtvol živočichů, vody a jiných substrátů - v němž se však původce nákazy aktivně množí. Toto organizační členění nemocí (na antroponózy, zoonózy a sapronózy) není často přijímáno širší

odbornou veřejností, proto se objevuje jen málo prací, které aspekt sapronóz zdůrazňují. Kniha si našla velmi rychle své čtenáře, jak výmluvně naznačuje počet koupených (stáhnutých) kapitol knihy v nakladatelství Springer (viz Přílohy).

V roce 2011 jsme na oborové konferenci "Ticks and Tick-borne Diseases" v Zaragoze představili přehled arbovirů přenášených klíšťaty v Evropě. Pro velmi kladný ohlas mezi kolegy, kteří nás žádali o powerpointovou prezentaci, nás napadlo připravit review v tištěné formě (**PRÁCE 39**). Review je jedinečné tím, že zahrnuje všech 27 dosud popsaných arbovirů přenášených klíšťaty v Evropě: flaviviry středoevropské klíšťové encefalitidy (TBEV), louping-ill (LIV), Tyuleniy (TYUV), a Meaban (MEAV); orthobunyaviry Bahig (BAHV) a Matruh (MTRV); fleboviry Grand Arbaud (GAV), Ponteves (PTVV), Uukuniemi (UUKV), Zaliv Terpeniya (ZTV), a St. Abb's Head (SAHV); nairoviry Soldado (SOLV), Puffin Island (PIV), Avalon (AVAV), Clo Mor (CMV), krymsko-konžské hemoragické horečky (CCHFV); bunyavirus Bhanja (BHAV); coltivirus Eyach (EYAV); orbiviry Tribeč (TRBV), Okhotskiy (OKHV), Cape Wrath (CWV), Mykines (MYKV), Tindholmur (TDMV), a Bauline (BAUV); dva thogotoviry (Thogoto THOV Dhori DHOV); a jeden asfivirus (virus africké horečky prasat ASFV). Zaměřili jsme se zejména na taxonomické zařazení virů, přenašeče, obratlovčí hostitele, onemocnění člověka, diagnostiku a dostupnou terapii, případně vakcinaci. Geografické rozšíření virů je přehledně znázorněno na mapkách. Cenné jsou především tabulky, které shrnují jednak experimentální patogenitu virů na zvířecích modelech a jednak citlivost jednotlivých buněčných kultur k dané škále virů. Na základě dosavadní citovanosti lze říci, že review si během let získává své čtenáře.

Toto review se posléze stalo podkladem pro kaiptolu (**PRÁCE 40**) v knize *Climate Change and Vector-borne Viral Diseases* (editor: Sunit K. Singh), která ve 32 kapitolách pojednává o zejména emergentních patogenech, nových hrozbách v rámci infekčních chorob pro lidstvo a to zejména v souvislosti s globálními změnami environmentálními (změna klimatu, povodně, hurikány) či socio-ekonomickými (demografické změny včetně migrace a mobility, mezinárodní obchod, dovoz zvířat a zvířecích produktů včetně ilegálního dovozu viz např. 'bushmeat trade' nebo 'pet animals import'). Mezi komentovanými patogeny se objevují např. SARS, zoonotické orthopoxviry, alfavirus O'nyong-nyong, zoonotické genotypy hepatitidy E, hantaviry, astroviry, rotaviry a noroviry, arboviry Thogoto, Usutu, Henan nebo Heartland.

Posledních 20 let se ve světové odborné literatuře neobjevila souborná práce mapující arboviry patogenní pro zvířata. Navíc s globálním obchodem se zvířaty narůstá zejména pro

Evropu riziko introdukce dosud exotických infekcí jako je např. virus africké horečky prasat (nyní zaznamenán zvýšený výskyt v zemích východní Evropy viz PROMED-MAIL: PRO/AH/EDR> African swine fever - Europe (18): Ukraine, Russia, Baltic, Poland, spread; Archive Number: 20150822.3595512) nebo flebovirus horečky Rift Valley (který je přenášen komáry *Ae. vexans* a který by v případě introdukce mohl mít katastrofální dopad zejména na chov hospodářských zvířat v Evropě). Nesmíme opomenout ani zcela nový bunyavirus Schmallenberg, objevený v roce 2011, který zejména v západní Evropě během epizootie způsobil nemalé škody u domácích přežvýkavců (Wernike a kol., 2015). V poslední době byly v Evropě zaznamenány také epidemie Q horečky v Nizozemí nebo epidemie horečky bluetongue v severozápadní Evropě (Maclachlan a kol., 2015; Morroy a kol., 2016). To vše bylo motivací k sestavení tohoto přehledu (**PRÁCE 41**), který byl posléze velmi dobře přijat i širší odbornou obcí a otištěn ve velmi prestižním virologickém časopise. Review podává detailní informace (taxonomie, geografické rozšíření, přenašeči, hostitelé, onemocnění) o 55 arbovirech patogenních pro zvířata, které náležejí do 7 čeledí: *Togaviridae* (komáry přenášené viry východní, západní a venezuelské encefalomyelitidy koní, viry Sindbis, Middelburg, Getah a Semliki Forest), *Flaviviridae* (komáry přenášené viry žluté zimnice, japonské encefalitidy, encefalitidy Murray Valley, West Nile, Usutu, izraelské meningoencefalitidy krůt, Tembusu a Wesselsbron; klíšťaty přenášená klíšťová encefalitida, louping ill, Omská hemoragická horečka, horečka Kyasanurského pralesa a Tyuleniy), *Bunyaviridae* (klíšťaty přenášené viry jako Nairobi sheep disease, Soldado a Bhanja; komáry přenášené viry horečky Rift Valley, La Crosse, Snowshoe hare a Cache Valley; tiplíky-přenášené viry Main Drain, Akabane, Aino, Shuni, a Schmallenberg), *Reoviridae* (tiplíky-přenášené viry afrického moru koní, Kasba, bluetongue, epizootická hemoragická nemoc jelenců, Ibaraki, koňská encefalóza, peruánská nemoc koní a Yunnan), *Rhabdoviridae* (flebotomy a komáry přenášené bovinní epizootická horečka, vezikulární stomatitida-Indiana, vezikulární stomatitida-New Jersey, vezikulární stomatitida-Alagoas and Coccal), *Orthomyxoviridae* (klíšťaty přenášený virus Thogoto), a *Asfarviridae* (klíšťaty přenášený virus africké horečky prasat).

Poslední souborná studie (**PRÁCE 42**) vznikla jako kolaborativní projekt v rámci evropského projektu EDENext (*Biology and control of vector-borne infections in Europe*), kterého se naše laboratoř zúčastnila v letech 2011 až 2015, konkrétně skupiny zabývající se emergentními nákazami přenášenými klíšťaty. Hlavním cílem review bylo rekapitulovat dostupné údaje o emergentních nákazách přenášených klíšťaty a to v urbánních a peri-urbánních biotopech. Zdá se totiž, že právě tyto biotopy se stávají významnými z hlediska tzv.

'public health', i když dosud byl tento aspekt spíše opomíjený. V review jsou shromážděna data o patogenech (virus středoevropské klíšťové encefalitidy, *B. burgdorferi*, *A. phagocytophilum*, '*Candidatus* Neoehrlichia mikurensis', *Rickettsia* spp., *Babesia* spp.) přenášených klíštětem *I. ricinus*, hlavních obratlovčích hostitelích a dále údaje o prevalencích sledovaných patogenů v klíšťatech. Důležitým aspektem je zdůraznění socio-ekonomických faktorů (např. "outdoorové" aktivity, reforestace a tvorba nových zelených příměstských oblastí, zahradničení, chov drobných zvířat), které mohou zvyšovat expozici člověka klíšťatům potažmo klíšťaty přenášeným patogenům. Práce také neopomíjí zdůraznit pojmy jako eko-epidemiologie a One-health, které jsou dnes velmi propagovány.

5. Závěr

Perspektivy výzkumu emergentních nákaz přenášených hematofágními členovci aneb nové hrozby i výzvy

Ještě jednou si vypůjčím trefnou větu doc. Luďka Daneše, kterou zmiňuji v úvodní pasáži: "Nové studie přinášejí nejen zprávy o rozšíření některých arbovirů a jejich příbuzných do míst, o nichž se dosud nevědělo, ale ukazují, že je nutno počítat i se vznikem kombinací virů s novými vlastnostmi, které mohou kdykoli přinést velká překvapení. Viry, které jsou dnes málo významné, se mohou stát velkými patogeny, mohou měnit svá působiště, hostitele i přenašeče. Je na místě skromnost a zapotřebí smířit se s tím, že všechny vědecké poznatky mohou platit jen dočasně, protože příroda a přírodní ohniska se vyvíjejí a mění dál, i když velmi pomalu (Daneš, 2003). Tato slova významného českého virologa nelze jistě brát na lehkou váhu a lze si jen přát, aby se komplexní výzkum arbovirů včetně dalších bakteriálních či protozoárních agens, jež přenášejí, nadále rozvíjel". Je však nezbytné tato slova doplnit. Nejenom varianty patogenů, ale dokonce nové patogeny jsou díky progresivním molekulárním metodám stále objevovány. Jmenujme alespoň pár nových patogenních agens přenášených hematofágy z poslední doby: arboviry Heartland, Henan či Bourbon, baktérie '*Candidatus* Neoehrlichia mikurensis' anebo *Borrelia myiamotoi* a další (Hubálek a Rudolf, 2011).

Výzkum ekologie zoonóz u nás má jistě na co navazovat. Studium jejich biologie a ekologie, které intenzívně probíhalo v 50. až 80. letech 20. století v bývalém Československu, posunulo tuto vědní disciplínu významně vpřed. Mnozí čeští a slovenští badatelé (většinou virologové) (abecedně: Vojtech Bárdoš, Rudolf Benda, Dionýz Blaškovič, Luděk Daneš, Vlasta Danielová, Elo Ernek, Milota Grešíková, Jaroslava Holubová, Jiří Januška, Jan Mária Kolman, Otto Kožuch, Milan Labuda, Helena Libíková, Doubravka Málková, Josef Nosek, Zdeněk Hubálek a mnozí další) patří k průkopníkům jak v evropském, tak i světovém měřítku, a některé jejich práce jsou stále pro svou platnost hojně citovány. Navíc navazující výzkum Parazitologického ústavu AVČR, zejména skupin vedených prof. Liborem Grubhofferem, prof. Janem Kopeckým či doc. Danielem Růžkem, jsou zárukou, že výzkum onemocnění přenášených hematofágy má své pokračovatele.

Naše pracoviště ve Valticích se díky několika evropským projektům stala provázána z řadou špičkových pracovišt v Evropě (Pasteur Institute v Paříži, Fridrich Loffler Institute v Greiswaldu, Institute for Tropical Medicine v Mnichově, Veterinary University ve Vídni,

Public Health England v Porton Down) a zejména cenné jsou těsné kolaborace s Evropským centrem pro prevenci a kontrolu nemocí (ECDC) ve Stockholmu a Centrem pro kontrolu nemocí (CDC) v americkém Fort Collins. Dovolím si říct, že dnes stojíme v první linii v záchytu emergentních nákaz přenášených hematofágními členovci v rámci České republiky. Navíc poloha naší laboratoře (v nejjižnějším cípu Česka), tedy teplomilné oblasti s masivním výskytem klíšťat a komárů, umožňuje včasný záchyt nových patogenů a dokonce invazivních vektorů, což se nám v minulosti podařilo několikrát prokázat.

V budoucnu nás jistě čekají nové hrozby, kterým budeme muset čelit a na které bychom měli být připraveni (import exotických arbovirů z tropických oblastí v důsledku migrace obyvatel a zvířat, posun vektorů do vyšších zeměpisných šířek v důsledku změn klimatu, introdukce nových patogenů v důsledku mezinárodního obchodu se zvířaty (včetně tzv. pet animals) (Tatem a kol., 2006; Hubálek a Rudolf, 2011). Evropská unie (narozdíl od České republiky) si uvědomuje tato reálná rizika, a proto posílila svoji podporu financováním projektů 6. a 7. rámcového programu se zaměřením na re-emergentní nákazy včetně těch přenášených hematofágními členovci (projekty EDEN, EDENext, VECTORIE, Antigone, EuroWestnile nebo Vmerge). Doufejme, že nové výzvy nás čekají i v programu HORIZON2020. Historie nedávných epidemií západonilské horečky v Americe (Nasci a kol., 2001), horečky chikungunya na ostrovech Indického oceánu (Mauritius, Seychelly, Mayotte a Reunion) včetně první evropské autochtonní epidemie horečky chikungunya v Itálii v okolí Ravenny (Rezza a kol., 2007) až po nynější rozsáhlou epidemii horečky Zika v Jižní a střední Americe (Wikan a Smith, 2016) budiž nám mementem, že boj s epidemiemi zdaleka nekončí. A současně pro nás také výzvou a motivací jak tento boj i nadále svádět.

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7. Přílohy – tištěné publikace autora předkládané jako součást habilitační práce

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PRÁCE 1

Rudolf I., Hubálek Z. 2003. Effect of salivary gland and midgut extract from *Ixodes ricinus* and *Dermacentor reticulatus* (Acari: *Ixodidae*) on the growth of *Borrelia garinii in vitro*. *Folia Parasitol*. 53: 159–160.

Stručná charakteristika: interakce mezi patogenem a vektorem (klíštětem) je určující při přenosu agens přenášených klíšťaty (stejně jako i jinými hematofágními členovci), zejména potom střevo, hemolymfa a slinné žlázy vektora hrají při přenosu patogena nezastupitelnou roli. Ve studii jsme zkoumali efekt extraktů slinných žláz (SGE) a střeva (MGE) nenasátých klíšťat *Ixodes ricinus* (kompetentního vektora *Borrelia burgdorferi*) a klíšťat *Dermacentor reticulatus* (nekompetentního druhu pro přenos *B. burgdorferi*) na motilitu a růst patogenní *Borrelia garinii in vitro*. Zatímco SGE a MGE *I. ricinus* stimulovaly signifikantně růst borrelií, extrakty *D. reticulutus* neovlivňovaly zpočátku významně růst borrelií, a dokonce působily inhibičně při delší kultivaci.

Hlavní přínos práce: práce vhodně doplňuje ostatní mezinárodní biochemické a molekulární analýzy vtom, že obě orgánové soustavy klíšťat disponují stimulujícími/inhibičními složkami (např. defensiny a také baterií dalších imunomodulačních molekul), z nichž některé ovlivňují kompetenci vektora pro přenos daného agens.

Příspěvek autora k dané práci: autor se podílel na sběru a pitvách klíšťat, přípravě extraktů slinných žláz a střeva klíšťat, kultivaci borrelií, *in vitro* experimentech v mikrodestičkách včetně jejich vyhodnocování a také na přípravě rukopisu.

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RESEARCH NOTE

EFFECT OF THE SALIVARY GLAND AND MIDGUT EXTRACTS FROM *IXODES RICINUS* AND *DERMACENTOR RETICULATUS* (ACARI: IXODIDAE) ON THE GROWTH OF *BORRELIA GARINII* IN VITRO

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The interaction between pathogen and tick vector seems to be crucial for vector-borne pathogens. For instance, *Borrelia burgdorferi* s.l., the Lyme disease agent, must overcome at least two main barriers in the tick vector body to be effectively transmitted: the midgut and the salivary glands. It has been found that salivary gland extract (SGE) of ixodid ticks affects the immune system of vertebrate hosts (Ribeiro J.M.C. 1989: Exp. Appl. Acarol. 7: 15–20; Kuthejlová M., Kopecký J., Štěpánová G., Macela A. 2001: Infect. Immun. 69: 575–578) and also contributes to the transmission of *B. afzelii* to the host (Pechová J., Štěpánová G., Kovář L., Kopecký J. 2002: Folia Parasitol. 49: 153–159).

In earlier studies it was observed that borreliae did not occur in host-seeking *Dermacentor reticulatus* (Fabricius, 1794) in contrast to *Ixodes ricinus* (L., 1758) (Hubálek Z., Halouzka J., Juřicová Z. 1998: Folia Parasitol. 45: 67–72), and that *B. garinii* survived in *I. ricinus* after experimental inoculation while it rapidly disappeared from *D. reticulatus* (Mátlová L., Halouzka J., Juřicová Z., Hubálek Z. 1996: Folia Parasitol. 43: 159–160).

The aim of this study was to examine the effect of SGE and midgut extract (MGE) from both *I. ricinus* and *D. reticulatus* on the growth, motility and morphology of the *B. garinii* spirochaete *in vitro*. This might address the question of vector competence of these two tick species for *B. burgdorferi* s.l. at the level of their compartments (salivary glands and midgut).

Salivary glands and midgut were removed from 40 unfed female *I. ricinus* and 20 unfed female *D. reticulatus*. This study attempted to simulate the effect of SGE and MGE on borrelial growth under conditions of host-seeking ticks, and therefore unfed individuals only were used. Dissected organs were homogenized with a small glass blender in phosphate-buffered saline pH 7.0 (PBS; Oxoid), placed in microtubes, and centrifuged at 9,000 *g* for 10 min. Clarified extracts were sterilized by filtration through the 0.2 µm Nanosep MF centrifugal device (Pall Corporation) and stored at –20°C. Final protein content (µg/ml) of the extracts was estimated (Bradford J. 1976: Anal. Biochem. 72: 248–254) as 32.8 (SGE) and 30.9 (MGE) in *I. ricinus*, and 29.3 (SGE) and 39.2 (MGE) in *D. reticulatus*. In the experiments, 100 µl of each extract (or PBS in the control) were mixed with 100 µl of a 3-day culture of *B. garinii* strain BR 14 (about 10^7 spirochaetes per ml) in BSK-H medium with 6% of rabbit serum (Sigma) in 96-well U-bottomed sterile microplates (Sarstedt), and covered with a sterile sealing film (Denville Scientific). The microplates were placed in a 33°C incubator for 11–12 days. Concentration of motile spirochaetes (the number of motile cells/ml of medium) was determined at intervals (0, 2, 4, 7, 9 and 11 days in *I. ricinus*, and 0, 2, 5, 7, 9 and 12 days in *D. reticulatus*), using darkfield microscopy. (1) Estimation of per cent motility was determined in 3 wells per variant, when 100 randomly selected spirochaetes were screened for motility per well. (2) Concentration of all spirochaetes (motile plus non-motile) was estimated in 10-µl volumes of appropriately diluted cultures on a microscope slide with a 20 × 20 mm coverslip (Hubálek Z., Halouzka J., Heroldová M. 1998: J. Med. Microbiol. 47: 929–932); for each variant, 3 wells with 5 counts (total, 15 repetitions) were used. The data were analysed with the two sample *t*-test using SOLO (BMDP Statistical Software). Significant differences in the concentration of motile spirochaetes were considered at $P < 0.01$.

The results are summarised in Tables 1 to 3. The proportion of motile spirochaetes decreased more slowly with the extracts of *Ixodes ricinus* than in corresponding control (C) since day 9 post inoculation (p.i.). On the other hand, the percentage of spirochaetal motility decreased more rapidly with SGE and MGE from *D. reticulatus* than in C since day 9 p.i. With *I. ricinus*, the concentration of motile spirochaetes increased significantly from days 2 to 11 (p.i.) with both SGE and MGE compared to C. In addition, the growth of spirochaetes was enhanced to a greater degree with SGE than with MGE on days 4, 7 and 9 p.i. With *D. reticulatus*, a significant increase in concentration of motile spirochaetes was only detected with SGE (compared to C) on day 5 p.i., while a marked decrease in concentration of motile spirochaetes was observed on day 9 p.i. with MGE, and on day 12 p.i. with both extracts compared to C. Moreover, many spirochaetes grown in the presence of *D. reticulatus* MGE were morphologically changed (compared to C and SGE) by 9 days p.i.; the cells were damaged (e.g., less discernible walls), shorter, and with a lower number of coils.

The effect of SGE and MGE on the growth of *B. garinii* spirochaetes *in vitro* thus differed between the two tick species tested. While extracts derived from *I. ricinus* (a competent vector for Lyme borreliosis) stimulated growth significantly, extracts from *D. reticulatus* (a non-competent species) did not affect the growth of borreliae markedly, or even inhibited their growth on days 9 (MGE) and 12 p.i. (MGE and SGE). Our results therefore indicate that the tick compartment extracts surprisingly need not be inhibitory for pathogen survival in the body of even non-competent tick species like *D. reticulatus* in a short-term exposure. In such species, the role of a barrier in

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Table 1. Per cent motility of *Borrelia garinii* spirochaetes in BSK-H medium with salivary gland (SGE) or midgut (MGE) extracts (compared to control, C) from ticks.

Ixodes ricinus				*Dermacentor reticulatus*			
Days p.i.	C	SGE	MGE	Days p.i.	C	SGE	MGE
0	100.0	100.0	100.0	0	100.0	100.0	100.0
2	99.3	99.3	98.3	2	99.3	99.7	98.7
4	97.3	98.0	96.3	5	97.7	99.7	97.0
7	92.0	94.7	88.3	7	91.0	94.3	88.0
9	47.3	84.0	77.0	9	87.7	80.0	52.7
11	18.3	26.7	50.0	12	51.0	24.3	5.7

Table 2. Effect of SGE or MGE from *Ixodes ricinus* on the growth of *B. garinii* in BSK-H medium (compared to control, C).

Days p.i.	Concentration of motile spirochaetes [$\times 10^6$/ml of medium]						Differences (*t*-values)		
	C		SGE		MGE				
	AVG	SD	AVG	SD	AVG	SD	C vs. SGE	C vs. MGE	SGE vs. MGE
0	9.08	1.88	9.24	1.36	9.35	1.83	0.26	0.40	0.19
2	27.30	6.58	33.77	6.74	36.29	7.10	2.66*	3.60**	1.00
4	40.37	6.45	80.53	9.80	50.61	9.73	13.25**	3.39*	8.39**
7	49.49	13.48	96.24	20.37	60.48	11.25	7.41**	2.42*	5.95**
9	19.38	9.81	87.93	15.90	45.72	10.81	14.21**	6.99**	8.50**
11	2.35	2.64	15.09	5.95	12.56	4.70	7.58**	7.34**	1.29

* P < 0.01; ** P < 0.001; AVG – arithmetic average; SD – standard deviation

Table 3. Effect of SGE or MGE from *Dermacentor reticulatus* on the growth of *B. garinii* in BSK-H medium (compared to control, C).

Days p.i.	Concentration of motile spirochaetes [$\times 10^6$/ml of medium]						Differences (*t*-values)		
	C		SGE		MGE				
	AVG	SD	AVG	SD	AVG	SD	C vs. SGE	C vs. MGE	SGE vs. MGE
0	10.92	2.30	13.72	3.78	13.44	2.41	2.45	2.93*	0.24
2	47.22	11.66	44.12	9.69	41.59	7.42	0.79	1.58	0.80
5	59.03	12.39	80.30	19.58	69.56	23.29	3.27*	1.41	1.37
7	75.26	12.77	85.40	11.48	74.31	17.53	2.29	0.17	2.05
9	78.77	20.48	80.13	4.52	46.62	3.67	0.19	4.99**	5.76**
12	36.24	11.21	17.53	7.59	4.43	1.25	5.35**	10.92**	6.60*

Explanations as for Table 2.

the pathogen transmission could be another tick compartment, e.g. the haemolymph (Johns R., Ohnishi J., Broadwater A., Sonenshine D.E., De Silva A.M., Hynes W.L. 2001: J. Med. Entomol. 38: 99–107), or non-specific immune compounds like lectins (Grubhoffer L., Jindrák L. 1998: Folia Parasitol. 45: 9–13).

A stimulatory chemotactic effect of SGE from *Ixodes scapularis* on *B. burgdorferi* s.s. *in vitro* was found in a recent study (Shih C.M., Chao L.L., Yu C.P. 2002: Am. J. Trop. Med. Hyg. 66: 616–621). Our work considered the effect of SGE and MGE on spirochaetal growth, but further experiments with other genomic species of borreliae (especially *B. afzelii*, *B. burgdorferi* s.s.) are required.

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PRÁCE 2

Rudolf I., Golovchenko M., Šikutová S., Rudenko N., Grubhoffer L., Hubálek Z. 2005. *Babesia microti* (Piroplasmida: *Babesiidae*) in nymphal *Ixodes ricinus* (Acari: *Ixodidae*) in the Czech Republic. *Folia Parasitol.* 52: 274–276.

Stručná charakteristika: lidská babezióza se dnes řadí mezi tzv. emergentní nákazy. V práci jsme se pokusili detegovat patogenní babesie v nenasátých klíšťatech *I. ricinus* v oblasti jižní Moravy.

Hlavní přínos práce: podařilo se nám poprvé na našem území detegovat v nenasátých klíšťatech lidského patogena *B. microti*, původce humánní babeziózy, který především na východě USA způsobuje těžší formy lidské babeziózy a často bývá spojován s rizikem přenosu při transfúzích krve nebo orgánových transplantacích.

Příspěvek autora k dané práci: autor se podílel na sběru klíšťat v terénu, jejich zpracování v laboratoři včetně molekulární analýzy, konečném vyhodnocení výsledků včetně přípravy rukopisu.

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RESEARCH NOTE

BABESIA MICROTI (PIROPLASMIDA: BABESIIDAE) IN NYMPHAL *IXODES RICINUS* (ACARI: IXODIDAE) IN THE CZECH REPUBLIC

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Abstract. A total of 350 nymphs of the common tick *Ixodes ricinus* (Linnaeus, 1758) were collected in an endemic focus of Lyme borreliosis (South Moravia, Czech Republic) and examined for the presence of the protozoan *Babesia microti* (França, 1909) by polymerase chain reaction (PCR), using primers specific for the *B. microti* gene encoding small subunit rRNA. The assay revealed five positive pools (out of 70 pools examined); the corresponding prevalence rate was about 1.5%. Sequence analysis of the PCR products confirmed their 100% homology with that of *B. microti*. The study represents the first evidence of *B. microti* in ixodid ticks in the Czech Republic.

Babesiosis is an emerging, tick-transmitted zoonotic disease caused by intraerythrocytic parasites of the genus *Babesia*. These piroplasmas are transmitted by ixodid ticks and are capable of infecting a wide variety of vertebrate hosts which are competent in maintaining the transmission cycle. Babesiae include at least three species pathogenic for humans: *Babesia bovis*, *B. divergens* and *B. microti* (Homer et al. 2000). Whereas the bovine parasite, *B. divergens*, is responsible for most European cases of human babesiosis, especially in splenectomized patients (Gorenflot et al. 1998), *B. microti* has not yet been implicated as a cause of autochthonous human illness in Europe (Foppa et al. 2002). Most human cases caused by *B. microti* have occurred in the north-eastern states of the USA and are transmitted by *Ixodes scapularis* (Spielman 1994).

However, *B. microti* is also present in European countries. First findings of *B. microti* in central Europe were reported in the blood from *Microtus arvalis*, *M. agrestis*, *Clethrionomys glareolus*, *Apodemus flavicollis* and *A. sylvaticus* rodents (Aeschlimann et al. 1975, Šebek 1975, Šebek et al. 1977). The occurrence of *B. microti* in rodents has been then reported from other European countries (Šebek et al. 1977, Šebek et al. 1980, Walter 1984, Telford et al. 2002). Thereafter, three species of the genus *Ixodes* have been found to carry and/or transmit *B. microti* in Europe: (1) *I. trianguliceps* in England (Hussein 1980) and Russia (Telford et al. 2002); (2) *I. ricinus* in Germany (Weber and Walter 1980, Walter 1981, Walter and Weber 1981), Slovenia (Duh et al. 2001), Switzerland (Foppa et al. 2002), England (Gray et al. 2002), Poland (Skotarczak and Cichocka 2001, Kuźna-Grygiel et al. 2002, Skotarczak et al. 2003) and Hungary (Kálmán et al. 2003); and (3) *I. persulcatus* in Lithuania (Alekseev and Dubinina 2003) and European Russia (Alekseev et al. 2003).

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The occurrence of *B. microti* in *I. ricinus* ticks has not yet been investigated in the Czech Republic. The purpose of the present study was to determine the prevalence of *B. microti* in *I. ricinus* ticks in one area of South Moravia (Czech Republic), where Lyme borreliosis is endemic. A total of 350 host-seeking nymphs of *I. ricinus* were collected in the surroundings of Valtice (South Moravia, Czech Republic) during 2003 by flagging the vegetation. The habitat was described in another paper (Hubálek et al. 1994). All tick specimens were frozen at –60°C until further processing. Immediately before DNA isolation, nymphs were pooled. Different pool sizes were used for two groups of ticks. We started with pools consisting of three nymphs, but after the first results obtained we shifted to pools of ten individuals (for technical reasons). All ticks were surface sterilized with 70% ethanol (PCR quality) and mechanically disrupted using a glass microblender. The total genomic DNA was extracted with QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR detection of *B. microti* DNA was performed as described by Persing et al. (1992), including primers bab 1 and bab 4 specific for the *B. microti* gene encoding small subunit rRNA (ss-rDNA). Each reaction tube contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.001% Tween 20, 2.5 mM $MgCl_2$, 200 mM mixture of dNTPs, 2.5 U Taq purple DNA polymerase and 25 pmol of each primer. PCR reaction was performed in a PTC-200 Gradient Thermal Cycler (MJ Research, USA) under the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 2 min of extension at 72°C consisting of 40 cycles. The PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualised by UV light. DNA extraction and PCR handling were done in two separate rooms to avoid possible cross-contamination of the samples. Specific PCR products were further characterized by sequence analysis. DNA fragments were precisely excised from the gel and purified with the Gel Extraction Kit (Qiagen, Hilden, Germany). To ensure the specificity, the PCR products were sequenced twice in both directions using bab 1 and bab 4

Table 1. Prevalence of *Babesia microti* in *Ixodes ricinus*, South Moravia, 2003.

Sample	No. of nymphs	Pool size	No. of pools	No. of pools positive	MIR[1]	MLE[2]
I	150[3]	3	50	1	0.67%	0.67%
II	200[4]	10	20	4[5]	2.00%	2.21%
Total	350		70	5	1.43%[6]	1.55%[6]

[1]minimum infection rate; [2]maximum likelihood infection rate; [3]coll. September; [4]coll. April (100 specimens) and September (100 specimens); [5]three of the September collection; [6]weighted average

Table 2. Prevalence of *Babesia microti* in host-seeking *Ixodes ricinus* in Europe.

Country/Reference	Larvae	Nymphs	Adults	Total[1]
GERMANY				
Walter 1981	–	2/375 (0.5)[2]	–	2/375 (0.5)
SLOVENIA				
Duh et al. 2001	–	9/69 (13.0)	4/70 (5.7)	13/139 (9.4)
POLAND				
Skotarczak and Cichocka 2001	12/385 (3.1)	49/1160 (4.2)	69/550 (12.5)	118/1710 (6.9)
Kuźna-Grygiel et al. 2002	–	8/412 (1.9)	0/49 (0.0)	8/461(1.7)
Skotarczak et al. 2003	4/19 (21.1)	26/234 (11.1)	41/280 (14.6)	67/514 (13.0)
SWITZERLAND				
Foppa et al. 2002	–	14/408 (3.4)	–	14/408 (3.4)
HUNGARY				
Kálmán et al. 2003	–	–	–	4/452 (0.9)

[1]nymphs and adults, total; [2]no. positive / no. examined (% positive) individuals

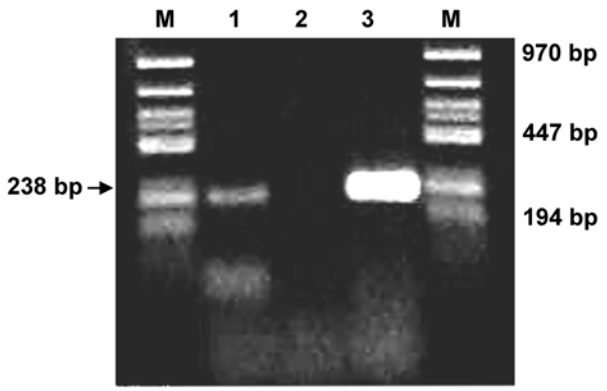

Fig. 1. PCR product of *Babesia microti* DNA from nymphal *Ixodes ricinus*. M – ladder; lanes 1, 2 and 3 – positive sample, negative control, and positive control (*B. microti* DNA), respectively.

primers. CEQ 2000 Dye terminator Cycle sequencing Kit was used, sequences were analysed on an ABI Prism 877 ITC automated DNA sequencer (Beckman Coulter, USA) using DNASTAR software (DNASTAR, London, UK), and compared with those in the GenBank. BLAST programs of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches. Minimum infection rate (MIR) was estimated as the ratio of the number of positive pools to the total number of individual ticks tested (in per cent). The other method of estimating the proportion of infected individuals in pooled samples was the maximum likelihood estimation (MLE), which gives results approaching the real situation more precisely than MIR (Gu et al. 2003). The corresponding software program MIR-IR, obtained from the authors, was used for the latter estimation.

In total, 5 of 70 pools (350 nymphal *I. ricinus*) were positive, giving MIR 1.43% (0.14 infected tick per 1,000 ticks). The alternative estimation by MLE approach yielded a very similar value, 1.55% (Table 1). Fig. 1 shows an example of one positive PCR specimen. The PCR products, subjected to sequence analysis, showed a 100% nucleotide homology with other *B. microti* strains deposited in GenBank: M 93660 (USA), AF 373331 (Slovenia), AF 231348 (Germany), AY 056017 (Switzerland) and AB0 83375 (Japan).

The prevalence of *B. microti* in *I. ricinus* found in this study (about 1.5% with both MIR and MLE calculations) is close to the infection rates reported in other European countries like Germany, Switzerland and Hungary, while considerably higher values were occasionally found in Slovenia or Poland (Table 2). European data show that *B. microti* occurs in all stages of the *I. ricinus* vector. This study has confirmed the presence of *B. microti* in the Czech Republic, where it had been earlier microscopically observed in rodents (Šebek 1975, Šebek et al. 1977). The lack of recognized human pathology associated with European strains of *B. microti*, despite exposure to infectious tick bites, may be a consequence of a lower virulence of European strains compared to the North American babesiae. Disease episodes due to *B. microti*, on the other hand, may be overlooked by general practitioners because of the relatively nonspecific symptoms (at least in mild cases) and a common presumption that this agent rarely, if at all, infects *I. ricinus* (Foppa et al. 2002).

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PRÁCE 3

Hubálek Z., Halouzka J., Juřicová Z., Šikutová S., **Rudolf I.** 2006. Effect of forest clearing on the abundance of *Ixodes ricinus* ticks and the prevalence of *Borrelia burgdorferi* s.l. *Med. Vet. Entomol.* 20: 166–172.

Stručná charakteristika: *B. burgdorferi*, původce lymské borreliózy, je dnes celosvětově nejvýznamnějším humánním patogenem přenášeným klíšťaty. Ve tříleté ekologické studii jsme se zabývali efektem vymýcení části lesního porostu na abundanci klíšťat a jejich promořenost borreliemi ve srovnání s kontrolním nevymýceným úsekem – tj. vlivem především antropogenních faktorů. Na vykáceném úseku došlo po dobu 2 let k redukci klíšťat (tedy včetně klíšťat infikovaných borreliemi) a tedy ke snížení rizika nákazy lymskou boreliózou. Promořenost klíšťat borreliemi se významně nelišila u vymýceného transektu ve srovnání s kontrolním nezasaženým transektem.

Hlavní přínos práce: existuje velmi omezený počet eko-epidemiologických studií zabývajících se konkrétními antropogenními vlivy na výskyt klíšťat a jejich promořenost vybranými patogeny.

Příspěvek autora k dané práci: autor se podílel na sběru klíšťat na vybraných transektech, jejich vyšetření v laboratoři na přítomnost *B. burgdorferi* pomocí molekulárních metod a také na přípravě rukopisu.

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Effect of forest clearing on the abundance of *Ixodes ricinus* ticks and the prevalence of *Borrelia burgdorferi* s.l.

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Abstract. Questing *Ixodes ricinus* L. (Acari: Ixodidae) ticks were collected on a forest trail that had been completely cleared of shrubs and ground vegetation in winter 2002 and on a nearby control uncleared forest transect in South Moravia (Czech Republic). Samples were collected each May in 2003, 2004 and 2005. Nymphal ticks were 3.4 times, 1.9 times and 1.2 times less frequent on cleared forest than on uncleared forest trails in the three respective years, whereas adult tick abundance was 27.2 times, 4.0 times and 2.2 times lower, respectively. The ticks were examined for borreliae by dark-field microscopy: prevalence of nymphal ticks infected with *Borrelia burgdorferi sensu lato* (12.6% to 20.0%) did not differ significantly between the cleared and uncleared trail during the 3 years. In conclusion, the habitat modification appeared to result in a decreased abundance of *I. ricinus* as well as a reduced frequency of infected ticks (and thus indirectly a lower potential risk of Lyme borreliosis), which lasted, however, for only 2 years. Eight cultures of borreliae isolated from the ticks were all identified as the 'ornithophilic' genomic species *Borrelia garinii*, possibly indicating a greater role of forest birds than that of forest rodents as the hosts of immature *I. ricinus* in the tick (and borrelial) colonization of the cleared part of the forest.

Key words. *Borrelia burgdorferi sensu lato*, *Borrelia garinii*, *Ixodes ricinus*, environmental management, forest clearing, habitat manipulation, habitat modification, Lyme borreliosis risk, tick, vegetation reduction, Czech Republic.

Introduction

Habitat modification, especially vegetation reduction, is regarded as one of the few useful techniques to control tick vectors of Lyme borreliosis, in addition to their chemical or biological control or reduction of host availability (Wilson, 1986; Schulze *et al*., 1988; Spielman, 1988; Jaenson *et al*., 1991; Ginsberg, 1994; Schulze *et al*., 1995; Tälleklint & Jaenson, 1995; Mount *et al*., 1997; Stafford & Kitron, 2002; Ward & Brown, 2004). The habitat manipulation usually involves mowing and/or removal of leaf litter, brush and shrubs in wooded residential areas, and covering the soil surface with dry substrates such as tree bark and wooden chips, or even pumice or gravel.

In South Moravia (Czech Republic), long-term efforts to evaluate abundance of *Ixodes ricinus* L. ticks and their infection rate with *Borrelia burgdorferi* sensu lato have been based on regular monitoring of the tick population in a study area along four fixed trails each May since 1991 (Hubálek *et al*., 2003). However, in winter of 2002–2003, one of the four trails was completely cleared of small trees, shrub and low herbaceous and grassy vegetation, and only solitary tall trees were left. This situation caused seemingly hostile conditions for ixodid ticks, and presented a challenging ecological experiment.

The aim of this study was to evaluate the impact of the forest clearing on the abundance, infection rate and frequency of infected nymphal and adult *I. ricinus*, and in turn, to assess indirectly the potential for risk of Lyme borreliosis in particular years after the forest habitat

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modification. To the best of the authors' knowledge, no similar experiment of a forest habitat manipulation has been published. A few North-American studies have tested the effect of mowing or removal of leaf litter in wooded residential areas on the density of the tick *Ixodes scapularis* Say (e.g. Wilson, 1986; Schulze *et al*., 1995), but the impact on the abundance of ticks infected with *B. burgdorferi* s.l. (and thus indirectly the potential risk of Lyme borreliosis) has not been evaluated. A related study (Mather *et al*., 1993) analysed the effect of controlled burning, not clearing, of woodland understory on abundance and infection prevalence in ixodid ticks.

Materials and methods

Study plot

Ixodes ricinus L. ticks were collected on a 10-ha study plot in a deciduous broad-leaved forest at the Rendezvous hunting lodge near Valtice (South Moravia, Czech Republic: 48°45′ N, 16°47′ E; 198 m above sea level). The study site was described previously in detail, including geological, pedologic, climatic, vegetational and faunal conditions (Hubálek *et al*., 1994, 2003). Briefly, the tree story of the forest on the study plot is composed of oaks [*Quercus cerris* L., *Quercus petraea* (Matt.) Liebl], lindens (*Tilia platyphyllos* Scop., *Tilia cordata* Mill.), common maple (*Acer campestre* L.), ash (*Fraxinus excelsior* L.), elm (*Ulmus laevis* Pall.), hornbeam (*Carpinus betulus* L.), service-tree (*Sorbus torminalis* [L.] Cr.) and white poplar (*Populus alba* L.). Shrub (*Acer campestre*, *Crataegus monogyna* Jacq., *Euonymus europaeus* L., *Prunus spinosa* L., *Tilia cordata*, *Rhamnus cathartica* L., *Rubus caesius* L., *Salix caprea* L., *Salix fragilis* L., *Ligustrum vulgare* L., *Sambucus nigra* L.) and herb strata are well developed, and an ample leaf litter normally covers the soil surface from autumn till spring. Local hosts of adult and nymphal *I. ricinus* ticks involve large and medium-sized mammals, mainly roe deer *Capreolus capreolus* (L.), wild boar *Sus scrofa* L., fox *Vulpes vulpes* (L.), squirrel *Sciurus vulgaris* L., and hedgehog *Erinaceus concolor* Martin, whereas common small mammalian hosts of larval ticks are the bank vole *Clethrionomys glareolus* (Schreber), yellow-necked mouse *Apodemus flavicollis* (Melchior), wood mouse *Apodemus sylvaticus* (L.), common shrew *Sorex araneus* L. and mole *Talpa europaea* L. The bird community is composed of about 60 breeding species, and common avian hosts of immature *I. ricinus* are blackbird *Turdus merula* L., song-thrush *Turdus philomelos* Brehm, robin *Erithacus rubecula* (L.), chaffinch *Fringilla coelebs* L., wren *Troglodytes troglodytes* (L.), yellowhammer *Emberiza citrinella* L., great tit *Parus major* L., nuthatch *Sitta europaea* L. and jay *Garrulus glandarius* (L.).

The forest clearing and plant succession

In winter of 2002–2003, one of the four study transects was completely cleared of small trees, shrub and low herbaceous and grassy vegetation, while most tall trees remained untouched (Fig. 1a). Leaf litter was also destroyed, and the surface was covered by wooden chips and tree bark splits (Fig. 1b). The cleared part of the forest (approximately 150-m long, and 15-m wide) was then followed for the vegetation cover (a broad eyeball estimate), succession, and occurrence of ixodid ticks during the first three years (2003, 2004, 2005) after the habitat modification.

Tick sampling and examination

Host-seeking *I. ricinus* were sampled in parallel both on the cleared forest trail (treated forest, TF) and on another, control trail (untreated forest, UF; situated in parallel with TF, 20 m apart: Fig. 1c) by flagging low vegetation and soil surface with white flannel cloths (0.9 × 0.6 m) always before noon in the second half of May, i.e. during the seasonal peak activity of adult and nymphal *I. ricinus* in the area (Hubálek *et al*., 1994). The cloth was examined every 5 m, and all ticks were removed into cork-stoppered glass tubes with a few grass blades, transported to the laboratory and maintained alive at +5°C until examined. Larval *I. ricinus* were sporadic in May and were therefore discarded. Approximately 100 nymphal and as many as possible (optimum, about 100 of both sexes) adult *I. ricinus* were examined for borreliae from the cleared and control transect each year from 2003 to 2005.

Individual ticks were dissected on a microscope slide, their midgut suspended in a drop of saline and examined for spirochaetes by dark-field microscopy at 600 ×. All fields of view of the preparation were screened and spirochaetes were counted (Hubálek *et al*., 1994).

Isolation and identification of borreliae

Attempts were made to cultivate borreliae only from those individual ticks containing their high numbers (> 100), by inoculating the suspension (about 200 μL) into tubes with 4 mL of BSK-H complete medium (Sigma, St Louis, MO, U.S.A.) supplemented with rifampicin (50 μg/mL) and phosphomycin (100 μg/mL). Culture tubes were incubated at 33°C and examined for spirochaetes by dark-field microscopy at regular intervals for up to 6 weeks. Identification of isolated borreliae into genomic species was done by polymerase chain reaction–restriction fragment length polymorphism analysis using primers directed against ribosomal spacer genes *rrf* and *rrl* (Postic *et al*., 1994).

Quantitative analyses

Relative abundance of the ticks was expressed as the frequency (*F*), i.e. the number of individuals collected per person-hour of flagging. Infection prevalence (*P*) was given

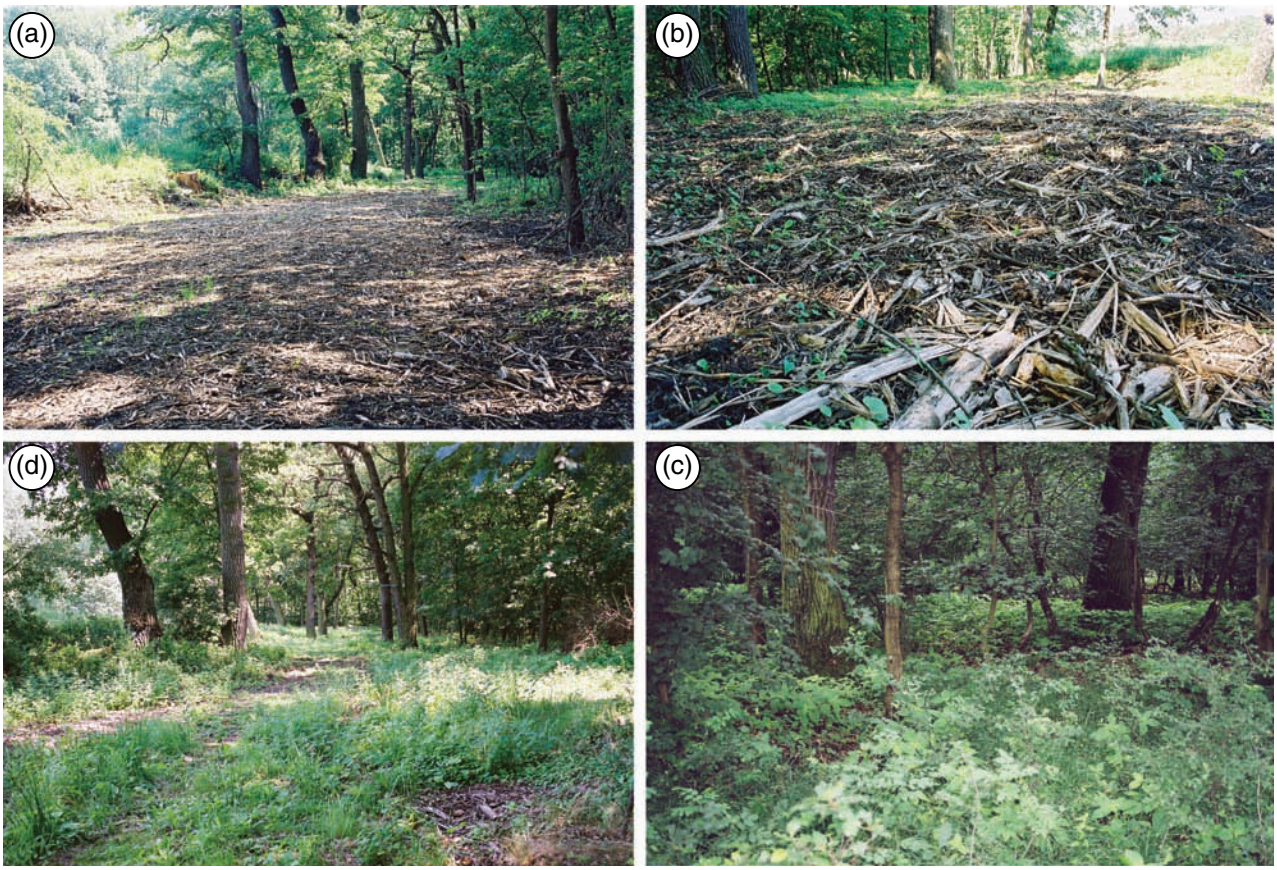

Fig. 1. (a) The cleared forest trail (May 2003), (b) wooden chips and bark splits on the soil surface of the cleared trail (May 2003), (c) the control forest transect (May 2003) and (d) the cleared forest trail: the same view as in Fig. 1(a) two years later (May 2005).

as the proportion (in per cent) of the ticks containing borreliae out of all examined ticks. Frequency of infected ticks (F_i) was calculated as the product $F \times P/100$, i.e. the number of infected ticks per flag-hour. Infective encounter time was then the average time (in minutes) to encounter one (the first) infected tick, i.e. $IET = 60/F_i$ (Hubálek *et al.*, 1994). The infection measures were determined separately for nymphal and adult ticks containing at least one spirochaete, and/or those with > 100 spirochaetes (i.e. the ticks with a higher spirochaetal load).

Contingency 2×2 tables with the chi-square were applied for comparative tests on homogeneity of proportions, and the Fisher exact test (SOLO – BMDP Statistical Software, Los Angeles, CA) was used for confirmation in cases with a small sample size.

Results

Vegetation cover and succession

The ground vegetation cover of the TF trail was barely 10% in May of 2003 – shortly after clearing (Figs 1a and b), but it reached about 50% in May 2004 and increased to approximately 75% in 2005 (Fig. 1d). However, the wooden chips and bark splits were still scattered over the TF study plot even in 2005.

In 2004, the ground layer included 25 herb and 16 grass species (see Appendix). A few tree seedlings, 10–20 cm high, also appeared: *Acer campestre*, *Carpinus betulus*, *Crataegus monogyna*, *Populus alba*, *Prunus spinosa*, *Quercus cerris*, *Quercus petraea* and *Tilia cordata*. In 2005, already 69 herbs and 22 grass species occurred on the TF trail (see Appendix).

Relative abundance of Ixodes ricinus ticks

Frequency of *I. ricinus* differed considerably between the cleared (TF) and the control (UF) trail in the spring following the clearing, but the difference became smaller with time (Table 1): *I. ricinus* nymphs were 3.4 times (a decrease by 71%), 1.9 times (a decrease by 47%) and 1.2 times (a decrease by 18%) less frequent on the cleared trail in 2003, 2004 and 2005, respectively. Adult ticks were considerably less frequent on TF transect than on control UF transect: 27.2 times (a decline by 96%), 4.0 times (a decline by 75%) and 2.2 times (a decline by 54%) in the respective years.

Table 1. Abundance of *Ixodes ricinus* ticks and their infection rate with borreliae

Year:	May 2003		May 2004		May 2005	
Trail*:	TF	UF	TF	UF	TF	UF
Total person-hours:	5.5	4.0	3.0	2.0	4.0	4.0
Frequency (*F*, no. ticks/h)						
Nymphs	19.6	67.5	34.5	65.0	26.5	32.5
Females	0.4	14.0	2.5	10.5	7.8	19.2
Females + males	0.9	24.7	6.5	26.0	20.7	45.0
Infection prevalence (*P*)						
Nymphs	20%	20%	13%	17%	14%	13%
n	100	101	101	101	106	103
Females	(0%)	7%	(25%)	19%	(0%)	8%
n	1	99	4	100	17	77
Females + males	(0%)	10%	17%	23%	9%	11%
n	3	197	12	201	58	177
Intense infection prevalence (*P* for > 100 borreliae/tick; *n* as above)						
Nymphs	8%	5%	4%	2%	2%	5%
Adults	(0%)	4%	(0%)	2%	5%	2%
Encounter time for infected ticks (*IET*, min)						
Nymphs	15	4	14	5	16	15
Females	(1667)	61	96	3	77	40
Females + males	(659)	24	56	10	34	12

*TF, cleared part of the forest; UF, uncleared forest trail (control). Percentage values in parentheses are approximate only (low numbers); the IET values in parentheses have been based on the P-values on the UF trail of that year.

The difference in the tick frequency between the cleared and uncleared trail was thus very low already in the third year after clearing, due to natural succession.

Prevalence of infection of ticks with borreliae

The prevalence of infected nymphal ticks on the plot ranged from 12.6% to 20.0% (Table 1 gives rounded values) and did not differ significantly between the TF and UF transects in the three years 2003 ($\chi^2 = 0.01$; $P = 0.97$), 2004 ($\chi^2 = 0.63$; $P = 0.47$) and 2005 ($\chi^2 = 0.11$; $P = 0.75$). The interannual differences in the prevalence of nymphal infection among the three years were also insignificant for both the cleared trail (2003 vs. 2004: $\chi^2 = 1.86$, $P = 0.17$; 2004 vs. 2005: $\chi^2 = 0.07$, $P = 0.79$; 2003 vs. 2005: $\chi^2 = 1.25$, $P = 0.26$) and control UF trail (2003 vs. 2004: $\chi^2 = 0.30$, $P = 0.59$; 2004 vs. 2005: $\chi^2 = 0.72$, $P = 0.40$; 2003 vs. 2005: $\chi^2 = 1.94$, $P = 0.16$).

Prevalence of infection in adult ticks varied from 8.6% to 23.4%, and it was significantly higher on UF in the year 2004 compared to the other two years, 2003 ($\chi^2 = 12.44$; $P = 0.0004$) and 2005 ($\chi^2 = 10.45$; $P = 0.001$), whereas there was no significant difference between the pair 2003 and 2005 ($\chi^2 = 0.03$; $P = 0.85$). Comparisons of prevalence of infection between the TF and UF trails in adult ticks was impossible to carry out in 2003 because of very low numbers of these ticks caught on the cleared transect; insignificant differences were revealed in the two other years: 2004 ($\chi^2 = 0.29$; $P = 0.59$), and 2005 ($\chi^2 = 0.21$; $P = 0.64$).

An average of 1.9% to 8.0% nymphal and 2.0% to 5.2% adult ticks contained > 100 borreliae (Table 1). The prevalence of these highly infected nymphal ticks did not differ significantly between the cleared and control trail in any year (2003: $\chi^2 = 0.77$, $P = 0.38$; 2004: $\chi^2 = 0.69$, $P = 0.41$; 2005: $\chi^2 = 1.42$, $P = 0.23$). It was irrelevant to evaluate the differences for adult ticks because of their too low numbers collected on the TF trail in 2003 and 2004 (Table 1); in 2005, the difference between TF and UF was insignificant ($\chi^2 = 1.28$; $P = 0.26$). Inter-annual fluctuations in nymphal prevalence of the intense infection (> 100 borreliae per tick) on the UF trail proved to be insignificant for all pairs 2003 vs. 2004 ($\chi^2 = 1.33$; $P = 0.25$), 2004 vs. 2005 ($\chi^2 = 1.27$; $P = 0.26$) and 2003 vs. 2005 ($\chi^2 = 0.01$; $P = 0.97$). Analogical comparison of highly infected nymphs on the TF trail revealed insignificant differences for the pairs 2003 vs. 2004 ($\chi^2 = 1.46$; $P = 0.23$), 2004 vs. 2005 ($\chi^2 = 0.79$; $P = 0.37$), whereas it was significant for the pair 2003 vs. 2005 ($\chi^2 = 4.16$; $P = 0.04$; confirmed with Fisher exact 2×2 test: $P = 0.041$). For highly infected adults on the UF trail, all differences between years were insignificant: 2003 vs. 2004 ($\chi^2 = 0.90$; $P = 0.34$), 2004 vs. 2005 ($\chi^2 = 0.03$; $P = 0.86$) and 2003 vs. 2005 ($\chi^2 = 0.55$; $P = 0.46$).

The frequency of infected nymphal ticks (F_i) was lower on TF transect than on the control transect 3.4 times (by 71%), 2.5 times (by 59%) and 1.1 times (by 9%) in 2003, 2004 and 2005, respectively. Similarly, the frequency of infected adult ticks was lower on TF vs. UF about 27.6 times (by 96%), 5.6 times (by 82%) and 2.7 times (by 63%) in 2003, 2004 and 2005, respectively (data not shown in

Table 1). The frequency of nymphal and adult ticks infected with > 100 borreliae was also lower on the cleared transect, but the result is less representative because of generally low numbers of these highly infected ticks.

Encounter time for infected ticks was longer (indicating a potentially lower risk of Lyme borreliosis) on TF than on the control transect: the *IET* values (in minutes) for nymphs on UF vs. TF were 5 vs. 15, 6 vs. 14 and 15 vs. 16 in 2003, 2004 and 2005, respectively. For adult *I. ricinus*, the respective *IET* differences were much higher: 24 vs. 659, 10 vs. 56 and 12 vs. 33, respectively. The *IET* values for ticks with a high spirochaetal load (> 100 borreliae) differed between the two trails 2.1 times, 0.9 times and 3.2 times in 2003, 2004 and 2005, respectively, for nymphs, and 32.6 times, 4.0 times and 1.0 times, respectively, for adults (data not shown in Table 1).

Identification of isolated borreliae

A total of 16 isolation attempts were carried out from individual ticks with a high spirochaetal burden: eight borrelial cultures were obtained, and identified as the genomic species *Borrelia garinii*. Five isolates of *B. garinii* were from nymphal *I. ricinus* collected on the cleared trail, and one each from nymphal, male and female *I. ricinus* collected on the control trail.

Discussion

The general frequency (*F*) of *I. ricinus* in the study area before clearing was described in a previous study (Hubálek *et al.*, 2003; Table 1): the overall average (and range) from 1991 to 2001 was 33.7 (17.8–58.5) nymphs/h and 44.7 (23.5–90.5) adults/h. In May 2002, the frequency of *I. ricinus* was 21.5 nymphs/h and 46.0 adults/h over all four trails (unpublished). These figures are lower than those in the present study for the UF trail, but higher than those for the TF transect. Although we cannot present exact pre-clearing data on the tick frequency from particular trails in that we usually pooled the ticks from all four trails prior to examination, we never observed considerable differences between the two trails (corresponding to TF and UF in the present study) in the number of ticks caught. The average prevalence of borreliae was 16.8% (range, 11.7% to 24.2%) in nymphs and 25.5% (range, 16.8% to 32.0%) in adults from 1991 to 2001; in May 2002, it was 15.6% in nymphs and 23.9% in adults. These figures are compatible with the respective figures for the years 2003–2005 in both the TF and UF trails for nymphs, but slightly higher for adults in 2003 and 2005.

Identification of spirochaetes that were isolated previously from local microscopically positive ticks always resulted in *B. burgdorferi* sensu lato, namely genomic species *B. afzelii*, *B. garinii*, and sporadically also *B. lusitaniae* and *B. burgdorferi* sensu stricto (Hubálek *et al.*, 1998; unpublished data). Therefore the terms 'borreliae' or 'spirochaetes' used in this study are regarded synonymous with *B. burgdorferi* s.l.

It is known that *B. garinii* is largely an 'ornithophilic' genomic species, associated with forest birds and ticks parasitizing them, whereas forest rodents more often harbour *B. afzelii*, a 'rodentophilic' genomic species in Eurasia (Nakao *et al.*, 1994; Hu *et al.*, 1997; Kurtenbach *et al.*, 1998; Humair *et al.*, 1999; Hanincová *et al.*, 2003a,b). The predominance of *B. garinii* in the present study indicates that most of the borreliae could have been introduced to the cleared transect with larval *I. ricinus* parasitizing birds, and less likely by *I. ricinus* parasitizing forest rodents. Conditions for small mammals in the modified habitat were far from optimal, and the rodents largely avoided it (absence of rodent traces on TF compared to their presence on UF), whereas some bird species known to be parasitized by larval *I. ricinus* visited ground on the cleared trail quite often: blackbird, songthrush, robin, chaffinch, great tit, nuthatch and yellowhammer were all observed here in the years 2003–2005.

Controlled forest burning has been considered as another measure of habitat modification to suppress the vector population. For instance, 92% (12.5 times), 53% (2.1 times), and 67% (3 times) decrease in the abundance of adult *I. scapularis* was observed 1, 2 and 3 years, respectively, after controlled burning of woods in Florida (Rogers, 1955). Similarly, 73% (2.7 times) reduction in numbers of adult *I. scapularis* was detected a year after wood burning on Great Island, Massachusetts, U.S.A. (Wilson, 1986). Forest fires in the Far Russian East removed *I. persulcatus* Schulze taiga ticks and reduced their abundance (frequency) for up to 3 years – e.g. still 4.3 times vs. unburned plots in the second year (Gorelova & Kovalevski, 1985; Gorelova, 1987). Following a controlled burning of woodland understory on Shelter Island (New York, U.S.A.), abundance of nymphal *I. scapularis* decreased twice 2 months later, but the risk of encountering nymphs infected with *B. burgdorferi* s.s. remained surprisingly the same as in the unburned wood (Mather *et al.*, 1993). Marked effects of controlled burning on nymphal (74% to 97% reduction in abundance) and especially larval *I. scapularis* were confirmed in a Connecticut study (Stafford *et al.*, 1998). However, the woodland burning technique would not be feasible in most countries, especially in residential and suburban areas.

The habitat modification followed in this study, i.e. the nearly complete (except for taller trees) forest clearing, did not affect significantly the prevalence of infection with borreliae in *I. ricinus* ticks. However, it reduced considerably the abundance (frequency, density) of *I. ricinus*, largely in the first and second year after the clearing and, in turn, infective encounter time. The potential risk of Lyme borreliosis was therefore reduced for the two years but already not in the third year after the habitat modification and ongoing natural succession of vegetation, compared to the nearby control, uncleared forest trail.

The adverse effect of the habitat modification was much more pronounced on adult than on nymphal ticks. This is

understandable, in that female *I. ricinus* need a higher vegetation layer (usually 20–80 cm) than nymphs to seek the host. The study of succession of ground herb/grass stratum revealed its considerable spatial spread (from *c.* 10% to *c.* 75% cover between the first and third year after clearing) combined with an increasing species diversity, most probably improving the life conditions for the host-seeking adult ticks. Nevertheless, questing *I. ricinus* nymphs, and not females, are the principal vector stage of Lyme borreliosis in Europe (Hubálek *et al.*, 1991, 2003; Matuschka *et al.*, 1992), and therefore a much better quantitative marker of the potential risk for Lyme borreliosis.

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Appendix

Plant succession on the cleared forest trail (TF)

2004

Herb species:

Alliaria officinalis Andrz., *Anthriscus sylvestris* (L) Hoffm., *Arctium lappa* L., *A.minus* (Hill) Bernh., *Campanula patula* L., *Centaurea jacea* L., *Cerastium holosteoides* Fries, *Chaerophyllum temulum* L., *Epilobium parviflorum* Schreb., *Galeopsis pubescens* Bess., *Galium aparine* L., *G.mollugo* L., *Geranium robertianum* L., *Geum urbanum* L., *Hypericum hirsutum* L., *Impatiens parviflora* DC., *Ranunculus acris* L., *Rubus caesius* L., *Rumex conglomeratus* Murr., *Scrophularia nodosa* L., *Stachys silvatica* L., *Stellaria media* (L) Vill., *Symphytum officinale* L., *Urtica dioica* L., *Veronica anagalis-aquatica* L.

Grass species:

Arrhenatherum elatius (L) Presl, *Brachypodium silvaticum* (Huds.) Beauv., *Calamagrostis epigeios* (L) Roth, *Carex remota* (L) Grufb., *Dactylis glomerata* L., *Deschampsia caespitosa* (L) Beauv., *Festuca gigantea* (L) Vill., *F.pratensis* Huds., *Juncus conglomeratus* L., *J.effusus* L., *Luzula nemorosa* (Poll.) E.Mey., *Melica uniflora* Retz., *Milium effusum* L., *Poa annua* L., *P.nemoralis* L., *Roegneria canina* (L) Nevski.

2005

Newly occurring herb species:

Achillea millefolium L., *Artemisia vulgaris* L., *Aster lanceolatus* Willd., *Atriplex patula* L., *Ballota nigra* L., *Bidens frondosa* L., *Carduus acanthoides* L., *Conyza canadensis* (L) Cronq., *Eupatorium cannabinum* L., *Fallopia convolvulus* (L) A.Löve, *Ficaria verna* Huds., *Galeopsis tetrahit* L., *Glechoma hederacea* L., *Humulus lupulus* L., *Hypericum perforatum* L., *Inula britannica* L., *Lamium album* L., *L.maculatum* L., *L.purpurem* L., *Lapsana communis* L., *Leontodon hispidus* L., *Lychnis flos-cuculi* L., *Lysimachia nummularia* L., *L.vulgaris* L., *Mentha aquatica* L., *Ornithogalum gussonei* Ten., *Persicaria hydropiper* (L) Spach, *Plantago major* L., *Potentilla reptans* L., *Primula veris* L. em. Huds., *Prunella vulgaris* L., *Pulmonaria officinalis* L., *Ranunculus repens* L., *Rumex obtusifolius* L., *Silene* sp., *Solanum nigrum* L., *Taraxacum officinale* Web., *Torilis japonica* (Hout.) DC., *Trifolium ochroleucum* Huds., *T.pratense* L., *T.repens* L., *Veronica hederifolia* L., *Viola odorata* L., *V.riviniana* Rchb.

Newly occurring grass species:

Agrostis stolonifera L., *Carex hirta* L., *Juncus tenuis* Willd., *Lolium perenne* L., *Poa pratensis* L., *Scirpus sylvaticus* L.

PRÁCE 4

Šikutová S., **Rudolf I.**, Golovchenko M., Rudenko N., Grubhoffer L., Hubálek Z. 2007. Detection of *Anaplasma* DNA in *Ixodes ricinus* ticks: pitfalls. *Folia Parasitol.* 54: 310–312.

Stručná charakteristika: rickettsie *Anaplasma phagocytophilum*, původce humánní anaplazmózy se dnes řadí vedle *B. burgdorferi* a viru středoevropské klíšťové encefalitidy mezi další emergentní patogeny přenášené klíšťaty ve středoevropském regionu. Rickettsie *A. phagocytophilum* se v přírodě vyskytuje v řadě genetických variant, z nichž pouze některé jsou patogenní pro člověka. V práci jsme se pomocí 'diagnostických' primerů pokoušeli stanovit prevalenci *A. phagocytophilum* v nenasátých klíšťatech *I. ricinus*.

Hlavní přínos práce: práce naznačuje metodický problém, který se ve své době mnoho vědců bálo vyslovit či šířeji komentovat. Šlo o průkopnickou studii, která naznačovala možná rizika při molekulárních detekcích patogenních anaplasem v klíšťatech a diskrepancích při stanovování prevalencí tohoto patogena jinde v Evropě – tj. jednotlivé molekulární záchyty *A. phagocytophilum* v klíšťatech je nutné dále sekvenovat (nejlépe verifikovat více lokusů) či jinak typizovat, aby byla odlišena patogenní varianta *A. phagocytophilum* od nepatogenních variant, případně jiných podobných rickettsií ('*Candidatus* Neoherlichia mikurensis', *Ehrlichia walkerii*). Tím by došlo ke zpřesnění prevalenčních údajů a skutečnému vyhodnocení rizika nákazy humánní anaplazmózou v různých lokalitách.

Příspěvek autora k dané práci: autor se podílel na sběru klíšťat v terénu, jejich zpracování v laboratoři včetně molekulární analýzy, konečném vyhodnocení výsledků včetně přípravy rukopisu.

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RESEARCH NOTE

DETECTION OF *ANAPLASMA* DNA IN *IXODES RICINUS* TICKS: PITFALLS

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Abstract. A total of 150 nymphal *Ixodes ricinus* (L., 1758) (Acari: Ixodidae) from the Czech Republic were examined for *Anaplasma phagocytophilum* (Foggie, 1949) Dumler et al., 2001 by PCR using EHR521/747 primers: 22 of 50 pools were positive (minimum prevalence, 14.7%). However, sequencing of the PCR products did not show complete homology with *A. phagocytophilum* (91%) while the closest relationship (95%) was found to "*Candidatus* Ehrlichia walkerii". The results indicate a need for care in interpretation of *Anaplasma* PCR results and for PCR optimization for detecting *A. phagocytophilum* in ticks.

The causative agent of human granulocytic anaplasmosis (HGA) (formerly called human granulocytic ehrlichiosis, HGE) is *Anaplasma phagocytophilum* (Foggie, 1949) Dumler et al., 2001 (formerly called *Ehrlichia phagocytophila*) (Anaplasmataceae, Rickettsiales), a gramnegative obligate intracellular bacterium with tropism to leukocytes in the vertebrate host. This is an emerging zoonotic disease transmitted by ixodid ticks and first described in the USA, where several hundred cases have been reported since 1994 (Bakken and Dumler 2006). A limited number of laboratory-confirmed cases of human anaplasmosis due to *A. phagocytophilum* have been reported from countries in Europe, including Austria, Italy, Latvia, the Netherlands, Norway, Poland, Czech Republic, Slovenia, Spain, and Sweden (Bakken and Dumler 2006), and the common tick *Ixodes ricinus* (L.) has been identified as the principal vector of this rickettsial agent in Europe (Parola and Raoult 2001). In Europe, prevalence of *A. phagocytophilum* in *I. ricinus* differs considerably according to various authors (Table 1).

The purpose of this study was to assess prevalence of *A. phagocytophilum* in nymphal *I. ricinus* ticks in an area of South Moravia (Czech Republic) where Lyme borreliosis is endemic (Hubálek et al. 2003). Host-seeking nymphal *I. ricinus* were collected by flagging low vegetation during September 2003. All tick specimens were frozen at –60°C until examination. Immediately before DNA isolation, nymphs were surface-sterilized with 70% ethanol (PCR quality), then pooled (3 nymphs per pool) and mechanically disrupted using a sterile glass microblender. The total genomic DNA was extracted with QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR detection of *A. phagocytophilum* was performed as described previously including primers EHR521 (5'-TGT AGG GGG TTC GGT AAG TTA AAG-3') and EHR747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') which amplify a 247 bp partial sequence of *A. phagocytophilum* 16S rRNA gene (Pancholi et al. 1995). Each reaction tube contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.001% Tween 20, 2.5 mM $MgCl_2$, 200 mM mixture of dNTPs, 2.5 U Taq purple DNA polymerase and 25 pmol of each primer. PCR technique was performed in a PTC-200 Gradient Thermal Cycler (MJ Research, USA) under the following conditions: 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 1 min of extension at 72°C consisting of 40 cycles. The PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualised under UV light. DNA extraction and PCR handling were done separately in two rooms to avoid possible cross-contamination of the samples. Specific PCR products were further characterized by sequence analysis. DNA fragments were precisely excised from the gel and purified with the Gel Extraction Kit (Qiagen, Hilden, Germany). The nucleotide sequences were determined by direct sequencing of PCR products. To ensure the specificity, the PCR products were sequenced twice in both directions using EHR521 and EHR747 primers. CEQ 2000 Dye terminator Cycle sequencing Kit was used, sequences were analysed on the ABI Prism 877 ITC automated DNA sequencer (Beckman Coulter, USA) using DNASTAR software (DNASTAR, London, UK), and compared with those in the GenBank. BLAST programs of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches.

A total of 150 nymphal *I. ricinus* in 50 pools were screened. Specific products of *A. phagocytophilum* were detected in 22 pools, which gives a minimum prevalence of 14.7%. Randomly selected PCR products from positive specimens were subjected to sequence analysis for confirmation and compared with sequences deposited in the GenBank database. Surprisingly, all sequences demonstrated only 91% nucleotide identity with the *A. phagocytophilum* AF481855.1, which was detected in cervids in Slovenia (Petrovec et al. 2002). The highest homology (95% nucleotide identity) was shown to a new "*Candidatus* Ehrlichia walkerii" (AY098730.1), which was detected in *I. ricinus* removed from asymptomatic patients in Belluno, Italy (Brouqui et al. 2003, Sanogo et al. 2003), followed by *Ehrlichia*-like sp. "Schotti variant" (AF104680; 95% nucleotide identity) and "*Candidatus* Neoehrlichia mikurensis" (AB074460.1; 95% nucleotide identity). Furthermore, another sequencing of 16S rRNA gene has confirmed the first results.

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Table 1. Minimum prevalence of rickettsiae declared as *Anaplasma phagocytophilum* in *Ixodes ricinus* in Europe according to various authors.

Country	Nymphs	Adults	Total[a]
Finland (Makinen et al. 2003)	0/111 (**0.0**)[b]	0/343 (**0.0**)	0/454 (**0.0**)
Norway (Jenkins et al. 2001)	1/185 (**0.5**)	2/156 (**1.3**)	3/341 (**0.9**)
Denmark (Skarphédinsson et al. 2007)	10/69 **(14.5)**	15/37 **(14.6)**	25/106 **(23.6)**
Estonia (Makinen et al. 2003)	–	3/100 (**3.0**)	3/100 (**3.0**)
Poland (Grzeszczuk et al. 2002)	1/74 (**1.4**)	59/302 (**19.5**)	60/376 (**16.0**)
Austria (Sixl et al. 2003)	–	12/235 (**5.1**)	12/235 (**5.1**)
Czech Republic (Hulínská et al. 2002)	–	–	2/90 (**2.2**)
Slovakia (Derdáková et al. 2003)	0/20 **(0.0)**	5/40 (**12.5**)	5/60 (**8.3**)
Hungary (Srétér et al. 2004)	–	6/452 (**1.3**)	6/452 (**1.3**)
Slovenia (Petrovec et al. 1999)	–	3/93 (**3.2**)	3/93 (**3.2**)
Republic of Moldova (Koči et al. 2007)	–	–	18/198 **(9.1)**
Italy (Mantelli et al. 2006)	100/1014 (**9.9**)	–	100/1014 (**9.9**)
United Kingdom (Ogden et al. 1998)	5/135 (**3.7**)	5/114 (**4.4**)	10/249 (**4.0**)
The Netherlands (Wielinga et al. 2006)	–	–	4/1580 (**0.3**)
France (Ferquel et al. 2006)	4/1065 **(0.4)**	2/171 (**1.2**)	6/1236 (**0.5**)
Switzerland (Pusterla et al. 1999)	3/575 (**0.5**)	18/1092 (**1.6**)	21/1667 (**1.3**)
Germany (Baumgarten et al. 1999)	–	6/275 (**2.2**)	6/275 (**2.2**)
Baltic Region (Russia) (Alekseev et al. 2001)	–	–	3/295 (**1.0**)
Bulgaria (Christova et al. 2003)	10/42 (**23.8**)	56/185 (**30.3**)	66/227 (**29.1**)
Portugal (Santos et al. 2004)	6/142 (**4.2**)	–	6/142 (**4.2**)
Spain (Oteo et al. 2000)	?/? **(24.1)**	–	?/? **(24.1)**

[a]nymphs and adults, total; [b]no. positive/no. examined (% positive) individuals.

These findings indicate potential difficulties in molecular detection of the HGA agent in ixodid ticks, when the primer pair EHR521 and EHR747 is used. According to a comparative study (Massung and Slater 2003), the primers EHR521 and EHR747 were found to be highly sensitive, but with a poor specificity, since they detected in addition to *A. phagocytophilum* also *Rickettsia rickettsii*, *Bartonella henselae*, *Ehrlichia chaffeensis*, and probably other rickettsial endosymbionts of ticks. Moreover, Massung et al. (2003) found a non-pathogenic (in mouse model) variant "Ap-1" of *A. phagocytophilum* occurring more often (about 10 times) than the pathogenic variant "Ap-ha"; at the same time, genetic difference between both variants was found to be negligible (only two nucleotides) in 16S rRNA gene sequence. Furthermore, sequence of a non-pathogenic variant of *A. phagocytophilum* was amplified from *I. ricinus* ticks collected in Spain (Portillo et al. 2005). Recent data suggest that Ap-1 is restricted to ruminant species and represents a lineage distinct from Ap-ha, which infects humans and numerous other mammals (Massung et al. 2006).

Our results, in accord with those of Shukla et al. (2003), emphasize the importance to sequence rickettsial PCR products for confirmation of their specificity. A very high prevalence of *A. phagocytophilum* in *I. ricinus* ticks in Europe was reported in, e.g., Bulgaria (Christova et al. 2003), Denmark (Skarphédinsson et al. 2007), Poland (Grzeszczuk et al. 2002), Slovakia (Derdáková et al. 2003) or Spain (Oteo et al. 2000). Some of these figures might have been over-estimated due to missing confirmation of PCR products by sequencing (Shukla et al. 2003). The relatively very low incidence of reported clinical cases of HGA (much lower than that of Lyme borreliosis) in Europe should be a reflection of the lower actual prevalence of the human pathogenic variant of *A. phagocytophilum* (compared to *Borrelia burgdorferi* sensu lato) in *Ixodes ricinus* ticks, but this contrasts with some data reported (Table 1). Our findings as well as reports of other authors (Massung et al. 2003, Shukla et al. 2003) indicate clearly that a high caution is necessary for correct interpretation of the PCR-based results of *A. phagocytophilum* presence in ixodid ticks.

In conclusion, standardisation of molecular detection of the HGA agent seems to be desirable. Most importantly, a novel, more specific primer pair, which would differentiate the human-pathogenic variant of *A. phagocytophilum* from the non-pathogenic one by PCR, is highly needed.

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PRÁCE 5

Jarošová V., **Rudolf I.**, Halouzka J., Hubálek Z. 2009. *Borrelia burgdorferi* s.l. v klíšťatech na ostravských haldách. 58: 90-97. *Epidemiol. Mikrobiol. Imunol.*58: 90–97.

Stručná charakteristika: práce se zabývá frekvencí klíšťat *I. ricinus* (nymf a dospělců) včetně zjištění prevalence *B. burgdorferi* na 2 ostravských haldách hlušinách (částečně porostlých vegetací) vzniklých po těžbě černého uhlí a jedné kontrolní (lesní) lokalitě. Překvapivě bylo zjištěno, že ostravské haldy hlušiny, pokud jsou porostlé vegetací a navštěvovány lidmi, představují stejné potenciální riziko nákazy lymskou borreliózou jako běžné lesní biotopy.

Hlavní přínos práce: další ojedinělá eko-epidemiologická studie zkoumající vliv antropogenních vlivů (těžba uhlí a následná rekultivace krajiny) na výskyt klíšťat a jejich promořenost borreliemi.

Příspěvek autora k dané práci: autor se podílel na molekulárních analýzách (PCR-RFLP identifikace borrelií) a přípravě rukopisu.

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Borrelia burgdorferi s.l. v klíšťatech na ostravských haldách

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Souhrn

V letech 2005 a 2006 byly prováděny periodické sběry klíštěte obecného *Ixodes ricinus* na ostravských haldách Oskar (A) a Emma (B), částečně porostlých vegetací včetně dřevin, a na kontrolní lesní lokalitě (C) u nedalekého města Hlučín. Průměrná frekvence klíšťat (počet nasbíraných klíšťat za hodinu vlajkování) byla vysoká, na haldě A 35,3 nymf a 12,7 dospělců, na haldě B 23,3 nymf a 26,0 dospělců, a na lokalitě C 25,4 nymf a 16,8 dospělců. Mikroskopií v zástinu bylo na přítomnost borrelií *Borrelia burgdorferi* sensu lato vyšetřeno z každé lokality 100 nymf a 100 dospělců (50 samic a 50 samců). Průměrná prevalence *B. burgdorferi* s.l. v klíšťatech byla na haldě A 10,0 % u nymf a 12,0 % u dospělců, na haldě B 10,0 % u nymf a 24,0 % u dospělců, a na lokalitě C byl tento podíl 13,0 % u nymf a 17,0 % u dospělců. Rozdíly v celkové prevalenci borrelií v klíšťatech mezi haldami A nebo B a kontrolní plochou bez ohledu na stadium klíštěte nebyly statisticky průkazné, i když dospělá klíšťata z haldy B obsahovala borrelie signifikantně četněji než dospělci z haldy A. Lokality se mezi sebou lišily ve frekvenci průměrného počtu klíšťat infikovaných borreliemi za hodinu sběru: na haldě A to bylo 3,3 nymf a 1,2 dospělců, na haldě B 1,5 nymf a 2,9 dospělců, a na kontrolní lokalitě 3,1 nymf a 2,6 dospělců. Izolační pokusy byly provedeny u 16 klíšťat s přítomností většího počtu spirochét, a u 8 z nich se podařilo vykultivovat borrelie, které byly pomocí PCR-RFLP identifikovány jako *B. garinii* (3 izoláty: 1 Hlučín; 2 halda B), *B. afzelii* (4 izoláty: 1 halda A; 3 halda B) a *B. burgdorferi* s.s. (1 izolát halda A). Výsledky poněkud překvapivě naznačují, že ostravské haldy hlušiny, pokud jsou porostlé vegetací včetně dřevin a navštěvovány lidmi, mohou teoreticky představovat přibližně stejné potenciální riziko nákazy člověka lymskou borreliózou jako běžné lesní biotopy.

Klíčová slova: *Ixodes ricinus* – výsypky hlušiny – lymská borrelióza – riziko přenosu.

Summary

Jarošová V., Rudolf I., Halouzka J., Hubálek Z.: *Borrelia burgdorferi* sensu lato in Ixodid Ticks from Ostrava Slag Heaps

In 2005 and 2006, *Ixodes ricinus* ticks were collected on two slag (waste rock) heaps from coal mines in the Ostrava area (North Moravia/Silesia, Czech Republic), Oskar (site A) and Emma (site B), partially covered by vegetation including trees, and at a control forest site near Hlučín (site C). The mean numbers of *I. ricinus* nymphs and imagoes flagged per person-hour were high: 35.3 nymphs and 12.7 imagoes, at site A, 23.3 and 26.0, respectively, at site B, and 25.4 and 16.8, respectively, at control site C. Using dark-field microscopy, 100 nymphs and 100 imagoes (50 females and 50 males) from each site were examined for borreliae. The mean prevalence rates of *Borrelia burgdorferi* sensu lato in nymphs and imagoes were 10.0 % and 12.0 %, respectively, at site A, 10.0 % and 24.0 %, respectively, at site B, and 13.0 % and 17.0 %, respectively, at site C. Differences in the prevalence of borreliae in nymphal and adult ticks from the slag heaps and control site were insignificant, but adult ticks from site B compared to site A contained borreliae significantly more frequently. The mean numbers of nymphs and imagoes infected with borreliae flagged per person-hour were 3.3 and 1.2, respectively at site A, 1.5 and 2.9, respectively, at site B, and 3.1 and 2.6, respectively, at site C. Isolation experiments for borreliae were carried out only in 16 ticks containing higher numbers of borreliae, with eight of these being culture-positive. The cultured borreliae were identified by PCR-RFLP as *B. garinii* (3 isolates: two from site B, one from site C), *B. afzelii* (4 isolates: one from site A, three from site B) and *B. burgdorferi* s.s. (one isolate from site A). Surprisingly, the results suggest that slag heaps, when covered by woody vegetation and frequented by humans, could theoretically pose roughly the same LB transmission risk to humans as common forest biotopes.

Key words: *Ixodes ricinus* – slag heaps – Lyme borreliosis – transmission risk.

Lymská borrelióza (LB), jejímž původcem je *Borrelia burgdorferi* sensu lato [2], je jednou z nejvýznamnějších a nejhojnějších zoonóz holarktické oblasti [6, 28, 30, 31]. Komplex *B. burgdorferi* s.l. zahrnuje t.č. minimálně 12 genomických druhů (genomospecies, nomenklatoricky nepřesně zvaných genospecies), které se částečně liší také svou ekologií, epidemiologií, mírou patogenity i klinickým obrazem vyvolávané nemoci. Pro člověka patogenní a LB prokazatelně vyvolávající jsou především *B. burgdorferi* s.s., *B. afzelii* a *B. garinii*, ojediněle jsou však popisovány také případy LB vyvolané *B. valaisiana* a *B. lusitaniae* [3, 27].

V České republice byly případy LB člověka laboratorně (sérologicky) potvrzeny poprvé ve druhé polovině 80. let [4, 21], a borrelie přítomné v tkáních pacientů byly u nás pozorovány nedlouho poté [15, 29]. V tomto období byla také v Česku *B. burgdorferi* s.l. poprvé mikroskopicky detekována v klíšťatech *Ixodes ricinus* [20] a následně z klíšťat izolována [14]. LB je u nás nejhojnější klíšťaty přenosnou zoonózou: během let 1993–2007 bylo v ČR hlášeno průměrně 3680 (rozsah 2138–6302) LB případů ročně [18, EpiDat – Státní zdravotní ústav Praha]. V roce 1995 bylo zaznamenáno rekordních 6302 případů onemocnění, což podle epidemiologů souviselo s nadnormálním výskytem klíšťat. Signifikantní vztah mezi početností nymf klíštěte obecného a incidencí LB byl prokázán např. na jižní Moravě [12].

Ekosystém, v němž se vektor LB – klíště komplexu *Ixodes ricinus* – převážně vyskytuje, jsou listnaté a smíšené lesy mírného klimatického pásu. Popsán je ovšem také častý výskyt borrelií v klíšťatech evropských městských parků [1, 7, 10, 19, 22–24]. Doposud však nebyla věnována dostatečná pozornost možnosti výskytu klíšťat infikovaných borreliemi v tak vyhraněně antropogenním biotopu, jaký představují odvaly karbonské hlušiny z černouhelných dolů, které se nacházejí např. na Ostravsku. Mnohé z těchto ostravských hald byly sukcesně osidlovány vegetací, a postupně se na nich objevovaly také některé druhy endotermních obratlovců, potenciálních hostitelů klíšťat. Proto jsme se domnívali, že by se na haldách mohla vyskytovat i klíšťata, případně nakažená borreliemi. Klíště *I. ricinus* bylo ostatně trvale nalézáno na výsypkách hnědouhelných dolů na Mostecku zhruba 10 let po jejich rekultivaci [26].

Materiál a metody

Charakteristika lokalit

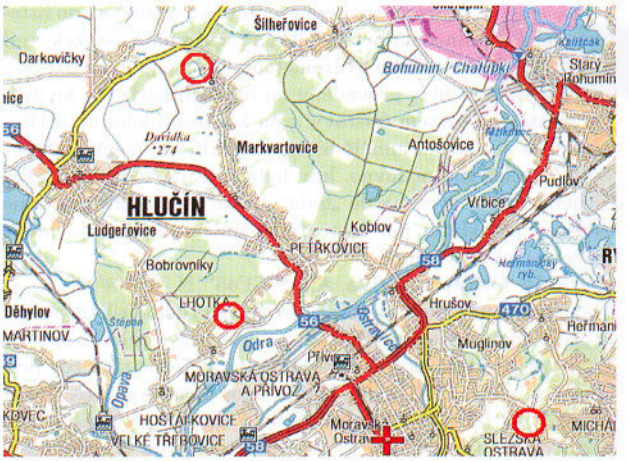
Všechny 3 sledované lokality se nacházejí na území Moravskoslezského kraje (obr. 1). V období 1961-1990 byl na tomto území průměrný roční souhrn srážek 677 mm, průměrná teplota vzduchu 8,6 °C, v měsících březen až listopad 11,7 °C. V roce 2005 byla průměrná teplota vzduchu od března do listopadu mírně nadnormální (12,4 °C), a roční souhrn srážek činil 699 mm (údaje Českého hydrometeorologického ústavu).

Sběry klíšťat byly prováděny na haldách patřících ke dvěma městským částem Ostravy, a na kontrolní lokalitě u Hlučína. Oba studované umělé biotopy odvalů na území Ostravy vznikly relativně nedávno, kdy byla hlušina (odpad z těžby černého uhlí) navezena do „hald" a tyto haldy pak postupně osídleny přírodním náletem dřevin anebo dřevinami uměle osazeny (rekultivovány).

Halda Oskar (lokalita A: 49°51'45" N, 18°14'10" E) je výsypkou nepravidelného tvaru z bývalého dolu Oskar (později přejmenovaného na důl Lidice), který byl založen v roce 1891 u západního okraje obce Petřkovice. Těžba uhlí zde probíhala v letech 1896–1967. Halda není termicky aktivní, má rozlohu 2,2 ha a objem hlušiny >1,5 mil. m^3. Výsypka byla rekultivována, vegetační kryt je přesto tvořen z velké části přírodním náletem dřevin (obr. 2). V druhové skladbě převládají ve stromovém patru bříza bradavičnatá, dub zimní a cer, javor klen, lípa malolistá, olše lepkavá; v keřovém patru bez černý, líska. Bylinné patro je zastoupeno např. druhy hluchavka bílá, kopřiva dvoudomá, sasanka hajní, jahodník obecný. Zdejší faunu obratlovců – hostitelů klíšťat – tvoří hraboš polní, norník rudý, myšice křovinná a lesní, rejsek obecný, ježek východní, zajíc, liška, prase divoké, srnec; z ptáků bažant obecný, kukačka, drozd zpěvný, kos, červenka, strnad obecný. Z klíšťat jsme prokázali pouze druh klíště obecné (*Ixodes ricinus*).

Halda Emma (lokalita B, halda označována také jako Terezie, Terezie-Emma či Emma-Terezie: 49°50'23" N 18°18'54" E) je bývalou výsypkou dolu Terezie (přejmenovaného na důl Petr Bezruč) a dolu Svaté Trojice, a nachází se v oblasti na pravém břehu řeky. Oba doly byly založeny ve 40. letech 19. století, byly posléze sloučeny, a těžba v dolu Petr Bezruč probíhala až do roku 1992. Haldu o rozloze asi 34 ha tvoří >4 mil. m^3 slehávající hlušiny. Tato více než 150 let stará halda je termicky aktivní, vyvěrají z ní bělostné obláčky plynů, obsahující zejména oxid siřičitý. Díky tomu se také na vrcholu odvalu nikdy neudrží sníh. Výsypka byla rekultivována: na severní straně je hustý les; jižní svah, který stále prohořívá, je porostlý řídčeji náletovými dřevinami (obr. 3). Halda je protkána několika značenými stezkami, a slouží dokonce jako výletní místo pro obyvatelstvo. V druhové skladbě převládají ve stromovém patru bříza bradavičnatá, dub zimní a cer, javor mléč, jasan ztepilý, jeřáb obecný; v keřovém patru bez černý, líska, dřín, lípa malolistá, svída. V bylinném patru jsou druhy kopřiva dvoudomá, svízel přítula, netýkavka malokvětá, starček obecný, kapraď samec a mnoho dalších. Ze savců se zde vyskytuje veverka, hraboš polní, myšice křovinná a lesní, rejsek obecný, ježek východní, zajíc; z ptáků např. bažant, kos, drozd zpěvný, červenka a strnad obecný. Z klíšťat jsme na lokalitě zjistili pouze klíště obecné.

Hlučín – porost Štípky (lokalita C: 49°55'25" N, 18°13'45" E; 286 m n.m.). Les Štípky, který jsme zvolili za kontrolní lokalitu, se nachází asi 2 km od předměstí Darkovičky, a je součástí bažantnice v Šilheřovicích. V druhové skladbě převládají ve stromovém patru dub letní, zimní a cer, javor klen a mléč, buk, bříza bradavičnatá, habr, olše lepkavá, smrk, jeřáb obecný; v keřovém patru pak bez černý, líska, ostružiník křovitý a maliník. V bylinném patru jsou zastoupeny druhy hluchavka bílá, kopřiva dvoudomá, sasanka hajní, netýkavka malokvětá, tuřice třeslicovitá, kapraď samec a mnoho dalších. Ze savců se běžně vyskytují hraboš polní, norník rudý, myšice křovinná, lesní a temnopásá, veverka, rejsek obecný, krtek, ježek východní, zajíc, liška, prase divoké, srnec. Pohybuje se zde velké množství bažantů a mnohé další druhy ptáků např. kukačka, kos, drozd zpěvný, červenka, střízlík, brhlík, sojka, straka, strnad obecný. Z klíšťat se zde nachází pouze druh klíště obecné.



Obr. 1. Mapka Ostravska (http://supermapy.centrum.cz) se třemi kroužky vyznačujícími lokality sběru (od severu k jihu studijní plochy Hlučín, halda A, a halda B)

Fig. 1. Map of the Ostrava area (http://supermapy.centrum.cz) with three encircled tick collection sites (from North to South: Hlučín, control site C, slag heap Oskar, site A, and slag heap Emma, site B)

Obr. 2. Halda A („Oskar")

Fig. 2. Site A (slag heap Oskar)

Obr. 3. Halda B („Emma"), svahy

Fig. 3. Site B (slag heap Emma), slopes

Obr. 4. Halda B („Emma"), horní partie

Fig. 4. Site B (slag heap Emma), upper part

Sběr klíšťat a jejich vyšetření na borrelie

Sběr. Hladové nymfy a imaga klíštěte obecného *Ixodes ricinus* byly sbírány vlajkováním nízké vegetace pomocí bílé flanelové látky (60x100 cm) od dubna do září v letech 2005 a 2006, a transportovány do laboratoře ve skleněných zkumavkách s korkovými zátkami a několika vloženými listy travin proti vyschnutí. Zkumavky s živými klíšťaty byly v laboratoři uchovávány při 5 °C, a listy travin podle potřeby obměňovány. Sběry klíšťat byly provedeny v 10 termínech: 4krát v roce 2005 a 6krát v roce 2006, všechny od dubna do července; podzimní sběry nebyly uskutečněny vzhledem k nízké početnosti klíšťat. Úhrnem bylo na borrelie mikroskopicky vyšetřeno 600 klíšťat – po 200 kusech (50 samic, 50 samců a 100 nymf) z každé lokality.

Tab. 1. Přehled použitých kontrolních kmenů borrelií s označením fragmentů vzniklých po restrikčním štěpení PCR produktu rrf (5S)–rrl (23S) [25]

Table 1. Control strains of 5 Borrelia species and lengths of restriction fragments of rrf (5S)–rrl (23S) intergenic amplicons [25]

Kmen	Genomický druh	Zdroj	Zeměpisná oblast	Donor	Amplikon	Fragmenty (bp)
B31^T	*B. burgdorferi* s.s.	*I. scapularis*	Shelter Island (N.Y.)	J.F.Anderson	254 bp	108, 51, 38, 29, 28
20047^T	*B. garinii*	*I. ricinus*	Bretaň (Francie)	I. Livey	253 bp	108, 95, 50
VS461^T	*B. afzelii*	*I. ricinus*	Valais (Švýcarsko)	I. Livey	246 bp	108, 68, 50, 20
VS116^T	*B. valaisiana*	*I. ricinus*	Valais (Švýcarsko)	I. Livey	255 bp	175, 50, 23, 7
BR 41	*B. lusitaniae*	*I. ricinus*	Valtice (ČR)	ÚBO AVČR	257 bp	108, 81, 39, 29

^{T} typový kmen příslušného genomického druhu.
^{T} type strain of the respective genomic species.

Mikroskopie. U jednotlivých klíšťat byla na mikroskopickém podložním sklíčku idiosoma oddělena od gnathosomy a končetin pomocí preparačních jehel. Vypreparované střevo bylo rozmělněno a střevní obsah homogenizován v kapce sterilního fyziologického roztoku, překryt krycím sklíčkem a vyšetřen mikroskopií v zástinu při zvětšení 160krát a 400krát. Prohlédnuta byla vždy celá plocha preparátu, a u pozitivních vzorků byly spirochéty spočítány. Spirochéty morfologicky shodné s borreliemi jsou považovány v této studii za *Borrelia burgdorferi* s.l., neboť nálezy spirochét jiných než náležejících ke komplexu *B. burgdorferi* s.l. v nymfách a dospělcích *I. ricinus* jsou ve střední Evropě zcela výjimečné (a tudíž zanedbatelné).

Izolace a kultivace. Byla-li infekce borreliemi podle mikroskopického vyšetření dostatečně intenzivní (>100 spirochét v klíštěti) nebo vykazovaly-li spirochéty výraznou motilitu, bylo přistoupeno k izolačnímu pokusu. Homogenát střeva klíštěte byl z krycího i podložního sklíčka smyt do malé (3 ml) skleněné zkumavky s médiem BSK-H (Sigma, Německo) s přídavkem fosfomycinu (100 μg/ml) a rifampicinu (50 μg/ml). Ve zvláštních případech (při rezistenci kontaminanty) byl použit také sulfametoxazol (50 μg/ml) a trimetoprim (10 μg/ml) (Sigma, USA). Inokuláty byly inkubovány až 4 týdny při 33 °C, průběžně mikroskopicky kontrolovány, a pozitivní vzorky pasážovány do čerstvého kompletního média BSK-H doplněného antibiotiky. Izolované kmeny byly pro uchování zmrazeny v plastových kryozkumavkách (Nunc, Dánsko) v médiu BSK-H s přídavkem 10% dimetylsulfoxidu (Sigma) jako kryoprotektiva při teplotě –60°C, případně i v kapalném dusíku při –196 °C.

Identifikace borrelií (PCR a polymorfismus délky restrikčních fragmentů, RFLP). Spirochetální buňky určené pro izolaci DNA byly kultivovány do logaritmické fáze růstu, zkoncentrovány centrifugací (8500 *g*, 30 min, 4 °C), 2krát promyty sterilním fyziologickým roztokem centrifugací za stejných podmínek, resuspendovány v odpovídajícím množství sterilního fyziologického roztoku (cca 0,5ml), a zmraženy při -20 °C. Bakteriální DNA byla izolována pomocí DNeasy® Tissue Kit (Qiagen, Německo) přesně podle návodu dodávaného výrobcem. Takto připravený vzorek DNA byl uchováván při –20 °C, a použit jako templát pro PCR. Primery byly vybrány tak, aby došlo k amplifikaci variabilního regionu mezi dvěmi konzervovanými strukturami, 3' koncem 5S rRNA (*rrf*) a 5' koncem 23S rRNA (*rrl*). Amplifikované fragmenty jsou u různých kmenů borrelií dlouhé 226 až 266 bp [25]. Byly připraveny vzorky našich izolátů a také typových kmenů borrelií, které byly pasážovány a uchovávány na oddělení medicínské zoologie ÚBO AVČR ve Valticích (tab. 1). V PCR byl použit 2krát koncentrovaný PPP Master Mix (Top-Bio, ČR): 15 mM Tris-HCl (pH 8,8); 40 mM $(NH_4)_2SO_4$; 0,02% Tween 20; 5 mM $MgCl_2$; 400 μM dATP, dCTP, dGTP, dTTP; 100 U/ml Taq purple DNA polymerázy; aditiva, stabilizátory. Reakční směs o celkovém objemu 25 ml se skládala z PPP Master Mix, PCR H_2O, primerů (Invitrogen, USA) o koncentraci 20 pmol, a templátové DNA. Program termocykleru PTC-200 (MJ Research, USA) zahrnoval celkem 30 cyklů s etapami 1 min/94 °C; 1 min/52 °C; a 2 min/72 °C. Izolace DNA, příprava PCR směsi, vlastní amplifikace stejně jako post-PCR kroky probíhaly odděleně (časově i prostorově) kvůli zamezení možné zkřížené kontaminace vzorků.

Amplikon mezerníkového úseku mezi geny *rrf* a *rrl* byl dále štěpen restrikčním enzymem *Mse*I (New England Biolabs, USA), který rozkládá DNA v cílovém místě 5'- T↓TAA- 3'; 3'- AAT↑T- 5' [25]. Po restrikčním štěpení PCR produktu byla provedena elektroforéza vzorků v aparatuře Biorad (Biorad Laboratories, USA) v 3% agarózovém gelu (Invitrogen, USA) při použití 0,5krát TBE pufru (SERVA, Německo) za standardních podmínek (napětí 70 V, 18 mA, 2,5 h). Gel byl barven ethidium bromidem (Top-Bio, ČR), a amplifikovaná DNA byla analyzována pod UV světlem v transluminátoru (Ultra-Cam, USA). Vizualizovaná DNA byla zpracována dokumentačním systémem Ultra-Cam (USA).

Kvantitativní údaje a jejich statistické zpracování

Abundance klíšťat a borrelií byly vyjádřeny indexy:

1) **Frekvence klíšťat (F) –** průměrný počet klíšťat za hodinu sběru vlajkou (neboli „na vlajkohodinu"). Charakterizuje momentální početnost klíšťat na lokalitě.

2) **Prevalence borrelií (P) –** průměrný podíl klíšťat s borreliemi z celkového počtu vyšetřených.

3) **Frekvence pozitivních klíšťat (F_p) –** průměrný počet klíšťat s borreliemi za hodinu vlajkování; $F_p = F \times P$ [13].

Pro hodnocení statistické průkaznosti rozdílů v proporcích (četnosti) podle tabulek 2x2 nebo 2x3 byl použit χ^2 test (program SOLO 4.0, BMDP Statistical Software, California, USA).

Výsledky

Početnost klíšťat

Celková průměrná frekvence klíšťat/h byla na kontrolní lokalitě 25,4 nymf a 16,8 dospělců; na haldě A 35,3 nymf a 12,7 dospělců; a na haldě B 23,3 nymf a 26,0 dospělců (tab. 2). Ze všech 3 lokalit byla nejvyšší frekvence nymf na haldě A při sběru dne 30.4.2005 (62,0), na haldě B 13.5.2005 (43,0), a v Hlučíně 16.4.2006 (rovněž 43,0). Frekvence dospělců na haldě A byla nejvyšší při sbě-

Tab. 2. Frekvence klíšťat (F, počet klíšťat/h), prevalence borrelií v nich (P, počet pozitivních/počet vyšetřených klíšťat), a frekvence pozitivních klíšťat (Fp, počet pozitivních klíšťat/h) na 3 lokalitách Ostravska; nt, netestováno

Table 2. Numbers of flagged ticks per person-hour (F), Borrelia prevalence rates (P = number of Borrelia-positives /number of investigated ticks), and numbers of flagged Borrelia-positive ticks per person-hour (Fp) at 3 sites in the Ostrava area; nt, not tested

	Nymfy			Samice			Samci		
Hlučín	F	P	F_p	F	P	F_p	F	P	F_p
17.4.2005	37,5	4/32	4,69	3	1/6	0,5	1,5	0/3	0
22.4.2005	36	nt	nt	3	1/3	1	5	0/5	0
30.4.2005	31	nt	nt	11	3/9	3,67	12	1/9	1,33
13.5.2005	34	nt	nt	12	nt	nt	13	nt	nt
7.4.2006	7	1/7	1	0	nt	nt	3	0/3	0
16.4.2006	43	5/28	7,68	4	1/4	1	14	0/14	0
28.4.2006	42	2/21	4	10	1/5	2	14	3/7	6
20.5.2006	10	0/8	0	5	2/5	2	13	1/4	3,25
17.6.2006	4	1/4	1	13	3/13	3	17	0/5	0
18.7.2006	9	nt	nt	5	0/5	0	9	nt	nt
Průměr	**25,35**	**13,0%**	**3,06**	**6,60**	**24,0%**	**1,65**	**10,15**	**10,0%**	**1,32**
Halda A	F	P	F_p	F	P	F_p	F	P	F_p
17.4.2005	44,7	0/28	0	0,67	0/1	0	1,33	1/2	0,75
22.4.2005	44	0/4	0	2	0/2	0	3	1/3	1
30.4.2005	62	0/10	0	4	0/4	0	3	0/3	0
13.5.2005	41	nt	nt	10	2/8	2,5	12	nt	nt
7.4.2006	27	3/13	6,23	7	0/7	0	6	0/6	0
16.4.2006	45	3/15	9	5	0/5	0	12	1/12	1
28.4.2006	24	0/8	0	10	0/5	0	12	0/6	0
20.5.2006	35	3/12	8,75	11	2/11	2	10	1/9	1,11
17.6.2006	25	1/10	2,5	4	0/4	0	8	3/8	3
18.7.2006	5	nt	nt	3	1/3	1	3	0/1	0
Průměr	**35,30**	**10,0%**	**3,31**	**5,67**	**10,0%**	**0,55**	**7,03**	**14,0%**	**0,76**
Halda B	F	P	F_p	F	P	F_p	F	P	F_p
17.4.2005	31,3	2/28	2,24	1,33	0/2	0	6	2/9	1,33
22.4.2005	25	nt	nt	14	nt	nt	16	4/14	4,57
30.4.2005	35	nt	nt	13	nt	nt	15	3/13	3,46
13.5.2005	43	nt	nt	19	nt	nt	26	nt	nt
7.4.2006	3	1/3	1	5	1/5	1	11	2/11	2
16.4.2006	18	1/16	1,12	12	5/12	5	14	nt	nt
28.4.2006	28	0/3	0	18	2/9	4	38	nt	nt
20.5.2006	30	6/30	6	11	3/11	3	16	nt	nt
17.6.2006	11	0/11	0	10	2/10	2	9	0/3	0
18.7.2006	9	0/9	0	1	0/1	0	5	nt	nt
Průměr	**23,33**	**20,0%**	**1,48**	**10,43**	**26,0%**	**2,14**	**15,60**	**22,0%**	**2,27**

rech 13.5.2005 a 28.4.2006 (pokaždé 22/h), na haldě B 28.4.2006 (56/h), a v Hlučíně 17.6.2006 (30/h). Sezonalita klíšťat *I. ricinus* byla posouzena pomocí hodnot jejich frekvence na všech 3 lokalitách během měsíců duben–červenec 2006: maximum frekvence nymf bylo na haldě A v dubnu (38/h), na haldě B v květnu (30/h) a na lokalitě C v dubnu (31/h); maxima frekvence dospělců bylo dosaženo na haldě A v květnu (22/h), na haldě B již v dubnu (33/h), a na lokalitě C až v červnu (30/h).

Prevalence borrelií v klíšťatech

Prevalenci ukazuje rovněž tab. 2. Celková průměrná prevalence *B. burgdorferi* s.l. v klíšťatech byla 11,0 % u nymf a 17,7 % u dospělců. Na lokalitě A bylo pozitivních 10,0 % samic, 14,0 % samců a 10,0 % nymf; na B 26,0 % samic, 22,0 % samců, 10,0 % nymf, a na kontrolní lokalitě 24,0 % samic, 10,0 % samců a 13,0 % nymf. Nejvyšší procento pozitivních dospělců, jak samic tak samců, bylo tedy zaznamenáno na haldě B. Pomocí χ^2 tes-

Tab. 3. Kategorie klíšťat podle počtu zjištěných borrelií. Z každé ze 3 lokalit bylo vyšetřeno 100 nymf, 50 samic a 50 samců

Table 3. Distribution of ticks by number of detected borreliae. A total of 100 nymphs, 50 males and 50 females from each of sites A, B and C were investigated. C, Hlučín (control site)

Lokalita	Hlučín			Halda A			Halda B		
Borrelií:	1–9	10–99	≥ 100	1–9	10–99	≥ 100	1–9	10–99	≥ 100
Nymfy	3	8	2	2	7	1	3	7	0
Samice	2	6	4	2	1	2	1	7	5
Samci	1	3	1	1	3	3	2	3	6

Tab. 4. Přehled kmenů borrelií izolovaných z klíšťat *Ixodes ricinus* na Ostravsku

Table 4. Borrelial strains isolated from *Ixodes ricinus* ticks at three sites of the Ostrava area

Izolát	Sex	Lokalita	Datum sběru	Genomický druh
BR V2	M	Hlučín	28.4.2006	*B. garinii*
BR V4	F	Halda B	20.5.2006	*B. afzelii*
BR V5	F	Halda A	20.5.2006	*B. afzelii*
BR V9	M	Halda A	17.6.2006	*B. burgdorferi* s.s.
BR V10	M	Halda B	20.5.2006	*B. garinii*
BR V11	M	Halda B	20.5.2006	*B. afzelii*
BR V12	M	Halda B	28.4.2006	*B. garinii*
BR V14	M	Halda B	28.4.2006	*B. afzeli*

tu nebyl zjištěn průkazný rozdíl v prevalenci borrelií mezi samicemi a samci v úhrnu ($\chi^2 = 1,12$; $P = 0,289$), ani na žádné ze 3 lokalit. Byla však zjištěna průkazně vyšší celková prevalence borrelií u dospělců než u nymf klíšťat ($\chi^2 = 5,43$; $P = 0,020$), která byla velmi výrazná zejména u klíšťat z haldy B ($\chi^2 = 6,94$; $P = 0,008$). Dospělá klíšťata z haldy B obsahovala také borrelie průkazně ($\chi^2 = 4,88$; $P = 0,027$) četněji (24,0%) než dospělci z haldy A (12,0%), ostatní rozdíly v prevalenci mezi lokalitami u imag ani u nymf průkazné nebyly.

Početnost infikovaných klíšťat

Průměrná frekvence klíšťat s borreliemi „za vlajkohodinu" byla na kontrolní lokalitě 3,1 nymf, 2,6 dospělců (1,6 samic a 1,3 samců); na haldě A 3,3 nymf, 1,2 dospělců (0,6 samic, 0,8 samců); a na haldě B 1,5 nymf, 2,9 dospělců (2,1 samic, 2,3 samců). Nejvyšší frekvence pozitivních nymf (tab. 2) byla na haldě A při sběru 16.4.2006 (9,0), na haldě B 20.5.2006 (6,0), a v Hlučíně 16.4.2006 (7,7). Frekvence pozitivních dospělců byla nejvyšší na haldě A při sběru 20.5.2006 (3,1), na haldě B 16.4.2006 (5,0), a v Hlučíně 28.4.2006 (8,0).

Počty borrelií v klíšťatech

Klíšťata byla rozdělena podle počtu zjištěných borrelií do 3 skupin (tab. 3). Nejvíce dospělců obsahujících >100 borrelií bylo nasbíráno na haldě B. Nymf obsahujících >100 borrelií bylo jen několik, a nymf s 10–99 borreliemi bylo nejvíce na kontrolní lokalitě C.

Izolace a identifikace borrelií

Z 16 izolačních pokusů v médiu BSK-H bylo úspěšných 8 pokusů (tab. 4). Amplifikací pomocí PCR primerů byl získán u vzorků pozitivních na přítomnost amplikonu *B. burgdorferi* s.l. fragment o velikosti 226-266 pb, který byl dále štěpen restriktázou *MseI*. Separací produktů štěpení na 3% agarózovém gelu byly získány fragmenty, které byly porovnány s již publikovanými restrikčními vzory [25]. Izolát BR-V2, který pocházel z lokality Hlučín, byl identifikován jako *B. garinii*. Izoláty BR-V5 a BR-V9 z lokality A byly identifikovány jako *B. afzelii* a *B. burgdorferi* s.s. Z haldy B pocházely vzorky BR-V4, BR-V10, BR-V11, BR-V12 a BR-V14. Ve třech případech byla identifikována *B. afzelii* a ve dvou případech *B. garinii* (tab. 4).

Diskuse

Tato studie byla zaměřena na opakovaný sběr klíšťat v antropogenně narušených ekosystémech ostravské aglomerace (2 starší haldy) a na kontrolním nenarušeném biotopu smíšeného lesa (Hlučínsko). Vybrané lokality jsou od sebe vzdáleny <10 km. Při výběru hald se muselo přihlížet jak k jejich dostupnosti, tak k možnosti provádět sběry. Halda A (Oskar) se nachází na okraji města, v její blízkosti jsou lesy a pole, a proto se u ní předpokládalo větší druhové zastoupení hostitelů a tedy i větší frekvence záchytu klíšťat než u haldy B (Emma), která se nachází v blízkosti centra města Ostravy. To ovšem bylo potvrzeno pouze u nymf, zatímco dospělých klíšťat bylo více na haldě B. Početnost klíšťat mezi lokalitami byla rozdílná, a vcelku výrazně převyšovaly nymfy nad dospělci.

Distribuce LB je vázána na výskyt klíštěte obecného a bývá proto spojena s lesními biotopy, avšak riziko nákazy představují také např. městské parky (viz úvod). Některé studie uvádějí, že v městských parcích dosahuje pozitivita klíšťat až 30 % u dospělců a 14 % procent u nymf – např. v Brně [10]. V literatuře jsme ovšem nenalezli práci, která by se zabývala vyšetřením na borrelie klíšťat z rekultivovaných hald hlušiny z černouhelných dolů. Naše nálezy překvapivě ukazují, že i v tomto extrémním antropogenním biotopu se klíšťata obsahující borrelie vyskytují, a to s početností, která dosahuje abundance v biotopech přirozených.

Všechny sběry byly uskutečněny v měsících duben až červenec. Podzimní sběry nebyly prováděny vzhledem k nízké početnosti klíšťat v této době. V popisu sezonality frekvencí se zaměřujeme pouze na rok 2006, poněvadž v r. 2005 byly provedeny jen 4 jarní sběry. Maximální početnost nymf byla zjištěna na lokalitě Hlučín a na haldě A v dubnu, zatímco na haldě B v květnu. Avšak maximální početnosti dospělců bylo dosaženo u Hlučína v červnu, na haldě A v květnu, na haldě B v dubnu. Zdá se, že na haldách je sezónní vývoj klíšťat rychlejší, pravděpodobně díky teplejšímu mezoklimatu odvalů (zvláště na haldě Emma). Na haldě A a také na haldě B během letních měsíců klesala početnost jak dospělců tak i nymf. To odpovídá studii, ve které v jarních měsících byla početnost klíšťat (dospělců i nymf) maximální a v následujících měsících klesala [13].

Průměrná prevalence *B. burgdorferi* s.l. v *I. ricinus* v Evropě dosahuje 1,9 % u larev, 10,8 % u nymf a 17,4 % u dospělců [9] a liší se podle metod použitých k detekci. Kultivace v BSK médiu je méně citlivá metoda než mikroskopie (v zástinu či fázovém kontrastu) a PCR [5, 9]. V naší studii byla celková průměrná prevalence *B. burgdorferi* s.l. v klíšťatech 11,0 % u nymf a 17,7 % u dospělců, což zcela odpovídá celoevropskému průměru, a neliší se nijak významně od dat uvedených v jiných pracech uskutečněných na území Česka. Např. na jižní Moravě byla zjištěna prevalence u nymf 17,2 % a u dospělců 23,2 % [11], na Olomoucku u nymf 7,0 % a u dospělců 11,8 % [17], v Praze 8,2 % u nymf a 15,9 % u dospělců [23]. V naší studii byla zjištěna průkazně vyšší celková prevalence borrelií u dospělců než u nymf klíšťat, která byla velmi výrazná zejména u klíšťat z haldy B. Je možné, že mikroklima tohoto termicky aktivního odvalu příznivě ovlivňuje vývoj klíšťat i borrelií, zejména v chladnějších obdobích roku. Rozdíly v prevalenci borrelií v klíšťatech mezi kontrolní plochou a haldami nebyly statisticky průkazné.

Lokality se mezi sebou mírně lišily v indexu průměrného počtu klíšťat infikovaných borreliemi za hodinu sběru: nejvyšší frekvence pozitivních nymf byla na haldě A (9,0) a největší frekvence pozitivních dospělců v Hlučíně (8,0), a obecně byla nejvyšší frekvence pozitivních klíšťat v květnu. Míra rizika, jakou představuje počet infikovaných klíšťat za hodinu pro potenciální návštěvníky daného stanoviště, je vysoká především u nymf na kontrolní lokalitě a haldě A, a u dospělců na haldě B, kde můžeme v průměru každých 20 min narazit na infikované klíště. Míra rizika je nejvyšší u haldy B, na které se vyskytovalo nejvíce dospělých klíšťat obsahujících >100 borrelií. Nymf obashujících >100 borrelií bylo jen několik, a nymf obsahujících 10–99 borrelií bylo nejvíce na kontrolní lokalitě Hlučín.

Pro identifikaci spirochét druhového komplexu *B. burgdorferi* s.l. se úspěšně používá PCR amplifikace *rrf* (5S)-*rrl* (23S) intergenového mezerníku ('spaceru') a následné analýzy tohoto amplikonu pomocí RFLP [25, 31], což bylo potvrzeno i v naší studii, v níž bylo pomocí této techniky určeno všech 8 získaných izolátů. Izolát z kontrolní lokality C byl identifikován jako *B. garinii*, izoláty z haldy A jako *B. afzelii* a *B. burgdorferi* s.s., a mezi kmeny pocházejícími z haldy B byla ve třech případech identifikována *B. afzelii* a ve dvou případech *B. garinii*. Toto zastoupení jednotlivých genomických druhů je typické pro Evropu, v níž převažují genomické druhy *B. garinii* (39,7%) a *B. afzelii* (37,1%), méně bývá zastoupena *B.burgdorferi* s.s. (15,9%) [8]. Frekvence výskytu *B. burgdorferi* s.s. se obecně snižuje od západu k východu, v České republice není nijak častá, a nevyskytuje se v Rusku [8, 25, 27]. Genomospecies *B. valaisiana* a *B. lusitaniae* jsou daleko méně obvyklé, i když byly zaznamenány i v zemích střední Evropy: *B. valaisiana* i u nás [16] a na Slovensku [5], a *B. lusitaniae* v Portugalsku [3], na Ukrajině, v České republice [11,16] a na Slovensku [5].

Poděkování

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PRÁCE 6

Šikutová S., Hornok S., Hubálek Z., Doležálková I., Juřicová Z., **Rudolf I.** 2009. Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. *Vet. Microbiol.* 135: 267–271.

Stručná charakteristika: v séroprevalenční studii jsme sledovali výskyt protilátek k arbovirům Bhanja a středoevropské klíšťové encefalitidy u ovcí, skotu a koní v severovýchodním Maďarsku. Hlavním cílem bylo pokusit se zmapovat především výskyt (formou presence protilátek) exotického viru Bhanja ve střední Evropě (byl totiž dříve izolován v sousedním Slovensku).

Hlavní přínos práce: ačkoliv jsme sérologicky neprokázali protilátky proti exotickému viru Bhanja u maďarských ovcí a krav, podařilo se nám potvrdit přítomnost protilátek viru klíšťové encefalitidy a to vůbec poprvé u domácích zvířat v severovýchodním Maďarsku. Z epidemiologického pohledu je nutné zmínit, že především pasené ovce a kozy mohou být zdrojem viru klíšťové encefalitidy a tedy se podílet na alimentárním přenosu infekce.

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Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary

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ABSTRACT

Blood sera collected from 400 domestic animals (260 cattle, 100 Merino sheep, and 40 Hutzul horses) in northeastern Hungary in 2005 were examined for antibodies against two tick-borne viruses, tick-borne encephalitis flavivirus (TBEV) and Bhanja bunyavirus (BHAV). Using ELISA as screening test and plaque-reduction neutralization as confirmatory test, seropositivity to TBEV was found to be 26.5% in cattle, 7.0% in sheep, and 0.0% in horses. Among cattle, the animals up to 3 years old had significantly lower seroprevalence rate than those in older age groups. Natural foci of tick-borne encephalitis are obviously present in northeastern Hungary. On the other hand, no antibodies neutralizing BHAV were detected in the domestic animals.

1. Introduction

Tick-borne encephalitis flavivirus (TBEV), family Flaviviridae, is the agent of tick-borne encephalitis (TBE), endemic in many European countries including Hungary. Three antigenically distinct subtypes cause TBE (Theiler and Downs, 1973; Randolph, 2008): the Western or Central European encephalitis subtype (W-TBE or CEE) has been isolated from most European countries and the European part of Russia, while the Siberian (S-TBE) and Far-Eastern (FE-TBE) subtype strains extend from European and Asian Russia (and Japan) to Finland and the Baltic countries. Thus all three subtypes circulate within Latvia, Estonia and Finland (Lundkvist et al., 2001; Golovljova et al., 2004; Jääskeläinen et al., 2006), but only W-TBE has been recorded in Lithuania (Mickiene et al., 2001). This pattern corresponds to the distributions of the competent tick vectors of TBE: the principal vector of the W-TBE subtype is the hard tick *Ixodes ricinus*, while the main vector of the S-TBE and FE-TBE subtype is *Ixodes persulcatus* (Randolph, 2008). Central European encephalitis (W-TBE) infection is usually subclinical in adult ruminants. Epidemiologically important, goat, sheep and cow excrete the virus in the milk (Van Tongeren, 1955; Grešíková, 1958a,b). Meningoencephalitis with ataxia, jumping, tremor and convulsions can affect lambs (Papadopoulos, 1980).

A very similar disease, caused by louping ill virus (LIV), occurs in sheep of the British Isles—the agent is very closely related to TBEV, in fact it is another (the westernmost) subtype of TBE, because antigenic and genomic similarity between LIV and W-TBE is higher than that between W-TBE and S-TBE (Shiu et al., 1991; Hubálek et al., 1995).

Bhanja virus (BHAV), family Bunyaviridae, causes meningoencephalitis in lambs and leucopaenia in cattle (Theiler and Downs, 1973) and is distributed in southern

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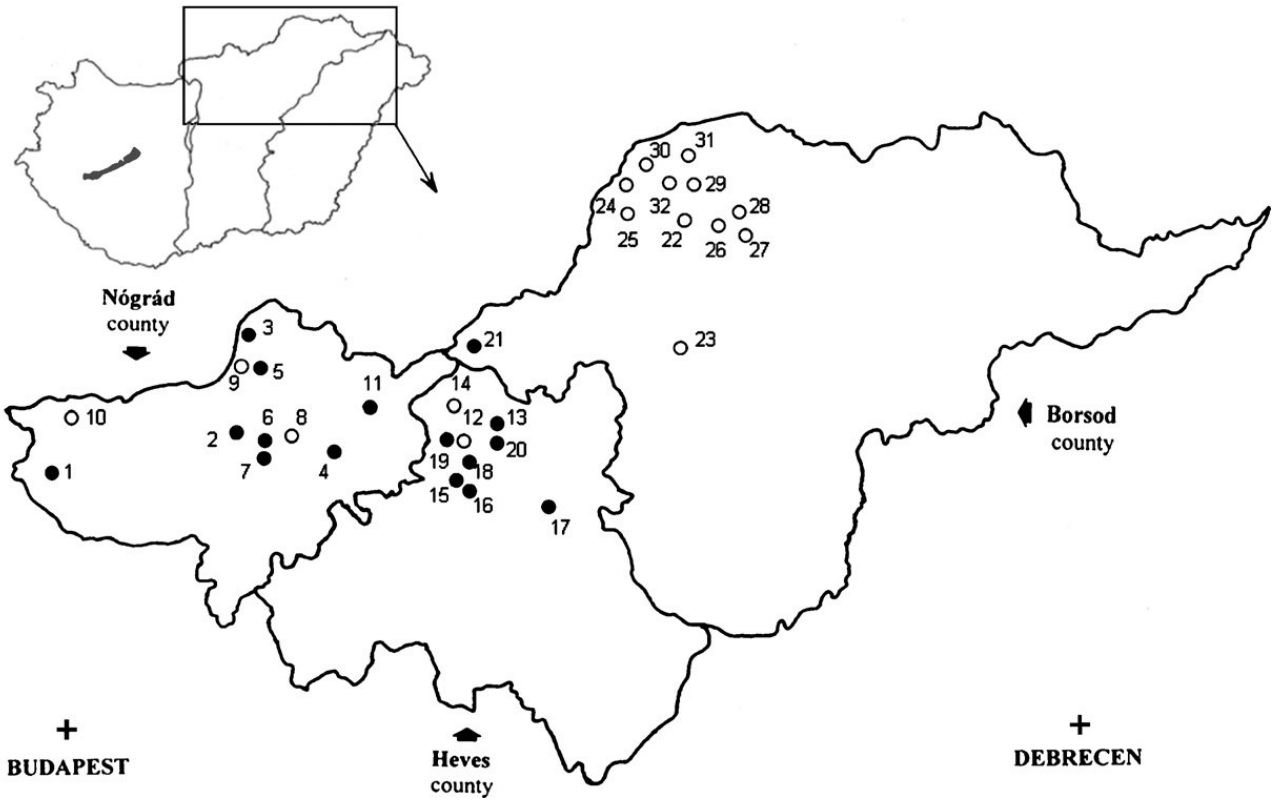

Fig. 1. Geographical distribution of animal sera investigated. Cattle sera were from the following locations: (1) Diósjenő; (2) Nógrádsipek; (3) Nógrádszakál; (4) Sámsonháza; (5) Piliny; (6) Zsunypuszta; (7) Felsőtold; (8) Kisbárkány; (9) Ludányhalászi; (10) Patak; (11) Kazár; (12) Pétervására; (13) Tarnalelesz; (14) Istenmezeje; (15) Mátraderecske; (16) Recsk; (17) Egerszólát; (18) Kisfüzes; (19) Ivád; (20) Fedémes; (21) Domaháza; (22) Alsótelkes; (23) Sajólászlófalva; (24) Aggtelek; (25) Trizs; (26) Szendrő; (27) Abod; (28) Galvács; (29) Perkupa; (30) Jósvafő; (31) Szögliget; (32) Szinpetri. Places where seropositivity was detected are marked by solid circles, lack of seropositivity is indicated by open circles. Horse sera came from location 30 and sheep sera came from location 21.

Asia, Africa and southeastern Europe (Hubálek, 1987). In Europe, BHAV is transmitted by metastriate ixodid ticks *Haemaphysalis punctata* and *Dermacentor marginatus*. Veterinary monitoring of sheep and goats in natural foci has been recommended (Hubálek and Halouzka, 1996).

2. Materials and methods

2.1. Serum samples

Blood samples were collected during the year 2005 from 400 grazed domestic animals in northern Hungary: 260 cattle sera from 32 localities (11 in Nógrád county, 9 in Heves county and 12 in Borsod-Abaúj-Zemplén county: Fig. 1), 100 Merino sheep sera from 5 flocks at Domaháza, and 40 horse (historical Hutzul breed) sera from Jósvafő.

2.2. Serological tests

2.2.1. TBEV

2.2.1.1. ELISA. Serum samples were tested in commercially available EIA TBEV-Ig kit (Test-Line, Ltd., Czech Republic) according to the instructions of the manufacturer. Native serum samples were inactivated at 56 °C for 30 min. The optical density was measured at 450 nm.

The test was regarded valid when the optical density (OD) value of the positive control was $\leq 0.5 \times$ average OD of the negative control, and when OD value of the negative control was ≥ 0.200. Results were expressed as a ratio of average OD value of the negative control/OD value of the sample. The cut-off value for positive sera was ≥ 1.9. All these positive sera were then tested in confirmatory assay, the plaque-reduction neutralization microtest (PRNμT).

2.2.1.2. PRNT. Plaque-reduction neutralization microtest (PRNμT) (Hubálek et al., 1979) on SPEV (porcine embryo kidney) cells, which is based on PRNT assay suggested by Madrid and Porterfield (1969), was used with the TBEV strain Hypr—a prototype of Central European encephalitis subtype of TBEV, isolated by Pospíšil et al. (1954) from a human patient. Sera were inactivated at 56 °C for 30 min, and diluted 1:5 in Leibovitz L-15 medium (Sigma, USA). Thirty microliter of diluted sera (in duplicate) were mixed with 30 μl of the TBEV suspension (containing about 30 plaque-forming units, PFU) in flat-bottomed microtiter plates (Sarstedt, USA), and incubated at 37 °C for 60 min. Then 60 μl of cell suspension (about 20,000 cells) in Leibovitz L-15 medium (Sigma, USA) with 2% foetal calf serum (Sigma, USA) and antibiotics were added to each well and incubated at 37 °C for 4 h. Thereafter 120 μl of a carboxy-methyl cellulose overlay was added to each well and incubated at 37 °C for 4 days. The cells were stained by naphthol blue–black solution for 50 min at room temperature. Sera reducing the number of PFU by 90%

($PRN\mu T_{90}$) at the screening dilution 1:10 were considered positive.

2.2.2. Bhanja virus

The serological assay was performed in analogy to $PRN\mu T$ used for TBEV. The BHAV strain applied was Bg 326 which was isolated from *Haemaphysalis* ticks in Bulgaria (Pavlov et al., 1978). The test was conducted on Vero E6 cells in flat-bottomed microtiter plates, and evaluated after an incubation at 37 °C for 3 days.

2.3. Statistical analysis

A SOLO statistical program (BMDP Statistical Software, Los Angeles, California, USA) with $2 \times n$ tables and χ^2-test was used to compare prevalence data between individual counties and among age categories. Differences in proportions were considered as significant when $p \leq 0.05$.

3. Results

3.1. Tick-borne encephalitis virus

Of the 260 cattle sera tested, 69 (i.e., 26.5%) were positive for TBEV in both ELISA and $PRN\mu T_{90}$: 29/105 (27.6%) in Nógrád county, 22/70 (31.4%) in Heves county, and 18/85 (21.2%) in Borsod-Abaúj-Zemplén county (Table 1). The difference in seroprevalence rate among counties was not significant ($\chi^2 = 2.175$; $p = 0.337$). Nevertheless, all positive bovine samples of Borsod-Abaúj-Zemplén county were collected in one place (Domaháza), where 69.2% of cattle were positive. No

Table 1
Antibodies against tick-borne encephalitis virus in cattle sera in northern Hungary.

County locality	Age (months)				Total
	≤36	37–60	61–96	97–212	
Nógrád					
(1) Diósjenő		**1/9**[a]	**1/1**		2/10
(2) Nógrádsipek	0/7	**1/3**			1/10
(3) Nógrádszakál	**1/2**	0/1	0/3	**1/4**	2/10
(4) Sámsonháza	0/5		0/2	**1/3**	1/10
(5) Piliny	0/1	**5/5**	**4/4**		9/10
(6) Zsunypuszta		**2/2**	0/3	**1/5**	3/10
(7) Felsőtold		**1/1**	**9/9**		10/10
(8) Kisbárkány	0/3		0/1	0/1	0/5
(9) Ludányhalászi		0/10			0/10
(10) Patak	0/1	0/7	0/2		0/10
(11) Kazár	0/6	**1/3**		0/1	1/10
Total	1/25	11/41	14/25	3/14	29/105 (27.6%)
Heves					
(12) Pétervására	0/10				0/10
(13) Tarnalelesz		**2/3**			2/3
(14) Istenmezeje	0/2				0/2
(15) Mátraderecske			**1/4**	**1/6**	2/10
(16) Recsk		0/1	**2/5**	**3/4**	5/10
(17) Egerszólát		0/2	**2/6**	**1/2**	3/10
(18) Kisfüzes	**1/5**	0/2	0/3		1/10
(19) Ivád	0/2	**2/4**	**2/3**	0/1	4/10
(20) Fedémes		**1/1**		**4/4**	5/5
Total	1/19	5/13	7/21	9/17	22/70 (31.4%)
Borsod-Abaúj-Zemplén					
(21) Domaháza		**6/6**	**2/4**	**10/16**	18/26
(22) Alsótelkes		0/1	0/9	0/2	0/12
(23) Sajólászlófalva	0/1	0/4	0/5		0/10
(24) Aggtelek		0/2	0/1	0/2	0/5
(25) Trizs			0/2	0/2	0/4
(26) Szendrő		0/2	0/1	0/4	0/7
(27) Abod				0/2	0/2
(28) Galvács	0/1		0/1	0/1	0/3
(29) Perkupa			0/2		0/2
(30) Jósvafő				0/1	0/1
(31) Szögliget			0/1	0/1	0/2
(32) Szinpetri	0/2	0/2	0/4	0/3	0/11
Total	0/4	6/17	2/30	10/34	18/85 (21.2%)
Total, northern Hungary	2/48 (4.2%)	22/71 (31.0%)	23/76 (30.3%)	22/65 (33.8%)	69/260 (26.5%)

Bold values indicate seropositive animals.
[a] No. positive/no.examined.

seropositivity was detected in the eastern part of the evaluated region (Fig. 1).

Distribution of positive cattle sera was also analyzed according to the age groups. The animals up to 36 months (3 years) old (A: young cattle) had significantly lower seroprevalence rate than those of the older age groups (B: 37–60; C: 61–96; D: 97–212-month-old age group) (A vs. B: $p = 0.0003$; A vs. C: $p = 0.0004$; A vs. D: $p = 0.0001$). No significant difference was found among the three older age categories (B vs. C: $p = 0.924$; B vs. D: $p = 0.722$; C vs. D: $p = 0.649$). Within particular counties, the seroprevalence in age categories was 4.0% (young cattle group), 26.8% (age group B), 56.0% (age group C), and 21.4% (age group D) in Nógrád county. In Heves county, seroprevalence against TBEV increased from 5.3% in the youngest cattle age group to 52.9% in the age group D. Seroprevalence in the whole Borsod-Abaúj-Zemplén county decreased from 35.3% in the age group B to 6.7% in the group C, and then increased to 29.4% (group D). All results are summarized in Table 1.

Of the 100 sheep samples, 7 were positive in both ELISA and PRNμT_{90}. All the seropositive sheep were at least 3 years old. There was no significant difference in TBEV seroprevalence between sheep age groups corresponding to those of cattle (A: 5.3%, B: 5.7%, C: 12.5%, and D: 10%). No seropositive horses were detected.

3.2. Bhanja virus

All serum samples (260 cattle, 100 Merino sheep and 40 Hutzul horses) examined in PRNμT against BHAV were negative.

4. Discussion

4.1. Tick-borne encephalitis virus

This is the first report on the seroprevalence of TBEV in domestic animals of northeast Hungary. The selection criteria of the study area were based on natural foci of TBE that have been described previously, taking into account the incidence of human cases (Molnár, 1982; Ferenczi et al., 2005; Rácz et al., 2006). In particular, for TBE risk-assessment a good correlation was demonstrated between the incidence of disease and the level of forestation (Rácz et al., 2006). In this way the region evaluated in the present study (northeast Hungary) was estimated to have a similar rate of exposure as the area (southwest Hungary) recognized with the highest risk of TBE. Furthermore, although the overall number of diagnosed human TBE cases in Hungary significantly decreased between 1991 and 2000, this could largely be attributed to a tendency of decline in the southern part of the country, whereas the incidence remained relatively constant in the northern region (Ferenczi et al., 2005).

At the same time, seropositivity of cattle or sheep to TBEV has only been evaluated in regions other than northeast Hungary (Molnár, 1982), and no similar data have been available on horses. Interestingly, the proportion of cattle showing seropositivity was lowest in the western part of the country (3–15.7%). However, the incidence of antibodies to TBEV in samples of cows (38.8%) from the southeastern region, and of sheep (19%) in northwestern Hungary (Molnár, 1982) exceeded the prevalence rates in northeast Hungary reported in the present study (i.e., 26.5% in cattle and 7% in sheep).

In neighbouring southeastern Slovakia, several serosurveys for TBEV were carried out among local domestic animals including sheep and cattle. For instance, Hubálek et al. (1985, 1986) found haemagglutination-inhibiting and neutralizing (PRNT) antibodies to TBEV in 8–25% of sheep, 44–54% of goats, and 2–14% of cattle sampled during 1982 and 1983.

As a plausible explanation for the high overall seroprevalence reported here for TBEV in cattle, all sampled animals were beef producers kept extensively, which usually implies a high level of repeated tick infestation (S. Hornok, personal observation). Thus the chances for TBEV transmission also become greater with the advance of age, as indicated by higher rates of seropositivity in older animals of the present study. Exposure to infected ticks is still regarded as the major risk factor in contracting TBEV, despite the fact that raw milk consumption has been implicated in human TBE epidemiology in several European countries (Grešíková, 1958b; Rieger et al., 1998). Potential of cow-to-calf natural transmission of TBEV should also deserve attention and evaluation, especially if the calving period coincides with the highest spring activity of ticks.

TBEV seroprevalence among sheep at Domaháza was approximately only one-tenth of that detected in local cattle. This suggests that although the age distribution, the annual period spent on pastures and the extent of grazed area was similar for herds and flocks in the relevant region, there still may have been differences in the rate of tick exposure between cattle and sheep. This could be, in part, attributed to their unique grazing habit or feeding preference, influencing contact with ticks which quest at certain heights on the vegetation. Cattle and sheep are also known to have variable predisposition for tick attachment, depending on body surface and predilection sites (Ogore et al., 1999).

Since ixodid ticks were found on horses during the present study (data not shown), their TBEV seronegativity cannot be explained by the lack of vector availability. On the other hand, horse samples were obtained in an area (Jósvafő) where cattle were also found seronegative. This result indicates that the eastern part of the evaluated region of northern Hungary appears to be non-endemic.

4.2. Bhanja virus

The seronegativity of grazed domestic animals (sheep, cattle, horse) for BHAV in northern Hungary has been surprising in that antibodies neutralizing this bunyavirus were detected some 30 years ago in the neighbouring Slovak territory in 63% of 19 examined goats as well as in 7% of 28 sheep (Bárdoš et al., 1977), later in 27% of 120 sheep (Hubálek and Juřicová, 1984; Hubálek et al., 1985, 1986) and then the virus was also isolated from *D. marginatus* ticks (Hubálek et al., 1988) in the Slovak Karst at Kečovo area, ecologically identical to, and the continuation of, the Hungarian Aggtelek Karst at Jósvafő. It is possible that either the BHAV activity decreased in this

region, or the domestic animals tested in Hungary did not have effective contact with the main European tick vector of this virus, *H. punctata* (Hubálek et al., 1985). Disappearance of *H. punctata* from formerly inhabited places of the three evaluated counties was recently reported (Hornok and Farkas, in press). In conclusion, an updated evaluation of the occurrence of BHAV in other endemic parts of eastern Europe is strongly encouraged.

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PRÁCE 7

Rudolf I., Mendel J., Šikutová S., Švec P., Masaříková J., Nováková D., Buňková L., Sedláček I., Hubálek Z. 2009. 16S rRNA gene-based identification of cultured bacterial flora from host-seeking *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* ticks, vectors of vertebrate pathogens. *Folia Microbiol.* 54: 419–428.

Stručná charakteristika: ačkoliv byla převážně klíšťata sledována z pohledu přenašečů řady humánních patogenů, nebyly zdaleka zkoumány další mikroorganizmy včetně nepatogenních či podmíněně patogenních, které se v klíšťatech vyskytují, ovlivňují jeho bakteriální diverzitu a mohou např. interagovat s patogeny (stimulačně či antagonisticky) a tím ovlivňovat vektorovou kompetenci klíšťat pro patogenní agens.

Hlavní přínos práce: práce poodhaluje střípek mikrobiálního spektra (pouze kultivovatelné baktérie) u medicínsky významných klíšťat *I. ricinus*, *D. reticulatus* a *H. concinna*. Práce patří mezi vůbec první, které se pokouší popsat kultivovatelné mikroby v klíšťatech včetně nepatogenních zástupců pomocí mol. metod. Dnes díky možnosti sekvenování nové generace samozřejmě popis mikrobiomu klíšťat i jiných hematofágů nabývá úplně jiných rozměrů.

Příspěvek autora k dané práci: autor se podílel na designu studie, jejím provedení (izolace a identifikace mikroorganizmů), analýze dat a přípravě publikace.

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16S rRNA Gene-Based Identification of Cultured Bacterial Flora from Host-Seeking *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* Ticks, Vectors of Vertebrate Pathogens

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ABSTRACT. A total of 151 bacterial isolates were recovered from different developmental stages (larvae, nymphs and adults) of field-collected ticks (67 strains from *Ixodes ricinus*, 38 from *Dermacentor reticulatus*, 46 from *Haemaphysalis concinna*). Microorganisms were identified by means of 16S rRNA gene sequencing. Almost 87 % of the strains belonged to G^+ bacteria with predominantly occurring genera *Bacillus* and *Paenibacillus*. Other G^+ strains included *Arthrobacter*, *Corynebacterium*, *Frigoribacterium*, *Kocuria*, *Microbacterium*, *Micrococcus*, *Plantibacter*, *Rhodococcus*, *Rothia*, and *Staphylococcus*. G^- strains occurred less frequently, comprising genera *Advenella*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas*, and *Xanthomonas*. Several strains of medical importance were found, namely *Advenella incenata*, *Corynebacterium aurimucosum*, *Microbacterium oxydans*, *M. schleiferi*, *Staphylococcus* spp., and *Stenotrophomonas maltophilia*. Data on cultivable microbial diversity in Eurasian tick species *D. reticulatus* and *H. concinna* are given, along with the extension of present knowledge concerning bacterial flora of *I. ricinus*.

Abbreviations

D.r. *Dermacentor reticulatus* *H.c.* *Haemaphysalis concinna* *I.r.* *Ixodes ricinus*

Ixodid and argasid ticks play an important role in transmission of a variety of zoonoses of viral, bacterial and protozoan origin (Beati 1996). The common tick, *I.r.*, the most prevalent tick species inhabiting the temperate zone of Europe, has been intensively studied because of its role in transmission of a wide range of human (*Flavivirus* of tick-borne encephalitis and *Orbivirus* Tribeč, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Rickettsia helvetica*, *Francisella tularensis*, *Babesia microti*) as well as animal (*Babesia divergens* and *B. ovis*) pathogens. Another tick species *D.r.* is involved in transmision of *F. tularensis*, *C. burnetii*, *B. canis*, and the *H.c.* tick could transmit *Flavivirus* of tick-borne encephalitis in European conditions (Hubálek and Rudolf 2007; Hulínská *et al.* 2007).

On the other hand, ixodid ticks harbor a wealth of microorganisms which have not been intensively studied so far. Only three relevant scientific publications describing cultivable tick bacterial flora have been published. Two of them outline briefly bacterial diversity in the American tick *Ixodes scapularis* and Australian ticks (*I. holocyclus*, *Boophilus decoloratus*, *Amblyomma triguttatum*, *Haemaphysalis longicornis* and *Aponomma fimbriatum*) (Martin and Schmidtmann 1998; Murrel *et al.* 2003). Stojek and Dutkiewicz (2004) demonstrated isolation and identification of several bacteria occurring in the European tick *I.r.* Unfortunately, none of these papers covers a wider microbial range and therefore gives only limited information. Moreover, there is an absence of data on microorganisms associated with other European tick species (*e.g.*, *D.r.* and *H.c.*). Several papers reported endosymbionts associated with ixodid ticks (Niebylski *et al.* 1997; Noda *et al.* 1997; Benson *et al.* 2004; Scoles 2004). Ticks also serve as hosts of obligatory intracellular bacteria belonging to the genera *Rickettsia*, *Ehrlichia*, *Anaplasma*, *Bartonella*, *etc*. (Hercík *et al.* 2007). Certain

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members of mentioned genera are symbionts, usually localized in the Malpighian tubules and/or ovaries and mostly non-cultivable; for that reason they were not included in this paper.

Our aim was to describe cultivable microflora from three species of medically important ticks (*I.r.*, *D.r.*, *H.c.*) by means of partial 16S rRNA gene-based sequencing.

MATERIALS AND METHODS

Localities and collection of ticks. Valtice area (48°41′28.62″N; 16°50′48.99″E; 198 m a.s.l.) is situated in the surroundings of Břeclav (South Moravia, Czech Republic) close to a hunting lodge Rendezvous (for details *see* Hubálek *et al.* 2003). In addition to *I.r.* ticks, *H.c.* and *D.r.* co-occur occasionally.

Obora Soutok near Lanžhot (48°38′31.93″N; 16°57′57.70″E; 151 m a.s.l.) is distinguished by typical flood-plain forest ecosystem and is located close to Austrian and Slovak national borders. Plant as well as animal species diversity is strongly influenced by periodic flooding (Hubálek *et al.* 1998). Ixodid tick community is composed of *I.r.*, *D.r.* and less frequent *H.c.*

Havraníky (48°49′6.63″N; 15°59′49.04″E; 330 m a.s.l.) belongs to unique European heath ecosystem characterized by thermophilic steppe flora and fauna. *H.c.* is the dominating tick species occurring in the habitat during June.

Tick collection, bacterial cultures and their maintenance (surface sterilization and homogenization of ticks, establishment of pure cultures and their long-term storage). Unfed ticks (larvae, nymphs and adults in *I.r.*; nymphs and adults in *D.r.* and *H.c.*) were collected by flagging low vegetation during seasons 2006 and 2007 (spring and autumn intervals). All tick specimens were sorted according to their species and stage and then stored alive and separately in sterile tubes at 4–6 °C. Ticks were carefully surface-sterilized under stringent conditions in a biohazard cabinet: they were first submerged in 3 % H_2O_2 followed by 70 % EtOH (the full effect of surface sterilization has been verified by fingerprinting of appropriately treated tick bodies directly on agar plates). Representative collection encompassed 42 specimens of *I.r.* (5 larvae, 10 nymphs, 14 males, 13 females), 19 of *D.r.* (1 nymph, 8 males, 10 females) and 25 of *H.c.* (10 nymphs, 5 males, 10 females).

Air-dried ticks were homogenized in sterile glass microblenders and appropriate dilutions of the whole-body homogenates were plated on different kinds of bacteriological media (*Oxoid*, UK): tryptone-soya agar for culturing common non-fastidious bacteria, brain–heart infusion agar and Columbia agar for recovering of fastidious and potentially pathogenic bacteria, kanamycin–aesculin azide agar for isolation of enterococci, and Lowenstein–Jensen agar for mycobacteria. The plates were incubated at 28 and 37 °C in parallel for ≈1 week (for mycobacteria, the incubation was prolonged for up to 1 month). Pure cultures were prepared by cross-streaks on particular media. The analyzed strains were maintained on glass beads at –70 °C (Jones *et al.* 1991) and isolated strains were then subcultured on brain–heart infusion agar for further analyses.

Phenotypic characterization. The morphological characteristics of isolates were determined using visual investigation of bacterial colonies on plates along with microscopic examination (Gram staining). Biochemical tests (cytochrome oxidase and catalase production, presence of hemolysis, conventional biochemical tests) were carried out.

Genomic DNA extraction and PCR amplification of partial 16S rRNA gene. The total genomic DNA from pure bacterial strains was extracted with QIAamp DNA Mini Kit (*Qiagen*, Hilden, Germany) according to manufacturer's instructions. PCR amplification of partial 16S rRNA gene was performed with unique primers

Div_{446} 5′-CTT AGT ATA AGC TTT TAT ACA GC-3′ and
Div_{1302} 5′-ATA GGT CAG AAA CTT GAA TGA TAC A-3′,

which were designed and specifically optimized. They were generated to amplify ≈800 bp specific regions of 16S rRNA gene of bacteria. The reaction tube contained 200 mmol/L mixture of dNTPs, 75 mmol/L Tris-HCl (pH 8.8), 20 mmol/L $(NH_4)_2SO_4$, 2.5 mmol/L $MgCl_2$, 10 ppm Tween 20, 2.5 U Taq purple DNA polymerase (*Top-Bio*, Czech Republic) and 25 pmol of each primer. The PCR reaction was performed in PTC-200 Gradient Thermal Cycler (*MJ Research*, USA) under these conditions: denaturation (1 min, 94 °C), annealing (30 s, 64 °C), and extension (2 min, 72 °C) consisting of 40 cycles. The products were then separated on 2 % agarose gel, stained with ethidium bromide and visualized by UV light. DNA extraction, PCR handling as well as post-PCR procedures were done in separate rooms to avoid possible cross-contamination. Specific products were further characterized by sequence analysis.

Sequence analysis of PCR products. The products were purified by means of precipitation with 26 % polyethylene glycol–6.5 mmol/L $MgCl_2 \cdot 6H_2O$–0.6 mol/L $NaOAc \cdot 3H_2O$ mixture. Direct sequencing of

purified products was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit ver. 1.1 (*Applied Biosystems*, USA) according to the manufacturer's instructions, and purified with EtOH–EDTA precipitation. The sequencing was performed on an ABI Prism 310 Genetic Analyzer (*Applied Biosystems*). PCR amplicons were multiply sequenced from both directions to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (*DNASTAR Inc*., USA) and also checked manually. The FASTA format and BLAST program (**http://www.ncbi.nlm.nih.gov/blast**) of the *National Center for Biotechnology Information* (Bethesda, MD, USA) were used for database searches. Unique sequences have been deposited in *Genbank* database under acces. no. FJ662445–FJ662476 for *I.r.* isolates, FJ662419–FJ662433 for *D.r.* ones and FJ662434–FJ662444 for *H.c.* ones.

RESULTS

Thirty-one isolation experiments were performed to describe cultivable bacterial microflora from three species of medically important ticks. A total of 151 bacterial isolates (67 strains from *I.r.*, 38 from *D.r.*, 46 from *H.c.*) were recovered from different developmental stages of field-collected ticks.

The yield of bacteria isolation attempts was quite low. Only 2–3 colonies were picked up from individual agar plates. The most fruitful media appeared to be Columbia agar (86 isolates) and brain–heart infusion agar (35), followed by tryptone-soya agar (24) and MacConkey agar (6). No strains were retrieved on kanamycin–aesculin azide agar and Lowenstein–Jensen agar.

G^+ bacteria represented ≈87 % of cultivable tick microflora (79 % in *I.r.*, 94 % in *D.r.*, 91 % in *H.c.*). In total, 67 strains from *I.r.* were sequenced (Table I). The most prevalent strains belonged to the genera *Bacillus* (22 strains) and *Paenibacillus* (19). Remaining G^+ strains inhabiting *I.r.* ticks belonged to the genera *Arthrobacter*, *Corynebacterium*, *Dietzia*, *Microbacterium* and *Rhodococcus*. G^- bacteria occurred less frequently, comprising genera *Advenella*, *Pseudomonas*, *Stenotrophomonas* and *Xanthomonas*. Only one strain (IR29 – '*Bacillus bhargavae*') was isolated from larvae. Bacteria *Bacillus pumilus* (strains IR3, IR33, IR60) have been found in females and males of *I.r.* ticks. Other isolates assigned to the genera *Bacillus* (strains IR39, IR42, IR44, IR46, IR52) occurred in nymphs and adults as well as members of the *Paenibacillus* group (strains IR1, IR4–IR6, IR9, IR10, IR17, IR24, IR30, IR48, IR63, IR65). Four strains (IR58, IR64, IR66, IR67) failed in determination of partial gene sequence (probably due to mutation in primer sequence).

In *D.r.*, 38 strains were sequenced (Table II). The most prevalent belonged to the genus *Bacillus* (7 strains) and *Paenibacillus* (23). Other G^+ strains involved genera *Kocuria*, *Rothia* and *Staphylococcus*. G^- bacteria consisted only of the genus *Pseudomonas*. Only one strain (DR31 – *P. amylolyticus*) was recovered from nymphal tick. Bacteria *Paenibacillus* spp. were isolated from nymphs (strains DR31–DR33), males (DR14, DR15, DR18–DR20, DR22, DR34, DR35), and females (DR3, DR12, DR26, DR38). Only one strain (DR41) failed in the determination of partial gene sequence (probably due to mutation in primer sequence).

In *H.c.*, 46 strains were sequenced (Table III). The most prevalent belonged to the genera *Bacillus* (14 strains) and *Paenibacillus* (12). Other G^+ strains inhabiting *H.c.* ticks belonged to the genera *Frigoribacterium*, *Microbacterium*, *Plantibacter*, *Rhodococcus* and *Staphylococcus*. G^- bacteria occurred less frequently and involved only genera *Pseudomonas* and *Rahnella*. Bacteria *Paenibacillus* spp. were found in nymphs (HC25, HC42, HC43), males (HC1) and females (HC11, HC14, HC16, HC28, HC38, HC39) of *H.c.* ticks, while *B. simplex* occurred in females (HC27, HC29) and males (HC32), and *B. pumilus* was found only in nymphs (HC19, HC20, HC40). One strain (HC12) did not provide satisfactory output by direct sequencing.

Two bacterial species, *B. pumilus* (strains IR3, IR33, IR60, DR24, HC19, HC20, HC40) and *P. amylolyticus* (IR1, IR4, IR5, IR9, IR10, DR26, DR31, DR32, DR38, HC38, HC39, HC42) occurred throughout all species of ticks, suggesting common phylogenetic status. Strains assigned to the genera *Rhodococcus* and *Microbacterium* occurred in *I.r.* and *H.c.*

DISCUSSION

The bacterial diversity of three species of medically important ticks has been outlined. The 16S rRNA (rDNA) molecule is widely recognized and used as a conservative macromolecule that allows phylogenetic placement of bacterial species (O'Neill *et al.* 1992). Sequence analysis of the 16S rRNA gene represents a reliable method for inferring the bacterial taxa (Wang *et al.* 1999) and has recently been used to resolve the

Table I. Bacterial strains isolated from the tick *Ixodes ricinus*

Strain	Primoisolation medium[a]	Cultivation temperature, °C	Developmental stage[b]	Locality[c]	Length, bp[d]	Taxon[e]	Sequence homology, %
IR1	COL	28	♀	V	731	*Paenibacillus amylolyticus*	99.59
IR3	COL	37	♀	V	732	*Bacillus pumilus*	100
IR4	COL	37	♀	V	749	*Paenibacillus amylolyticus*	99.73
IR5	COL	37	♀	V	733	*Paenibacillus amylolyticus*	99.73
IR6	TSA	28	♀	V	732	*Paenibacillus* sp.	99.86
IR7	TSA	37	♀	V	733	*Paenibacillus* sp.	99.86
IR8	BHI	28	♀	V	733	*Paenibacillus* sp.	100
IR9	BHI	37	♀	V	721	*Paenibacillus amylolyticus*	99.86
IR10	BHI	37	♀	V	731	*Paenibacillus amylolyticus*	99.73
IR11	COL	28	♂	V	718	*Bacillus pumilus*	100
IR12	COL	28	♂	V	733	uncultured bacterium clone nbt67d11	100
IR13	COL	28	♂	V	696	*Pseudomonas brenneri*	100
IR14	TSA	28	N	V	734	*Microbacterium* sp.	99.86
IR15	COL	28	N	V	223	*Paenibacillus* sp.	99.55
IR16	COL	28	N	V	424	*Arthrobacter* sp.	99.29
IR17	BHI	28	N	V	727	*Paenibacillus* sp.	99.73
IR18	BHI	28	N	V	735	*Corynebacterium aurimucosum*	100
IR19	BHI	28	N	V	745	*Dietzia* sp.	99.87
IR20	COL	28	♂	V	737	uncultured bacterium clone nbt120d02	100
IR21	BHI	28	♂	V	712	*Pseudomonas* sp.	99.58
IR22	TSA	37	♂	V	445	*Paenibacillus* sp.	98.66
IR23	TSA	28	♂	V	726	*Pseudomonas* sp.	100
IR24	BHI	37	♂	V	714	*Paenibacillus* sp.	99.86
IR25	COL	28	♂	V	744	*Pseudomonas* sp.	99.87
IR26	COL	28	♂	V	727	*Pseudomonas* sp.	99.73
IR27	COL	28	♂	V	730	*Rhodococcus* sp.	100
IR28	COL	37	♂	V	711	*Paenibacillus* sp.	99.72
IR29	BHI	28	L	V	717	'*Bacillus bhargavae*'	99.72
IR30	BHI	37	♂	V	709	*Paenibacillus* sp.	99.58
IR31	COL	37	♂	V	698	*Bacillus weihenstephanensis*	99.86
IR32	COL	37	♂	V	716	uncultured *Paenibacillus* sp.	99.72
IR33	TSA	37	♂	V	729	*Bacillus pumilus*	100

IR34	COL	28	♂	V	715	*Paenibacillus* sp.	100
IR35	COL	28	♂	V	570	*Pseudomonas* sp.	99.65
IR36	BHI	28	♂	V	718	*Xanthomonas* sp.	99.72
IR37	COL	28	♂	V	717	*Microbacterium oxydans*	100
IR39	COL	28	♂	V	729	*Bacillus* sp.	100
IR40	COL	28	♂	V	731	*Pseudomonas* sp.	100
IR41	COL	28	♂	V	718	'*Bacillus bhargavae*'	99.86
IR42	COL	37	♂	V	730	*Bacillus* sp.	100
IR43	COL	28	♀	V	729	uncultured bacterium clone nbt120d02	100
IR44	COL	37	♀	V	728	*Bacillus* sp.	100
IR45	COL	37	♀	V	715	*Bacillus pumilus*	100
IR46	COL	28	N	V	730	*Bacillus* sp.	100
IR47	COL	28	N	V	732	*Bacillus* sp.	100
IR48	COL	28	♂	V	490	*Paenibacillus* sp.	99.80
IR49	COL	28	♂	O	727	*Bacillus cereus*	100
IR50	BHI	37	♂	O	728	*Bacillus cereus*	100
IR51	COL	37	♂	O	723	*Bacillus* sp.	100
IR52	COL	37	♀	O	734	*Bacillus* sp.	100
IR53	COL	37	♀	O	731	*Bacillus* sp.	99.86
IR54	COL	37	♀	O	752	*Bacillus cereus*	100
IR55	COL	37	♀	O	753	*Paenibacillus* sp.	99.47
IR56	BHI	37	♀	O	454	*Bacillus pumilus*	98.89
IR57	MC	37	♀	O	716	*Advenella incenata*	100
IR58	BHI	37	♀	O	–	sequence undetermined	–
IR59	COL	37	♀	O	760	*Bacillus subtilis*	99.86
IR60	BHI	28	♀	O	733	*Bacillus pumilus*	100
IR61	COL	28	♀	O	734	uncultured bacterium clone BH2_aao22g03	99.86
IR62	COL	28	♀	O	735	*Stenotrophomonas maltophilia*	100
IR63	BHI	28	♂	O	735	*Paenibacillus* sp.	100
IR64	MC	28	♂	O	–	sequence undetermined	–
IR65	BHI	37	♀	O	741	*Advenella incenata*	99.86
IR66	BHI	37	♀	O	–	sequence undetermined	–
IR67	BHI	28	♀	O	–	sequence undetermined	–
IR68	COL	28	♀	O	752	*Bacillus piatikordis*	100
IR69	BHI	28	♀	O	734	*Rhodococcus* sp.	100

[a]COL – Columbia agar, BHI – brain–heart infusion agar, TSA – tryptone soya agar, MC – MacConkey agar.
[b]L – larvae, N – nymph.
[c]V – Valtice, O – Obora soutok, H – Havraníky.
[d]Size of the sequenced part of bacterial 16S rRNA gene.
[e]The closest match in GenBank according to FastA search.

Table II. Bacterial strains isolated from the tick *Dermacentor reticulatus*

Strain	Primoisolation medium[a]	Cultivation temperature, °C	Developmental stage[b]	Locality[c]	Length, bp[d]	Taxon[e]	Sequence homology, %
DR1	COL	28	♀	V	727	*Bacillus* sp.	100
DR2	COL	28	♀	V	772	*Paenibacillus* sp.	96.13
DR3	TSA	28	♀	V	754	*Paenibacillus* sp.	100
DR4	TSA	28	♀	V	731	*Paenibacillus anaericanus*	96.72
DR5	TSA	28	♀	V	731	*Paenibacillus anaericanus*	97.62
DR6	COL	28	♀	V	733	*Rothia* sp.	100
DR7	COL	28	♀	V	723	uncultured bacterium clone nbt233f04	100
DR8	TSA	28	♀	V	733	*Staphylococcus* sp.	100
DR9	BHI	28	♀	V	713	*Paenibacillus anaericanus*	96.22
DR11	BHI	37	♀	V	752	*Staphylococcus equorum*	99.87
DR12	COL	37	♀	V	732	*Paenibacillus* sp.	100
DR14	COL	28	♂	V	749	*Paenibacillus* sp.	100
DR15	COL	37	♂	V	749	*Paenibacillus* sp.	100
DR16	COL	37	♂	V	719	*Bacillus* sp.	100
DR17	TSA	28	♂	V	716	*Paenibacillus amylolyticus*	99.72
DR18	TSA	37	♂	V	717	*Paenibacillus* sp.	100
DR19	TSA	37	♂	V	748	*Paenibacillus* sp.	100
DR20	BHI	28	♂	V	716	*Paenibacillus* sp.	100
DR21	BHI	28	♂	V	731	*Bacillus plakortidis*	99.86
DR22	BHI	37	♂	V	731	*Paenibacillus* sp.	100
DR24	COL	37	♀	V	759	*Bacillus pumilus*	100
DR25	COL	37	♀	V	732	*Paenibacillus* sp.	99.45
DR26	COL	37	♀	V	731	*Paenibacillus amylolyticus*	99.59
DR27	COL	37	♀	V	733	*Bacillus cereus*	100
DR28	COL	37	♀	V	732	*Bacillus* sp.	100
DR29	COL	37	♀	V	731	*Paenibacillus* sp.	100
DR30	COL	37	♀	V	717	*Paenibacillus* sp.	100
DR31	BHI	28	N	O	733	*Paenibacillus amylolyticus*	99.59
DR32	COL	28	N	O	738	*Paenibacillus amylolyticus*	99.86

DR33	BHI	37	N	O	732	*Paenibacillus amylolyticus*	100
DR34	COL	37	♂	O	708	*Paenibacillus* sp.	100
DR35	COL	37	♂	O	736	*Paenibacillus* sp.	99.86
DR36	COL	28	♀	O	751	*Pseudomonas graminis*	99.73
DR37	COL	28	♀	O	734	*Pseudomonas abietaniphila*	99.86
DR38	COL	28	♀	O	708	*Paenibacillus amylolyticus*	100
DR39	COL	28	♀	O	731	*Bacillus gibsonii*	99.76
DR40	COL	28	♀	O	701	*Kocuria* sp.	100
DR41	COL	28	♀	O	–	sequence undetermined	–

[a–e]*See footnotes* of Table I.

Table III. Bacterial strains isolated from the tick *Haemaphysalis concinna*

Strain	Primoisolation medium[a]	Cultivation temperature, °C	Developmental stage[b]	Locality[c]	Length, bp[d]	Taxon[e]	Sequence homology, %
HC1	BHI	37	♂	H	709	*Paenibacillus* sp.	99.86
HC2	TSA	37	♀	H	434	*Paenibacillus lautus*	100
HC3	COL	37	♀	H	700	*Bacillus* sp.	100
HC6	TSA	28	♂	H	729	*Rahnella* sp.	100
HC7	TSA	28	♀	H	734	*Bacillus simplex*	100
HC8	TSA	28	♀	H	702	*Bacillus* sp.	100
HC9	COL	28	♀	H	724	*Bacillus cereus*	100
HC10	COL	28	♀	H	534	*Pseudomonas* sp.	100
HC11	TSA	37	♀	H	744	*Paenibacillus* sp.	100
HC12	TSA	37	♂	H	–	sequence undetermined	–
HC13	TSA	37	♂	H	716	*Staphylococcus* sp.	100
HC14	COL	37	♀	H	572	*Paenibacillus* sp.	100
HC15	COL	28	♀	H	736	*Microbacterium oxydans*	100

continued

HC16	BHI	28	♀	H	546	*Paenibacillus* sp.	99.19
HC17	TSA	28	♀	H	649	*Rhodococcus* sp.	100
HC18	TSA	28	♀	H	776	*Microbacterium schleiferi*	100
HC19	COL	37	N	H	778	*Bacillus pumilus*	100
HC20	COL	28	N	H	739	*Bacillus pumilus*	100
HC21	COL	28	N	H	773	*Pseudomonas* sp.	99.22
HC22	TSA	28	N	H	773	*Pseudomonas* sp.	99.17
HC23	BHI	28	N	H	773	*Bacillus* sp.	100
HC24	BHI	37	N	H	776	*Bacillus* sp.	100
HC25	BHI	37	N	H	779	*Paenibacillus* sp.	99.87
HC26	COL	37	N	H	761	*Plantibacter* sp.	100
HC27	TSA	28	N	H	744	*Rhodococcus* sp.	100
HC28	COL	28	♀	H	534	*Paenibacillus* sp.	99.63
HC29	COL	28	♀	H	774	*Bacillus simplex*	100
HC30	COL	28	♀	H	478	*Bacillus* sp.	100
HC31	BHI	37	♂	H	778	*Staphylococcus* sp.	99.74
HC32	COL	28	♂	H	775	*Bacillus simplex*	100
HC33	COL	28	♂	H	764	*Paenibacillus* sp.	99.74
HC34	COL	28	♀	H	789	*Staphylococcus* sp.	100
HC35	COL	37	♂	H	763	*Micrococcus luteus*	100
HC36	MC	28	♀	H	768	*Staphylococcus* sp.	100
HC37	MC	28	♀	H	776	*Staphylococcus equorum*	99.87
HC38	COL	28	♀	H	774	*Paenibacillus amylolyticus*	100
HC39	COL	37	♀	H	758	*Paenibacillus amylolyticus*	99.86
HC40	MC	28	N	H	759	*Bacillus pumilus*	100
HC41	COL	37	N	H	760	*Bacillus pumilus*	100
HC42	COL	37	N	H	782	*Paenibacillus amylolyticus*	100
HC43	BHI	37	N	H	753	*Paenibacillus* sp.	100
HC44	BHI	28	N	H	709	*Bacillus pumilus*	100
HC45	COL	28	N	H	733	*Microbacterium oxydans*	100
HC46	COL	28	N	H	731	*Frigoribacterium* sp.	100
HC47	COL	28	♀	H	731	*Rhodococcus* sp.	100
HC48	MC	28	♀	H	685	*Staphylococcus* sp.	99.85

[a–e]*See footnotes* of Table I.

phylogenetic positions of a variety of microorganisms. This method was also used by us with a novel PCR primer pair which has never been used before for 16S rRNA sequencing in prokaryotes.

Our results correspond very well to similar studies which aimed at describing cultivable microflora of ixodid ticks. Martin and Schmidtmann (1998) partially determined 73 bacterial isolates recovered from the tick *I. scapularis* collected from vegetation and on vertebrate hosts. Seven *Bacillus* species were found in the tick interior, including *B. thuringiensis*, *B. brevis*, and *B. sphaericus*, which are known to affect adversely insects and ticks (Kati *et al.* 2007). Similar results revealed investigation of 21 bacterial isolates (18 G^+, 3 G^-) from adult ticks recovered from deer. The G^+ strains consisted of 2 *Corynebacterium* sp., 16 ones being identified to the genus *Bacillus.* G^- isolates were *Stenotrophomonas maltophilia* and *Kluyvera ascorbata*, while the others did not have a match in the database. Although no obvious differences were revealed between bacteria isolated from male or female ticks, or from the internal *vs*. the external part of the tick, differences in bacterial flora composition between nymphs and adults were evident: 73 % of G^- isolates were from nymphs.

Murrel *et al.* (2003) isolated and characterized bacteria from ticks, lice and fleas in Australia. They screened 5 species of ticks (*I. holocyclus*, *B. decoloratus*, *A. triguttatum*, *H. longicornis*, *A. fimbriatum*). A total of 239 bacterial strains from arthropods were isolated on nutrient agar, blood agar and ISP2 agar and identified based on their partial 16S rRNA gene sequences. In *I. holocyclus,* the species found were, *e.g.*, *Bacillus cereus*, *B. thuringienis*, *B. pumilus*, *Staphylococcus saprophyticus*, *S. xylosus*, *Pantoea stewartii*, *S. maltophilia*, *Pseudomonas putida*, *Burkholderia cepacia*, in *Amblyomma triguttatum*, *e.g.*, *B. licheniformis*, *B. cohnii*, *B. pumilus*, in *Aponoma fimbriatum*, *e.g.*, *P. putida*, *S. maltophilia*, and in *B. microplus* they were *B. cereus*, *B. thuringiensis*, *S. saprophyticus*, *P. putida*, *S. maltophilia*, *Enterobacter cloaceae*, *P. agglomerans*, *Serratia entomophila*, *S. rubidea*, *Klebsiella planticola*, and many others. Some genera were isolated from almost all of the specimen samples (*Stenotrophomonas*, *Pseudomonas*, *Bacillus*). On the other hand, some genera were restricted to one type of ectoparasite (*e.g.*, *Curtobacterium*, *Clavibacter*, *Renibacterium*, *Arthrobacter*, *Leucobacter* were only identified among isolates from the Australian ticks).

Schabereiter-Gurtner *et al.* (2003) evaluated broad-range identification of bacterial communities present in ticks by using a molecular approach. Therefore, 16S rDNA genotyping in combination with analysis of denaturing gradient gel electrophoresis was used for the detailed detection and identification of bacteria infecting ticks. For phylogenetic identification the obtained sequences were compared with 16S rDNA sequence of known bacteria listed in the *GenBank* database. Phylogenetic analysis revealed a limited variety of genera (*e.g.*, *Staphylococcus*, *Rhodococcus*, *Haemobartonella*, *Moraxella*, *Pseudomonas*, *Borrelia*). Some of these bacteria (*Staphylococcus*, *Rhodococcus*, *Pseudomonas*) have been found by us as well. According to the authors, this method seems to be well suited for the detection and identification of bacteria in ticks, regardless of whether these bacteria are fastidious, obligate intracellular or non-cultivable. However, this approach has insufficient taxonomical value because of its inability to reliably differentiate obtained isolates into the species level.

Stojek and Dutkiewicz (2004) examined 372 *I.r.* ticks for the presence of internal G^- bacteria other than *B. burgdorferi*. The G^- isolates were identified with the API systems 20E and NE microtests. Surprisingly, strains identified as belonging to *Pasteurella pneumotropica–haemolytica* complex proved to be the most commonly occurring G^- bacteria in *I.r.* ticks in Poland. Other G^- taxa were isolated less frequently, *viz*. *Aeromonas hydrophila*, *P. agglomerans*, *P. aeruginosa*, *S. maltophilia*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *S. marcescens*, and *S. plymuthica*. However, this paper has covered only a limited spectrum of cultivable microorganisms occurring in ticks. Furthermore, the API system may cause difficulties in reliable identification of environmental samples in contrast with medically important isolates.

Many of the acquired isolates in our study seem to be genetically similar with bacterial sequences recovered from various environmental sources (*e.g.*, plants, water, soil) and were submitted to the *GenBank* database only recently. There is no evidence that bacteria isolated by us create an integral part of tick internal autochthonous microflora; some of them might be ingested incidentally during tick feeding on a vertebrate host or during host seeking on vegetation and in soil litter, and they could survive in tick midgut. Only several strains with pathogenic potential (Lau *et al.* 2002; Coenye *et al.* 2005; Falagas *et al.* 2008) have been isolated by us: *Advenella incenata* (recently described species belonging to the family *Alcaligenaceae*), *Corynebacterium aurimucosum*, *Microbacterium oxydans*, *M. schleiferi*, *Staphylococcus* spp. and *Stenotrophomonas maltophilia*. All these microbes are occasionally found in various clinical materials of human as well as animal origin.

There is lack of detailed information concerning cultivable microflora associated with ixodid ticks along with possible antagonistic effects between particular members of tick microflora and pathogenic microorganisms that inhabit ticks. This is also valid for the European common ticks *I.r.*, *D.r.* and *H.c.* – important vectors of a variety of animal and human pathogens. Our work provides for the first time data on

microbial diversity in Eurasian tick species *D.r.* and *H.c.* and supplements data concerning *I.r.* Another finding that all three tick species share certain common microbes could have impact on phylogenetics of tick symbionts. Our paper contributes only partially to the incredible bacterial diversity in invertebrate world, further taxonomic studies being necessary for filling up this existing gap.

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PRÁCE 8

Rudolf I., Šikutová S., Kopecký J., Hubálek Z. 2010. Salivary gland extract from engorged *Ixodes ricinus* (Acari: *Ixodidae*) stimulates *in vitro* growth of *Borrelia burgdorferi* sensu lato. *J. Basic. Microbiol.* 50: 294–298.

Stručná charakteristika: studie navazuje na práci Rudolf a Hubálek (2003) a doplňuje ji o vliv SGE z nasátých klíšťat *I. ricinus* na růst tři genomických druhů borrelií. Slinné žlázy s nasátých klíšťat *I. ricinus* významně stimulují růst *B. garinii*, *B. afzelii* a *B. burgdorferi* sensu stricto.

Hlavní přínos práce: studie potvrzuje zjištění, že slinné žlázy hrají nezastupitelnou roli při sání a následném přenosu patogenních borrelií na obratlovce (viz. např. 'saliva assisted transmission').

Příspěvek autora k dané práci: autor se podílel na designu práce, *in vitro* experimentu zahrnujícím kultivaci borrelií s SGE v mikrotitračních destičkách, jeho vyhodnocení a přípravě publikace.

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Short Communication

Salivary gland extract from engorged *Ixodes ricinus* (Acari: *Ixodidae*) stimulates *in vitro* growth of *Borrelia burgdorferi* sensu lato

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In vitro effect of salivary gland extract from fed *Ixodes ricinus*, the competent vector of Lyme borreliosis in Europe, on the growth of *Borrelia burgdorferi* sensu lato (*B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto) was examined in BSK-H medium. Motility rate, concentration of motile spirochetes and their morphology were estimated at intervals of 0, 2, 4, 6 and 8 days using darkfield microscopy. Salivary gland extract derived from *I. ricinus* stimulated markedly the growth of three genomic species of borreliae. The results confirm a substantial role of salivary glands in the mechanism of pathogen transmission to vertebrate host.

Keywords: Salivary gland extract / *Ixodes ricinus* / Ticks / *Borrelia burgdorferi* sensu lato

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Introduction

Tick-borne spirochetes including borreliae circulate in a triangle of parasitic interactions between the spirochete, its tick vector and vertebrate host [1]. Pathogens share a very intimate relationship with the vectors and their interaction is crucial for transmission [2]. For instance, *Borrelia burgdorferi* sensu lato (s.l.), the Lyme borreliosis (LB) agent, must overcome at least two main barriers in the tick vector body to be effectively transmitted: the midgut and the salivary glands. Pathogens vectored by arthropods spend a significant part of their lifes in the alimentary canal and interactions between these microbes and arthropod gut epithelium are key elements in determining vector competence. From this point of view, the tick alimentary system has received much less attention than that of haematophagous insects [3]. On the other hand, salivary glands of haematophagous arthropods are intensively studied. It has been found that saliva of ixodid ticks has various pharmacological activities, especially it affects the immune system of vertebrate hosts [4] and contributes substantially to the transmission of pathogens to the host [5].

Much less attention has been given to direct effect of saliva on the growth of the pathogens. Schwan [6] suggested the presence of chemoattractans in tick salivary glands. More recently, Shih *et al.* [7] have shown an increased chemotactic migration of *B. burgdorferi* sensu stricto (s.s.) in U-tubes with salivary gland extract (SGE) from *Ixodes scapularis*, the competent vector of LB in North America. Rudolf and Hubálek [8] demonstrated effect of SGE and midgut extract (MGE) from unfed host-seeking ticks *I. ricinus* and *Dermacentor reticulatus* on the growth of *B. garinii in vitro*. SGE and MGE from the competent vector species (*I. ricinus*) stimulated the growth of spirochetes, whereas the extracts from non-competent tick species (*D. reticulatus*) were inhibitory under conditions of long-term cultivation.

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The purpose of the present study was to examine the effect of SGE from the fed female *I. ricinus* on the growth, motility and morphology of *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s. spirochetes *in vitro*.

Materials and methods

Bacteria

The spirochetal strains used in the study were: *B. garinii* BR14 isolated from *I. ricinus* at Valtice, Czech Republic, *B. afzelii* VS461 isolated from *I. ricinus* in Valais, Switzerland [9] and *B. burgdorferi* s.s. ZS7 isolated from *I. ricinus* in Freiburg, Germany [10]. They were cultivated in BSK-H medium with 6% rabbit serum (Sigma, USA) at 33 °C.

Salivary gland extract

Adult *I. ricinus* ticks maintained in the colony of the Institute of Parasitology, Academy of Sciences of the Czech Republic in České Budějovice have been screened routinely for *B. burgdorferi* s.l. by PCR with negative results. The ticks were fed in groups of mating pairs within retaining cells attached to the backs of guinea pigs. Engorged female ticks were removed after 5 d of feeding, their salivary glands were dissected and pooled. After washing in phosphate-buffered saline (PBS), the salivary glands were homogenised in 1 ml of PBS, sonicated and clarified by centrifugation at 10,000 *g* for 10 min. The protein concentration of clarified SGE was determined using an Protein estimation kit (BioRad, Richmond, USA). Aliquots of the SGE preparation were stored at −70 °C.

In vitro growth assay

In the experiments, 100 µl of SGE (25 µg of protein/ml) or PBS (control) were mixed with 100 µl of a 3-d culture of *B. garinii*, *B. afzelii* or *B. burgdorferi* s.s. (about 10^6 spirochaetes inoculated) in BSK-H medium in 96-well flat-bottomed sterile microplates (Sarstedt, Germany), and covered with a sterile sealing film (Denville Scientific Inc., USA). The microplates were placed in a 33 °C incubator for 6–8 d. Concentration of motile spirochetes (the number of motile cells/ml) was determined at intervals of 0, 2, 4, 6 and 8 d using darkfield microscopy: (i) estimation of motility (in per cent) was determined in 3 wells per variant, when 100 randomly selected spirochaetes per well were examined for motility; (ii) concentration of all (motile plus non-motile) spirochetes was evaluated in 10 µl volumes of appropriately diluted cultures on a microscope slide with a 20 × 20 mm coverslip [11] – for each variant, 3 wells with 5 counts (total, 15 replicates) were used.

Statistical analysis

The data were analyzed with the two sample *t*-test using SOLO (BMDP Statistical Software, USA). Significant differences in the concentration of motile spirochaetes were estimated at $P < 0.001$.

Results and discussion

The effect of SGE from the fed *I. ricinus* females on the growth of *B. burgdorferi* s.l. is summarized in Fig. 1 (A–C).

Effect of SGE on concentration of motile spirochetes was shown to be slightly different in the three genomic species of *B. burgdorferi* s.l. tested. SGE stimulated the growth of the strain BR14 markedly on day 2 ($t = 9.25$; $P < 0.001$), stimulatory effect was also observed on day 4 ($t = 8.58$; $P < 0.001$), 6 ($t = 7.25$; $P < 0.001$) and 8 ($t = 10.15$; $P < 0.001$) (Fig. 1A). On the other hand, in VS461 and ZS7 strains a significant increase in the concentration of motile spirochetes with SGE was only detected on day 4 ($t = 13.57$ in VS461, $t = 13.21$ in ZS7; $P < 0.001$) (Fig. 1B and Fig. 1C). The total number of spirochetes has increased about 21 times in *B. garinii*, 19 times in *B. afzelii* and 5 times in *B. burgdorferi* s.s., whereas the number of spirochetes in the control increased less apparently: about 11 times in *B. garinii*, 13 times in *B. afzelii* and 3 times in *B. burgdorferi* s.s.

In addition, spirochetes grown in the presence of SGE seemed to be morphologically typical, displaying contrast margins, regular coils and motility, while a number of partially damaged cells were found after long-term cultivation in the control.

The objective of this study was to investigate the effect of SGE from the competent vector of LB (*I. ricinus*) on the growth of selected *B. burgdorferi* s.l. genomic species *in vitro*. Borreliae are mainly located in apical surface of the tick gut epithelium while they are found rarely in the salivary glands after repletion and through the molting period. However, during feeding the spirochetes are able to penetrate gut epithelium and pass through the haemocoel to the salivary glands [12]. This process is associated with differential expression of several genes in spirochaetes. Specifically, downregulation of protein OspA gene and upregulation of OspC gene is needed for efficient migration of spirochetes from the midgut to the salivary glands [13]. Several borrelial genes required for tick colonization or transmission have been identified recently [14]. These

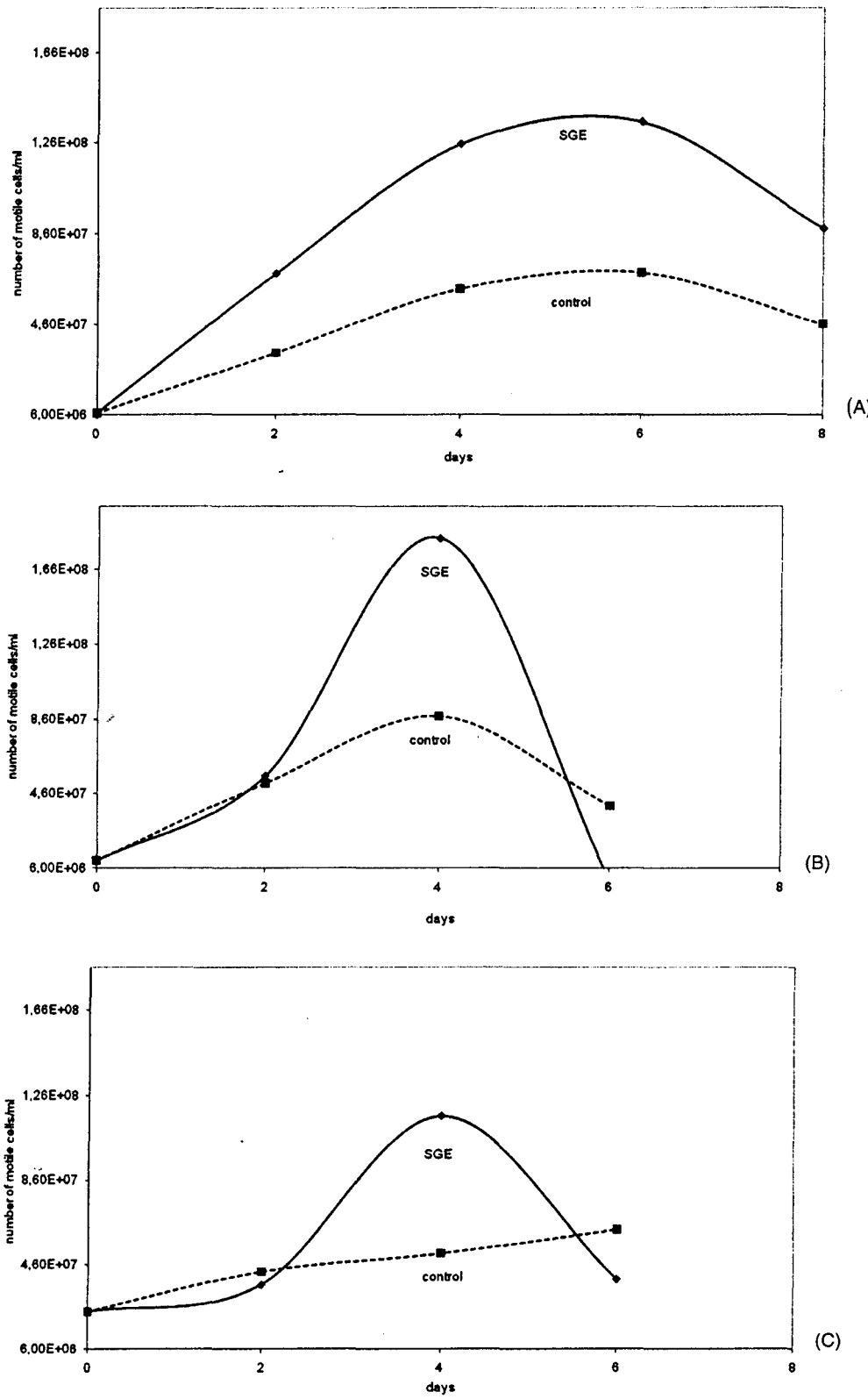

Figure 1. Concentration of motile spirochetes *B. garinii* BR14 in BSK-H medium with salivary gland extract (SGE) from fed *I. ricinus* female, compared to control; (B) Concentration of motile spirochetes *B. afzelii* VS461 in BSK-H medium with salivary gland extract (SGE) from fed *I. ricinus* female, compared to control; (C) Concentration of motile spirochetes *B. burgdorferi* s.s. ZS7 in BSK-H medium with salivary gland extract (SGE) from fed *I. ricinus* female, compared to control.

genes required for transmission are induced by a pathway controlled by the alternate sigma factors RpoN (sigma 54) and RpoS. A protein in the gut of *I. scapularis* ticks that functions as a receptor for *B. burgdorferi* has been also determined in that study.

Although the promotion of tick-borne pathogen transmission *via* the action of tick saliva components on the host (termed saliva-activated transmission, SAT), has been demonstrated [15], identity of the SAT factor is not known. The SAT phenomenon has been demonstrated for several tick borne pathogens including Thogoto virus [16], tick-borne encephalitis virus [17], *B. afzelii* [18], *B. burgdorferi* sensu stricto and *B. lusitaniae* [19], and *Francisella tularensis* [20]. The SAT factor candidates are frequently referred to be immunosuppressive and anti-inflammatory molecules [21–23], e.g. data on the impact of tick SGE on the inflammation induced by tick-transmitted pathogen were documented by Severinová *et al.* [24].

I. ricinus tick saliva-activated transmission of *B. burgdorferi* was also studied on the C3H/HeN mouse model [25]. Results have shown early effect of tick saliva on the proliferation and distribution of *Borrelia* spirochetes in the host, probably due to the effect of saliva on the host innate immunity mechanisms. In another study [26], SAT phenomenon of *B. burgdorferi* sensu stricto was demonstrated using real-time PCR and SGE from partially fed *I. ricinus* ticks (C3H/HeN mice were injected intradermally with spirochetes mixed with SGE). The accelerating effect of SGE on spirochete proliferation was demonstrated on day 1 post infection, when a 4.2-fold increase in spirochetes was found in the skin and a 10-fold increase in the blood, compared with control mice. The data represent the first direct evidence of a SAT effect of *I. ricinus* SGE on infection with the Lyme disease agent *B. burgdorferi*.

Rudolf and Hubálek [8] found a stimulatory effect of SGE from unfed *I. ricinus* on the growth of *B. garinii*. The present study confirmed that phenomenon in fed females of *I. ricinus* as well. It is interesting that the increase in the number of spirochaetes caused by SGE from fed *I. ricinus* ticks was higher than that caused by SGE from unfed ticks with approximately the same bacterial inoculum and protein concentration [8]. Certain compounds present in the salivary glands probably attract migration of spirochetes from the midgut to the salivary glands during tick feeding [6]. Other molecules apparently create favourable milieu for the growth and transmission of spirochaetes that reached the salivary glands. These molecules seem to be present throughout the unfed and fed stages of the ixodid tick [8]. Further research focusing on ixodid tick-pathogen interface could clarify the role of tick saliva molecules in the transmission of *B. burgdorferi* and other pathogenic microorganisms vectored by haematophagous arthropods.

Conclusions

In summary, many molecules are being involved in succesfull pathogen transmission from the haematophagous vector to vertebrate host. This study has demonstrated stimulatory effect of SGE on borrelial growth *in vitro* and thus indirectly supported idea about substantial role of salivary glands in pathogen transmission. SGE from engorged tick *I. ricinus* has stimulated markedly the growth of three proven pathogenic genomospecies: *B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto. This is only a preliminary step that might be essential for further identification of SAT compound in tick salivary glands.

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PRÁCE 9

Konvalinová J., **Rudolf I.**, Šikutová S., Hubálek Z., Svobodová V., Svoboda M. 2012. Likely emergence of canine babesiosis in the Czech Republic. *Acta Vet. Brno.* 81: 91–95.

Stručná charakteristika: psí babezióza patří mezi významné veterinární parazitární nákazy a způsobuje u psů život ohrožující onemocnění. Ačkoliv se psí babezióza vyskytuje u našich jižních sousedů, nebyly dosud u nás zaznamenány autochtonní případy. V poslední dekádě dochází k plíživému rozšiřování areálu této nákazy a k jejímu posunu na sever. Je tedy důležité provádět včasnou surveillance včetně vyšetření psů a klíšťat v rizikových oblastech. Poprvé byly na našem území detegované specifické protilátky proti *Babesia canis* u psů, kteří nevycestovali do endemických zemí výskytu onemocnění, což naznačuje možnost autochtonního přenosu této velmi závažné veterinární nákazy také v České republice. Nepodařilo se však prokázat patogenní babesie u klíšťat *D. reticulatus*, které jsou jejími primárními vektory.

Hlavní přínos práce: práce jako jedna z mála upozornila na možnost rizika šíření této nebezpečné nákazy na našem území.

Příspěvek autora k dané práci: autor se podílel na molekulárním vyšetření klíšťat *D. reticulatus* na přítomnost patogenní *B. canis* a na přípravě rukopisu.

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Contribution to canine babesiosis in the Czech Republic

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Abstract

From March to November 2010, a total of 68 samples of blood from 41 hunting and working dogs that never left the Czech Republic were examined. Some dogs were sampled repeatedly. Blood samples were examined by polymerase chain reaction for the presence of DNA of piroplasms with negative results. Specific IgG antibodies against *Babesia canis* were detected by indirect immunofluorescence test, and five dogs (12.21%) were seropositive. Titres ranged from 50 to 200. One dog was positive in two samplings within 3 months. The highest number of positive samples was taken in June. The results of this study suggest a likely contact of the examined dogs with the parasite; although in 2005, a total of 340 adult unfed *Dermacentor reticulatus* ticks in 34 pools screened by PCR for babesiae were negative.

Dogs, Babesia canis, antibodies, Dermacentor reticulatus

Canine babesiosis, one of the most important emerging tick-borne diseases of dogs with worldwide distribution, is transmitted by intra-erythrocytic protozoan of the genus *Babesia*. Traditionally, identification of species is based on morphology and host specificity. According to these criteria, canine piroplasms are divided into two distinct species, the large (4–5 µm) *Babesia canis* and the small (2.5 µm) *Babesia gibsoni*. Based on the differences in geographical distribution, vector specificity, antigenic properties, pathogenicity and ss-ribosomal RNA gene three subspecies of *B. canis* are distinguished, namely *B. canis canis* transmitted by *Dermacentor reticulatus* in Europe, *B. canis vogeli* transmitted by *Rhipicephalus sanguineus* in tropical and subtropical regions, and highly pathogenic *B. canis rossi* transmissible by *Haemaphysalis leachi* in South Africa (Uilenberg 2006). *B. canis canis* is the most importcant agent of babesiosis in Europe.

The incidence of *Dermacentor reticulatus* in the Czech Republic is limited to the basins of the Morava and Dyje rivers in the Břeclav and Hodonín regions and along the border with Slovakia (Kubelová and Široký 2010) (Fig. 1). The activity of adults has two peaks, with the first being in the spring from early March (however, ticks can be observed as early as late February, depending on weather conditions – adults are sometimes found even on snow) to mid April. The second peak of adults' activity starts in September. This tick species inhabits mainly lowland biotopes, waterlogged broadleaved forests, meadows, inundated areas of rivers and fringes of forests. Incidence of *Dermacentor reticulatus* is irregular and insular. In Central Europe, autochthonous canine babesiosis due to *B. canis* was recorded in several countries. Surprisingly, no autochthonous case of canine babesiosis was reported so far in the Czech Republic, although babesiosis is present in all the countries surrounding the Czech Republic and the competent vector of the disease frequently occurs (Svobodová and Svobodová 2004). In Slovakia, first cases of autochthonous babesiosis

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started to emerge in 1997; the first case of babesiosis in dog was documented in 2000 (Chandoga et al. 2002). The incidence of babesiosis nearest to the Czech Republic was observed in the neighborhood of Malacky, Slovakia. So far, only imported babesiosis has been observed in the Czech Republic – the first imported infection was described in 1992 (Kučera 1992).

Dynamics of the spreading of canine babesiosis in Europe markedly changed in the last few years. This is largely connected with the expanding area of *D. reticulatus* distribution. In fact, the expansion of the vector's area and the increasing number of clinical cases of babesiosis has been observed also in all adjacent countries. Babesiosis has spread to Germany, Austria, Hungary and Poland as well as Switzerland (Földvári and Farkas 2005; Sréter et al. 2005; Duh et al. 2006; Zygner and Wedrychowicz 2006; Zygner et al. 2008; Hornok and Farkas 2009).

Babesiosis is a serious dog disease. Typical symptoms of acute babesiosis include apathy, anorexia, fever and general weakness. The disease leads to haemolytic anaemia along with thrombocytopaenia, lymphadenopathy and splenomegaly. Jaundice and haematuria can occur as well. Clinical signs are often very variable and the disease can have mild to peracute course that results in death within 2 days. Incubation period of *B. canis* is 10 to 21 days (Boozer and Macintire 2003). The infection induces an antibody reaction which is usually not strong enough to eliminate all babesiae in a host organism. Animals therefore become chronic carriers of the infection (Vercammen et al. 1997). In most cases, antibodies occur within 8 to 10 days after the infection. Puppies under 2 months of age can have colostral antibodies. Poor immune reaction is typical for puppies under 8 months of age. Antibody levels start to decrease 5 to 8 months after the animal went through the infection. Protection of dogs that underwent the disease against reinfection with the same *Babesia* species lasts 5 to 8 months on average. Antibodies acquired after the infection with one *Babesia* species do not protect against the infection with other species (Boozer and Macintire 2003; Uilenberg 2006). In certain studies, parasitaemia was detected in up to 36% of serologically negative dogs (Taboada 1998). Animals that recover from the infection and live in endemic localities acquire the so-called pre-immunity, i.e. non-sterile immunity. This means that the parasite survives in the host organism and eliminates reinfections. To the best of the authors' knowledge, no comprehensive study on *B. canis canis* and its main tick vector *D. reticulatus* nor systematic survey of dogs from endemic localities for the presence of antibodies to *B. canis* was conducted in the Czech Republic.

The aim of our study was to examine a group of dogs living in the region where emergence of *B. canis* infection might be expected. The presence of *D. reticulatus* vector was confirmed in that locality. Moreover, it is located near to Slovakia where the disease commonly occurs. Examinations of dogs followed up the pilot study which was carried out to assess prevalence of *B. canis canis* in *D. reticulatus* ticks in the South Moravia region (Czech Republic), where the vector is widespread and enzootic focus of tularaemia occurs (Hubálek et al. 1996).

Materials and Methods

From March to November 2010, a total of 41 dogs of 11 breeds (Siberian husky being the most frequent breed) were examined. The sample included 21 males (one of them castrated) and 19 females, aged 1 to 12 years. The body weight of these dogs ranged from 6 to 42 kg. All animals came from the Břeclav district (Břeclav and Lanžhot localities) where *D. reticulatus* occurs. They were hunting and working dogs that never left this territory. As the dogs often worked in the field, they were more likely to be infested with ticks. All animals were clinically healthy. Blood samples of some of them were collected repeatedly. Of a total of 41 dogs, blood samples of 21 animals were collected once, 7 dogs were sampled twice and 13 animals thrice. A total of 68 blood samples were collected. Samples were taken at monthly intervals at the least. Blood was sampled in March, April, June and November.

Blood samples were taken from v. cephalica antebrachii. Samples of full blood (inserted in EDTA) and blood serum were obtained from each dog. Full blood samples were examined by PCR method. DNA was

extracted from the samples using the commercial kit QIAGEN NucleoSpin Blood (Machery-Nagel, Germany) as prescribed by the manufacturer. To amplify the diagnostic fragment of the 91 piroplasmid SSU rRNA gene, we designed the forward primer TB-F (5´-CTTCAGCACCTTGAGAGAAAT-3´) and the reverse primer TB-R (5´-TCDATCCCCRWCACGATGCRBAC-3´). Amplification condition were: 5 min at 94 °C, 39 cycles each of 94 °C for 45 s, 62 °C for 30 s, and 72 °C for 45 s, with the addition of a final extension period of 10 min at 72 °C. DNA isolated from the dog with confirmed imported *B. canis* infection (it was a patient at our clinic) was used as a positive control. Specific IgG antibodies against *Babesia canis* were detected by indirect immunofluorescence using the commercial Babesia canis IFA IgG Antibody Kit (Fuller Laboratories Fullerton, California, USA). The kit manufacturer states that titres 50 and more suggest recent or current infection. Host-seeking adult *D. reticulatus* were collected by flagging low vegetation during April 2005. All tick specimens were frozen at -60 °C until examination. Immediately before DNA isolation, ticks were surface sterilized with 70% ethanol (PCR quality), then pooled (10 ticks per pool) and mechanically disrupted using a sterile glass microblender. The total genomic DNA was extracted with QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Molecular detection of *B. canis* was performed as described previously (Jefferies et al. 2003) including primers PIRO-A1 (5´- AGGGAGCCTGAGAGACGGCTACC - 3´) and PIRO-B (5´- TTAAATACGAATGCCCCCAAC - 3´) which amplify an approximately 450 bp long conservative region of the 18S rRNA gene of babesiae.

Results

A total of 68 blood samples taken from 41 dogs were examined by PCR. No sample contained DNA of *B. canis*. Specific antibodies were detected in 5 dogs (12.2%). Serological examination based on indirect immunofluorescence detected 6 positive serum samples. Titres ranged from 50 to 200. One dog was positive in two samplings within 3 months. The highest number of positive samples was taken in June. The results are demonstrated in Table 1. A total of 340 adult *D. reticulatus* ticks (210 females and 130 males) in 34 pools were screened for babesiae. Specific PCR products of babesial DNA were not detected in any of the examined pools.

Table 1. Titres of specific antibodies against *Babesia canis* in positive dogs (IFA)

Breed	Sex	Age (years)	Blood examination and titre of specific antibodies		
			March	April	June
Dachshund	Male	3	50	NT	50
Jagdterrier	Female	11	Negative	100	NT
German Shepherd	Male	5	Negative	Negative	50
Dachshund	Male	8	Negative	NT	50
Siberian Husky	Male	8	NT	NT	200

NT- not tested

Discussion

Canine piroplasms are increasingly more frequently brought to the north (Gothe and Schmid 1995; Losson et al. 1999). The geographical distribution of the causative agent and thus the occurrence of babesiosis are largely dependent on the distribution of the competent vector and susceptible host, therefore being regarded as endemic for certain regions (Martinod et al. 1986). We encounter clinical cases of babesiosis increasingly more often at our clinic. So far, they have been only cases of imported babesiosis, mostly from Slovakia. One of the risk groups is represented by search and rescue dogs that are often used to work abroad. Autochthonous infection has not been observed in the Czech Republic yet.

Examination performed in April 2005 did not detect *B. canis* in *D. reticulatus* ticks picked up in the localities where they occur. In 2010, we examined a group of dogs coming

from the locality with incidence of *D. reticulatus* near the border with Slovakia. Those dogs often worked in the wild, which made them more likely to get into contact with ticks. None of the examined dogs ever left the Czech Republic. Serological examination proved that 5 dogs were positive for antibodies against *B. canis*. In one of these dogs positive titre was observed repeatedly after 3 months (March and June; titer 50 in both cases). In April, one positive result (titre 100) was recorded. The highest number (4) of positive results was observed in June when also the highest titre (200) was recorded. Spring activity of *D. reticulatus* spans over March throughout April, having the second peak in late summer. Antibodies start to be produced 1 to 2 weeks after contact with the infectious agent. All the examined animals were clinically healthy. Our results indicate a likely contact of the examined dogs with the parasite. If the infectious dose was low, the infection could induce only antibody reaction without the outburst of the disease. In such cases, the parasite's DNA in the samples could be under the detection limit, or the parasite was eliminated.

Babesiae were not detected in the blood of the examined dogs by PCR. This indicates that the parasite was either absent in the samples or there was such a low level of its DNA that it was not possible to detect it by this type of assay. Diagnostics of babesiosis is based on direct detection of the parasite in blood smear or on using PCR method. Serology is used rather for seroepidemiologic studies than clinical diagnostics. Certain studies indicate that up to 36% of dogs with parasitaemia can be serologically negative (Taboada 1998). In localities with babesiosis, serologically positive dogs should not be used for breeding, even if parasite was not detected in them. In these animals a low level of parasitaemia under the detection limit of microscopy or PCR cannot be ruled out. Subclinical infections of this kind cause problems in breeding kennels and pose a risk in cases of transfusion therapy (Taboada 1998; Birkenheuer et al. 2003; Boozer and Macintire 2003; Irwin 2005).

As far as incidence of babesiosis is concerned, the Czech Republic has a unique position nowadays compared to the adjacent countries where the vector's area is expanding and babesiosis is spreading out. Long-term incidence of *Dermacentor reticulatus* in the Czech Republic was confirmed only in a relatively small area around the Morava and Dyje rivers in the southeastern part of the country (Fig. 1). Although babesiosis is commonly detected in Slovakia near the Czech national border, no autochthonous clinical case of babesiosis has been confirmed in the Czech Republic yet. The examination of 340 ticks in 2005 did not demonstrate the presence of the parasite's DNA. In 2010, we detected antibodies

Fig. 1. Localities with the incidence of *Dermacentor reticulatus* – Hodonín, Břeclav, Lanžhot

against babesiosis in five dogs (12.2%). Although babesiae were not detected directly by PCR, the results of our study indicate that the presence of *B. canis* in the Czech Republic cannot be excluded. Epidemiological surveillance including distribution of competent vector, detection of the disease agent, seroprevalence study of dogs, and monitoring of acute and imported cases are needed to elucidate whether canine babesiosis could become established in the Czech Republic.

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PRÁCE 10

Venclíková K., **Rudolf I.**, Mendel J., Betášová L., Hubálek Z. 2014. Rickettsiae in questing *Ixodes ricinus* ticks in the Czech Republic. *Ticks and Tick-borne Dis.* 5:135–138.

Stručná charakteristika: celkem 1473 nenasátých klíšťat sbíraných s vegetace bylo pomocí mol. metod vyšetřeno na přítomnost pro člověka patogenních rickettsií (*Rickettsia monacensis*, *R. helvetica*, '*Candidatus* Neoehrlichia mikurensis' a *A. phagocytophilum*) ve dvou lokalitách ostravského regionu (urbánní a přírodní).

Hlavní přínos práce: práce přináší první relevantní data o výskytu patogenních rickettsií v nenasátých klíšťatech *I. ricinus* sbíraných s vegetace na našem území.

Příspěvek autora k dané práci: autor se podílel na designu studie, sběru klíšťat v terénu, jejich determinaci, molekulární analýze, zhodnocení získaných dat i přípravě rukopisu.

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Original article

Rickettsiae in questing *Ixodes ricinus* ticks in the Czech Republic

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ABSTRACT

Tick-borne rickettsiae are an important topic in the field of emerging infectious diseases. In the study, we screened a total of 1473 field-collected *Ixodes ricinus* ticks (1294 nymphs, 99 males, and 80 females) for the presence of human pathogenic rickettsiae (*Rickettsia helvetica*, *R. monacensis*, '*Candidatus* Neoehrlichia mikurensis', and *Anaplasma phagocytophilum*) in natural and urban ecosystems using molecular techniques. The minimum infection rate (MIR) for *Rickettsia* spp. was found to be 2.9% in an urban park and 3.4% in a natural forest ecosystem; for '*Candidatus* Neoehrlichia mikurensis', we observed MIRs of 0.4% in the city park and 4.4% in the natural habitat, while for *A. phagocytophilum* the MIR was 9.4% and 1.9%, respectively. Our study provides the first data on the occurrence of human pathogenic rickettsiae in questing *I. ricinus* ticks in the Czech Republic.

Introduction

Ixodid ticks (mainly *Ixodes ricinus* in Central Europe) present a significant health risk for humans. For many vertebrate species, these ticks are vectors of multiple pathogens; the most important are tick-borne encephalitis virus (TBEV) and *Borrelia burgdorferi* sensu lato (Hubalek and Rudolf, 2011). The order Rickettsiales taxonomically belongs to the α-Proteobacteria class (Dumler et al., 2001). Members of the order Rickettsiales are intracellular pathogens dependent on their eukaryotic host cell (in both vertebrate and invertebrate hosts). According to phylogenetic analysis, there are 6 clades of *Rickettsia* species, 2 of them associated with arthropods (Weinert et al., 2009). Most members are tick-borne, but some are transmitted by fleas, lice, or mites (Hubalek and Rudolf, 2011). Although they are widely distributed throughout the world, the species and associated human clinical diseases vary depending on the geographical locations. Many new potentially pathogenic rickettsiae, including '*Candidatus* Neoehrlichia mikurensis', have been identified during the past few years (Kawahara et al., 2004; Oteo and Portillo, 2012).

Monitoring tick vectors and the pathogens they transmit, within the scope of epidemiological surveillance, is an important tool for better prevention and control of tick-borne diseases. The recent emergence of tick-borne pathogenic rickettsiae is therefore attracting the attention of public health experts. In the Czech Republic, data on *Rickettsia* spp., *Anaplasma phagocytophilum*, '*Candidatus* Neoehrlichia mikurensis', their prevalence in ticks, and their medical importance are insufficient. We have therefore performed a molecular based survey on the prevalence of these selected tick-borne pathogens in nymphal and adult host-seeking ticks in a natural and an urban ecosystem in order to complete data on their occurrence in Central Europe.

Materials and methods

Study sites

Ixodes ricinus ticks were collected at two study sites: Ostrava city (49°47′ N 18°14′ E) and Proskovice (Ostrava surroundings, 49°44′ N 18°12′ E). The first study site is an urban park. The local fauna is represented by small mammals and birds and the vegetation by broadleaved deciduous trees and grass. The forest is surrounded by housing estates and used for leisure activities and dog walking. The second study site is a natural ecosystem outside the town. This mixed forest with dominant broad-leaved trees is rarely visited by people. The fauna consists of small and medium-sized mammals, roe deer, birds, and occasionally wild boar.

Ticks were collected by flagging low vegetation from April to September (a period of seasonal activity of *I. ricinus* in Central Europe) 2010. The sampled ticks were divided into test

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tubes according to sex and developmental stage and were pooled (5 nymphs per tube, 3 adults per tube) before being frozen at −60 °C.

Homogenization of ticks and genomic DNA isolation

All *I. ricinus* ticks were surface-sterilized with 70% ethanol (PCR quality) and mechanically disrupted using the TissueLyser apparatus (Qiagen, Hilden, Germany) in 245 μl of PBS (Oxoid, England). The total genomic DNA was extracted from 100 μl of the tick homogenate with a QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Detection of Anaplasma phagocytophilum

Real-time PCR for the detection of *A. phagocytophilum* was performed according to Courtney et al. (2004) using ApMSP2f/ApMSP2r primers and complementary ApMSP2 TaqMan probe labeled at the 5′ and the 3′ ends with BHQ1 and FAM, respectively. The PCR reaction was carried out on the real-time PCR machine ABIPRISM 7500 (Applied Biosystems, USA) by using QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany).

Detection of Rickettsia spp. and 'Candidatus Neoehrlichia mikurensis'

Single-step PCR was used for *Rickettsia* spp. detection. The method has been described by Regnery et al. (1991), the primers used for *gltA* gene detection were Rp877p (5′-GGGGGCCTGCTCACGGCGG-3′) and Rp1258n (5′-ATTGCAAAAAGTACAGTGAACA-3′). Conventional PCR was also used for '*Candidatus* Neoehrlichia mikurensis' detection (Fertner et al., 2012). The primers used for 16S rRNA gene detection were "micurensis 729F" (5′-GGCGACTATCTGGCTCAG-3′) and "micurensis 1016R" (5′-GCCAAACTGACTCTTCCG-3′). Selected samples positive by PCR for *Rickettsia* spp. and '*Candidatus* Neoehrlichia mikurensis' (20 amplicons for *Rickettsia* spp. and 20 amplicons for '*Candidatus* Neoehrlichia mikurensis') were subjected to sequence analysis.

Sequence analysis of PCR product

The PCR product was purified by precipitation with PEG/Mg/NaAc (26% polyethylene glycol, 6.5 mM $MgCl_2 \cdot 6H_2O$, 0.6 M $NaAc \cdot 3H_2O$). Direct sequencing of the purified PCR product was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) according to the manufacturer's instructions and purified with EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). PCR amplicons were bidirectionally sequenced once to ensure high-quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v.6.0 (DNASTAR Inc., USA) and also checked manually. The FASTA format and BLAST program (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches.

Statistical evaluation

Differences between minimum infection rates (MIR) were evaluated using contingency tables and chi-square test at the 5% probability level.

Table 1
Numbers of *I. ricinus* ticks tested.

	Males	Females	Nymphs	Ticks in total
Ostrava-Bělský les (urban site)	17/51[a]	16/45	36/180	69/276
Proskovice (natural site)	16/48	12/35	223/1114	251/1197
Total	33/99	28/80	259/1294	320/1473

[a] No. of pools/no. of individual ticks.

Results

We examined 1473 *I. ricinus* ticks (1294 nymphs, 99 males, and 80 females; Table 1). From 320 tick pools in total, 251 contained ticks from the natural ecosystem and 69 pools contained ticks from the urban park.

From the 320 *I. ricinus* pools, 49 were positive for *Rickettsia* spp. Eight positive pools contained ticks from the urban park, 41 pools were samples from the natural ecosystem (Table 2). In the urban park, the overall MIR was 2.9%. Differences in MIRs between stages were insignificant (females 4.4%, males 3.9%, nymphs 2.2%). In the natural ecosystem, the overall MIR rate was 3.4%; differences in MIRs between stages were also insignificant (nymphs 3.5%, females 2.9%, males 2.1%). There was no significant difference between the MIRs of either tick stage at the 2 localities. Overall, 6 *Rickettsia* spp.-positive amplicons were 100% identical to *R. monacensis* (GenBank accession no. JX003686) and 14 amplicons for *R. helvetica* (GenBank accession no. JX040636), respectively. Each of the 2 species was detected at both study sites.

Fifty-four pooled samples in total were positive for DNA of '*Candidatus* Neoehrlichia mikurensis'. In total, 20 amplicons have shown 100% identity to '*Candidatus* Neoehrlichia mikurensis' (GenBank accession no. GQ501090) detected recently in the blood of a 61-year-old man with signs of septicemia (Fehr et al., 2010). There was one positive sample (nymphs) from the urban park (MIR 0.6%; overall MIR 0.4%). The MIR of 4.4% for the natural ecosystem was significantly higher ($P = 0.017$ for nymphs, $P = 0.004$ for adults). Differences in MIR between stages (adults 8.4%; males 6.3%; females 11.4%; nymphs 4.1%) were insignificant ($P = 0.07$).

There were 49 samples that were positive for *A. phagocytophilum*, 26 of which were from the urban park and 23 of which from the study site representing a natural ecosystem. The MIR for the urban park was 9.4%. Adults (13.5%) were found more often infected than nymphs (7.2%), but the difference is not significant. The natural ecosystem's overall MIR was 1.9%. Most often infected were adult ticks (MIR 13.2%; males 14.6%, females 11.4%), whereas only 1.1% (MIR) of the nymphs were found infected. The difference between the stages is statistically significant ($P = 0.0001$).

Discussion

Rickettsioses are an important topic in the field of emerging tick-borne infections. Until recently, Mediterranean spotted fever was the only tick-borne rickettsiosis diagnosed in Europe. However, within the past 2 decades, several other rickettsial species have emerged as potential human pathogens (*Rickettsia slovaca*, *R. helvetica*, *R. raoultii*, *R. massiliae*, *R. mongolotimonae*, *R. monacensis*, or *R. rioja* (Oteo and Portillo, 2012).

Two rickettsial species with pathogenic potential were detected in our study: *R. helvetica* (human infections documented from France, Italy, Sweden, and Switzerland) and *R. monacensis* (human illness described in Spain) (Oteo and Portillo, 2012).

Rickettsia spp. prevalence varies greatly between study sites, years, tick vectors and their different life stages in Europe. It ranges from 0.5% to almost 66% (Oteo and Portillo, 2012) taking in account different methodological approaches used. The MIRs presented in this study are within the scale of prevalences published so far. For

Table 2
Prevalence (MIR, %) of rickettsiae in *I. ricinus* at the two study sites.

	Urban site (Bělský les)				Natural ecosystem (Proskovice)			
	Males	Females	Nymphs	Total	Males	Females	Nymphs	Total
Rickettsia spp.	3.9	4.4	2.2	2.9	2.1	2.9	3.5	3.4
'*Candidatus* Neoehrlichia mikurensis'	0	0	0.6	0.4	6.3	11.4	4.1	4.4
Anaplasma phagocytophilum	17.6	8.9	7.2	9.4	14.6	11.4	1.1	1.9

Explanation: Minimum infection rate (MIR) was calculated from the number of total ticks examined under assumption that every positive pool has contained only one infected tick.

instance, the MIRs with *R. helvetica* and with *R. monacensis* in pools of nymphal *I. ricinus* (consisting of 5 individuals each) in Hungary were 1.9% and 0.11%, respectively (Egyed et al., 2012). A recent study from Sweden documents the variability of prevalences of *R. helvetica* in different localities; the overall prevalence of *Rickettsia* spp. in individually examined adult *I. ricinus* ticks from 29 different study sites was found to be 9.5% (Wallménius et al., 2012). In another study, individually tested adult *I. ricinus* ticks from several study sites in Luxembourg showed a prevalence of 5.1%. They were identified as *R. helvetica* (98.6%) and *R. monacensis* (1.4%) (Reye et al., 2010). Prevalences of *Rickettsia* spp. in questing ticks in Germany differ substantially in comparison with our study. In Hanover, 33.3% of the individually examined *I. ricinus* ticks were found to be infected (98.9% with *R. helvetica* and 1.1% with *R. monacensis*) (Schicht et al., 2012), while in Thuringia, the prevalence was 9.3% in nymphs and 18.8% in adults (Hildebrandt et al., 2010).

'*Candidatus* Neoehrlichia mikurensis' is in a novel clade in the Anaplasmataceae family and has been found in small mammals (Kawahara et al., 2004; Pan et al., 2003; Schouls et al., 1999) and in *I. ricinus*, *I. ovatus*, and *I. persulcatus* ticks (Fertner et al., 2012; Kawahara et al., 2004; Richter and Matuschka, 2011; Schouls et al., 1999). This bacterium was first detected in ticks in the Netherlands in 1999 (Schouls et al., 1999), but it started to attract attention only recently because of its association with several human cases of disease (Fehr et al., 2010; Pekova et al., 2011; von Loewenich et al., 2010; Welinder-Olsson et al., 2010). That is why only a few prevalence studies are available so far. The prevalence in Eurasian regions varies and was reported to be 3.0% in pooled or unpooled [sic!] adults of *I. ovatus* in Japan (Kawahara et al., 2004), 0.2% in individually examined adults of *I. persulcatus* in Siberia (Rar et al., 2008, 2010), 1.7% in individually examined adults of *I. ricinus* in France (Richter and Matuschka, 2011), and 8.1% in individually examined adults of *I. ricinus* in Germany (Richter and Matuschka, 2011). PCR-DGGE analysis of the bacterial community of nymphal *I. ricinus* has shown that '*Candidatus* Neoehrlichia mikurensis' is among the most prevalent bacterial species present in these ticks (Van Overbeek et al., 2008). In our study, 1473 ticks were tested and the MIR in questing *I. ricinus* ticks was 0.4% in an urban park and 4.4% in a natural ecosystem. This difference between habitats could be caused by the fact that small mammals (*Myodes glareolus* acts as reservoir host for this bacterium) are more likely to be found in natural woodland than in urban parks (Andersson and Raberg, 2011). The significance of this finding is substantiated by 2 human cases of infection diagnosed in the Czech Republic in 2008 and 2009 (Pekova et al., 2011).

The obligate intracellular bacterium *A. phagocytophilum* is transmitted by *I. ricinus* ticks in Europe and causes granulocytic anaplasmosis in humans and several other mammalian species (Hubalek and Rudolf, 2011). *Anaplasma phagocytophilum* has been detected in ixodid ticks in most European countries (Šikutová et al., 2007). Its prevalence in ticks varies significantly between years (even in the same study site) and between different developmental stages of ticks (Overzier et al., 2013). Ixodid ticks from urban parks were tested for *A. phagocytophilum* also in other European countries. Its overall prevalence in individually examined questing *I. ricinus* ticks from several Bavarian public parks was 9.5% in 2009 (5.0% of nymphs and 12.5% of adults) and 6.6% in 2010 (3.9% of nymphs and 8.9% of adults) (Schorn et al., 2011). In Polish urban and suburban forests, the prevalence was 14% in individually examined *I. ricinus* ticks (2.0% of nymphs and 29.7% of adults) (Stańczak et al., 2004), while in a Slovak suburban forest, it was 8.3% in individually examined adults (Derdáková et al., 2003). Adult ticks were more often infected than nymphs in all these studies, including our study. Interestingly, the overall 1.9% MIR with *A. phagocytophilum* in our forest region representing a natural ecosystem was comparatively low. This figure differs from higher prevalences of individually examined ticks published for north-eastern Poland (8.7% of adults) (Grzeszczuk et al., 2004), Mid Germany (9.3% of nymphs and 18.8% of adults) (Hildebrandt et al., 2010), and Norway (3.3% of nymphs and 7.1% of adults) (Rosef et al., 2008).

Summing up, prevalence data discussed in our study should be interpreted with care due to different molecular approaches used, which may cause difficulties in comparison of such data. Moreover, the use of primers lacking specificity and sensitivity in many molecular studies might contribute to low comparability of prevalence data and their overestimation, most evidently seen in molecular detection of *A. phagocytophilum* (Massung and Slater, 2003; Shukla et al., 2003; Šikutová et al., 2007).

Despite the detection of tick-borne pathogenic rickettsiae in tick vectors, only a few clinical cases (except for human granulocytic anaplasmosis) of human rickettsiosis have been documented in the Czech Republic up to now. Whereas Lyme borreliosis, tick-borne encephalitis, and tularemia are notifiable diseases, other tick-borne infections such as human granulocytic anaplasmosis or other rickettsioses do not have to be reported. Thus, we currently do not know the true incidence of these neglected tick-borne diseases in the human population in Europe. Limited epidemiological data might indicate that mild cases of neglected tick-borne human rickettsiosis go unrecognized (Dumler et al., 2005; Cochez et al., 2011).

In conclusion, our molecular survey of neglected tick-borne pathogens complements data on the occurrence of *Anaplasma phagocytophilum*, '*Candidatus* Neoehrlichia mikurensis', and *Rickettsia* spp. (namely, *R. monacensis* and *R. helvetica*) in the tick *I. ricinus* in Central Europe.

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PRÁCE 11

Venclíková K., Betášová L., Šikutová S., Jedličková P., Hubálek Z., **Rudolf I.** 2014. Human pathogenic borreliae in *Ixodes ricinus* ticks in natural and urban ecosystem (Czech Republic). *Acta Parasitol.* 59: 717–720.

Stručná charakteristika: pomocí molekulárních metod (PCR, reverse-line blot) byla sledována prevalence patogenní *B. burgdorferi* v nenasátých klíšťatech na dvou lokalitách Ostravska (urbánní a přírodní). Poprvé se u nás podařilo detegovat emergentní spirochétu *B. spielmanii* v klíšťatech *I. ricinus* v urbánní lokalitě. Další patogenní borrelie představovaly tyto genomické druhy: *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s., *B. valaisiana* a *B. lusitaniae*.

Hlavní přínos práce: i urbánní lokality představují pro člověka značné riziko nákazy *B. burgdorferi*.

Příspěvek autora k dané práci: autor se podílel na designu studie, molekulárních analýzách, hodnocení i přípravě rukopisu.

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VERSITA

Human pathogenic borreliae in *Ixodes ricinus* ticks in natural and urban ecosystem (Czech Republic)

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Abstract

A total of 1279 field-collected *Ixodes ricinus* ticks were screened for *Borrelia burgdorferi* sensu lato (s.l.) in a natural and an urban ecosystem of Ostrava city (Czech Republic) by using molecular methods. Minimal prevalence rate for *Borrelia burgdorferi* s.l. in ticks for the urban park Bělský les was found to be 13.8% (17.6% in males, 17.8% in females and 11.7% in nymphs), similarly for the natural site Proskovice was minimal prevalence 15% (12.5% in males, 20% in females and 14.9% in nymphs). Six proven human pathogenic genomic species have been recorded in the study: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae*, and *B. spielmanii*. Emerging *B. spielmanii* was detected for the first time in *Ixodes ricinus* ticks in the region. Our results highlight the need for surveillance of zoonotic tick-borne pathogens even in urban areas.

Keywords,

Ixodes ricinus, *Borrelia burgdorgeri* s.l., genomic species, ixodid ticks

Introduction

Ixodid ticks (in Central Europe mainly *Ixodes ricinus*) represent a significant health risk for humans and many other vertebrate species as vectors of multiple pathogens of which the most important are flaviviruses of tick-borne encephalitis complex (TBEV), *Borrelia burgdorferi* sensu lato (s.l.), *Francisella tularensis*, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *R. slovaca*, *Babesia microti*, *B. divergens* and *B. venatorum* (EU1) (Hubálek and Rudolf 2011).

Borrelia burgdorferi s.l. is a complex of gramnegative bacteria in the Order *Spirochaetales* and contains 18 genomospecies. Spirochaete *B. burgdorferi* is prevalent in ixodid ticks in Europe, USA, Asia and North Africa, with variation in geographic and genetic distribution. Pathogenic genomic species present in Europe are: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. valaisiana*, *B. bissettii*, *B. spielmanii* and *B. bavariensis* (Stanek and Reiter 2011).

The aim of this study was to determine the minimum prevalence rate (MIR) of an important tick-borne pathogenic spirochaete *Borrelia burgdorferi* s.l. in nymphal and adult host-seeking *Ixodes ricinus* ticks in two different ecosystems: a natural (a deciduous mixed forest at Proskovice – a total of 1197 ticks were examined) and an urban (a municipal park Bělský les located in Ostrava- a total of 276 ticks were examined) by using molecular biology techniques (Real-time PCR and Reverse-line blotting) in order to assess public health risk of urban and natural site for acquiring Lyme borreliosis which is reportable disease in the Czech Republic (a total of 3304 human infections recorded in 2012 according to The National Institute of Public Health).

Materials and Methods

Study sites

Ixodes ricinus ticks were collected at two study sites: Ostrava city (49°47′N 18°14′E) and Proskovice (Ostrava surroundings, 49°44′N 18°12′E). The first study site is the urban park. Local fauna is represented by small mammals and birds, and vegetation by broadleaved deciduous trees and grass (grass cutting and long-term treatment of local trees is performed on irregular basis). The forest is surrounded by housing estates and used for leisure activities and dog-walking. The second study site is the natural ecosystem outside the town. This mixed forest with dominant broadleaved trees is rarely visited by people. The fauna consists of small and

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medium-sized mammals, roe deer, birds, and occasionally wild boars.

Ticks were collected by flagging low vegetation between April and September (a period of seasonal activity of *I. ricinus* in Central Europe) 2010. The sampled ticks were divided into test tubes according to sex and developmental stage and pooled (5 nymphs per tube, 3 adults per tube) before being frozen at –60°C.

Extraction of nucleic acids

Homogenization of ticks and genomic DNA isolation

All *I. ricinus* ticks were surface sterilized with 70% ethanol (PCR quality) and mechanically disrupted using the TissueLyser apparatus (Qiagen, Hilden, Germany) in 245 µl of PBS (Oxoid, England). The total genomic DNA was extracted from 100 µl of the tick homogenate with a QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Ticks were examined for the presence of *B. burgdorferi* s.l. using TaqMan Real-time PCR procedure and assigned to genomic species by using conventional PCR followed by Reverse line blotting.

Real-time PCR procedure

Real-time PCR for detection of *B. burgdorferi* s.l. was performed according to Courtney *et al*. (2004) using Bb23Sf/Bb23Sr primers (specific for the *B. burgdorferi* 23S rRNA gene) and Bb23Sp TaqMan probe. The fluorogenic labels at the 5´ and the 3´ ends of the probe were BHQ1 and FAM, respectively. The PCR reaction was carried out on the 7500 Real-Time PCR system (Applied Biosystems, USA) by using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). Cycling conditions as well as other PCR steps were performed according to Courtney *et al*. (2004) by using the QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany).

Touch-Down PCR, Reverse-line blotting (RLB)

Primers B-5SBor/23SBor were used for a PCR amplification of *B. burgdorferi* s.l. DNA. In addition, touchdown temperatures ranging from 60 to 52°C for *B. burgdorferi* s.l. were applied on samples (to avoid amplifying nonspecific sequences). The PCR conditions including specific concentrations of reaction mixture as well as complete RLB hybridization assay were determined according to Rijpkema *et al*. (1995) and Schouls *et al*. (1999). The RLB probes designed for *B. burgdorferi* s.l., *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, and *B. bissettii* were used (Gern *et al*. 2010). DNA extraction, PCR set-up as well as post-PCR processing were done in separate rooms to avoid possible cross-contamination of the samples.

Statistical evaluation

Differences between the MIR were evaluated using contingency tables and chi-square test at 5% probability level.

Results

A total of 1279 ticks were examined in 320 pools for human pathogenic tick-borne borreliae in the natural (251 pools) and the urban (69 pools) ecosystems in the surroundings of Ostrava. Results are briefly summarized in Table 1 and 2.

There were 217 pooled samples positive for *B. burgdorferi* s.l. (38 pooled samples from the city park and 179 pooled samples from the natural ecosystem). The overall MIR for Bělský les (the city park) was 13.8% (males 17.6%, females 17.8%, nymphs 11.7%). There was no significant difference in prevalence between males and females (P = 0.99) or between adults and nymphs (P = 0.17). The overall MIR for Proskovice (the natural ecosystem) was found to be 15% (males 12.5%, females 20%, nymphs 14.9%). There was no significant difference in the MIR between males and females (P = 0.35) or between adults and nymphs (P = 0.85) either.

B. burgdorferi s.l. genomic species were identified using reverse line blot assay (see Table 2). Overall six proven pathogenic genomic species have been recorded in the study: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae* and *B. spielmanii*.

We were unable to identify pathogenic genomic species in 17 *B. burgdorferi* s.l. positive samples, probably due to low DNA concentration. The most abundant *Borrelia* genomic species was *B. afzelii* (157 samples), followed by *B. valaisiana* (36 samples),

Table I. Numbers of tested *I. ricinus* ticks and *B. burgdorferi* s.l. prevalence (MIR ,%)

	Males	Females	Nymphs	Ticks in total
Ostrava-Bělský les (urban site)	17/51[a] (17.6)	16/45 (17.8)	36/180 (11.7)	69/276 (13.8)
Proskovice (natural site)	16/48 (12.5)	12/35 (20.0)	223/1114 (14.9)	251/1197 (15.0)
Total	33/99	28/80	259/1294	320/1473

[a]Number of pools/number of ticks tested
Explanation: Minimum infection rate (MIR) was calculated from the number of total ticks examined under assumption that every positive pool has contained only one infected individual tick

Table II. Numbers of positive samples for *B. burgdorferi* genospecies

	B. garinii	*B. afzelii*	*B. burgdorferi* s.s.	*B. valaisiana*	*B. lusitaniae*	*B. spielmanii*
Bělský les (urban site)	5	16	5	10	2	1
Proskovice (natural site)	6	141	14	26	4	0
Total	11	157	19	36	6	1

B. burgdorferi s.s. (19 samples), *B. garinii* (11 samples), *B. lusitaniae* (6 samples) and *B. spielmanii* (1 sample).

Discussion

B. burgdorferi s.l. is a worldwide complex of spirochaetes containing several genomic species. Some are recognized as human pathogens and cause Lyme borreliosis (Stanek and Reiter 2011). Here we present the MIR for *B. burgdorferi* in *I. ricinus* ticks in the natural and the urban ecosystms in the Czech Republic in order to assess the risk of acquiring the disease.

Rauter and Hartung (2005) calculated the overall prevalence of *Borrelia* species in Europe to be 13.7%. The most common genospecies were *B. afzelii* (38%), *B. garinii* (33%), *B. burgdorferi* s.s. (18%), *B. valaisiana* (19%), and *B. lusitaniae* (7%). These figures were calculated from results extracted from 154 records published from 1984 till 2003. Several studies concerning *B. burgdorferi* prevalence in ticks in the Czech Republic have been published so far. Bašta *et al*. (1999) established the prevalence of *B. burgdorferi* in *I. ricinus* ticks, collected in Prague (the capital of the Czech Republic) between years 1995–1998, to be 2.8–9.2%. *B. garinii* and *B. afzelii* were the most common genomic species detected. In city of Brno 12.1% of *I. ricinus* ticks tested positive for *B. burgdorferi* s.l. (Pejchalová *et al*. 2007). We report the MIR for the city park to be 13.8%, higher than both figures mentioned above. This might indicate a marked increase in *B. burgdorferi* prevalence in urban ecosystems in the Czech Republic or simply using more specific and sensitive molecular methods in our study.

Studies conducted in Slovakia reported identical group of genomic species as in the Czech Republic. *B. burgdorferi* MIR established by PCR was 30.2% (Smetanová *et al*. 2007). Another molecular study of Slovak *I. ricinus* ticks from 2012 yielded the MIR 25% and the first detection of *B. miyamotoi* in Slovakia (Subramanian *et al.* 2012).

Some other European countries reported prevalence of *B. burgdorferi* over 20%: Serbia – 42.5% (Milutinović *et al*. 2008), Latvia – 28% (Etti *et al*. 2003), Belgium – 23%; (Misonne *et al*. 1998), or Bulgaria – 30.7% (Christova *et al*. 2001), The prevalence in Poland varied – 5.4%, 12.3%, 22.2% and 22%, respectively (Cisak *et al.* 2006; Lenčáková *et al.* 2006; Stańczak *et al*. 2000; Sytykiewicz *et al*. 2012). All ticks tested in studies mentioned above were collected at woodland areas and forests and were analyzed individually (adults) or in pools (nymphs).

Figures reported from countries situated mostly in Western and Northern Europe only rarely exceeded 20% prevalence: Germany – 15.8% (Vögerl *et al.* 2012), Austria – 14.5% (Blaschitz *et al.* 2008), Luxembourg – 11.3% (Reye *et al.* 2010), Norway – 16% (Jenkins *et al.* 2001), Lithuania – 13.3% (Paulauskas *et al.* 2008), Denmark – 11% (Skarphédinsson *et al.* 2007), the Netherlands – 7.6% (Wielinga *et al.* 2006), Switzerland – 17.4% (Gern *et al*. 2010). In Ireland prevalence ranged between 11.5% – 28.9% according to study site (Kirstein *et al.* 1997).

Most common human pathogenic *B. burgdorferi* genomic species in Europe are *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana* (Rauter and Hartung 2005). The frequency of individual genomic species varies among countries. We reported here the presence of all genomic species mentioned above and the first time detection of *B. spielmanii* in the Moravian region. *B. spielmanii* has been detected rarely in Germany and Switzerland (Gern *et al.* 2010; Vögerl *et al.* 2012). Many studies fail to include *B. spielmanii* detection in their analysis therefore its prevalence in Europe might be in fact higher than suggest available data.

These results contribute to the surveillance of selected tick-borne pathogens in the surroundings of Ostrava city. Molecular survey represents scientific background for the comparison of prevalence data among other European countries and complements missing information concerning occurrence of *Borrelia burgdorferi* s.l. in the tick *I. ricinus* in urban ecosystem. Spirochaete *B. spielmanii* has been detected for the first time in *I. ricinus* ticks from urban locality, highlighting the need for surveillance of neglected tick-borne pathogens even in urban areas.

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PRÁCE 12

Venclíková K., Mendel J., Betášová L., Hubálek Z., **Rudolf I.** 2015. First evidence of *Babesia venatorum* and *Babesia capreoli* in questing *Ixodes ricinus* ticks in the Czech Republic. *Ann. Agric. Environ. Med.* 22: 212–214.

Stručná charakteristika: humánní babezióza patří mezi opomíjené zoonózy a v literatuře chybí data o jejím výskytu na našem území. Pomocí molekulárních metod jsme vyšetřili nenasátá klíšťata *I. ricinus* na přítomnost babezií ve dvou lokalitách (urbánní a přírodní).

Hlavní přínos práce: podařilo se poprvé na našem území detegovat v nenasátých klíšťatech *I. ricinus* sbíraných z vegetace *Babesia venatorum* (dříve *Babesia* sp. EU1) a také *B. capreoli* a tím prokázat možné riziko nákazy zoonotickými babesiemi.

Příspěvek autora k dané práci: autor se podílel na designu studie, molekulárních analýzách, hodnocení i přípravě rukopisu.

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SHORT COMMUNICATION

First evidence of *Babesia venatorum* and *Babesia capreoli* in questing *Ixodes ricinus* ticks in the Czech Republic

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Venclikova K, Mendel J, Betasova L, Hubalek Z, Rudolf I. First evidence of *Babesia venatorum* and *Babesia capreoli* in questing *Ixodes ricinus* ticks in the Czech Republic. Ann Agric Environ Med. 2015; 22(2): 212–214.

Abstract

Introduction and objective. *Ixodes ricinus* is the most common tick species occurring in Central Europe and it serves as a principal vector of emerging human pathogens. The aim of this study was to determine the prevalence of *Babesia* spp. in host-seeking *I. ricinus* in urban and natural habitats.

Materials and methods. PCR was applied on samples to assess prevalence of *Babesia* spp. in questing ixodid ticks. Sequencing was used for *Babesia* species determination.

Results. 1,473 *I. ricinus* ticks (1,294 nymphs, 99 males and 80 females) were examined for the presence of *Babesia* spp. at the two study sites. Minimum infection rate for *Babesia* spp. was found to be 0.5% (infected *I. ricinus* nymphs were only detected in the natural ecosystem). Two *Babesia* species were identified by sequencing: *B. venatorum* (formerly called *Babesia* sp. EU1) and *B. capreoli*.

Conclusions. The results obtained represent the first evidence of the occurrence of *B. venatorum* and *B. capreoli* in host-seeking *I. ricinus* ticks in the Czech Republic.

Key words

Babesia sp. EU1, *Babesia venatorum, Babesia capreoli, Ixodes ricinus,* ixodid ticks

INTRODUCTION

Ixodid ticks (mainly *Ixodes ricinus* in Central Europe) present a significant health risk for humans, being vectors of multiple pathogens, of which the most important are the tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Rickettsia* spp., and *Babesia* spp. [1].

Babesiae are protozoan intraerythrocytic organisms belonging to the phylum *Apicomplexa*. More than 100 *Babesia* species infect a wide variety of wild and domestic animals, such as horses, sheep and pigs, but only few have been documented to infect humans. The first human case of babesiosis (caused by *B. divergens*) was reported in 1957 in Europe [2] and today the disease shows a worldwide distribution. The typical host reservoirs for *Babesia* spp. in Europe are cattle, roe deer and small mammals. In Europe, many ixodid tick species can transmit babesiae to their natural hosts; however, *I. ricinus* is the most important human-biting tick involved and is the only species thought to transmit the main *Babesia* spp. (*B. microti*, *B. divergens* and *B. venatorum*) that cause human babesiosis. Most of the patients are asplenic and immunosuppressed. Interestingly, transplacental transmission and transmission through transfusion of blood and blood products have been documented in areas where babesiosis is endemic [3].

Monitoring tick vectors and the pathogens they transmit is an important tool within the scope of epidemiological surveillance. In Central Europe, however, data on the regional distribution and possible risk areas for acquiring babesiosis via tick infestation are not available.

Therefore, a molecular based survey was carried out on the prevalence of zoonotic babesiae in nymphal and adult host-seeking ticks in natural and urban ecosystems, in order to complete data on their occurrence in Central Europe.

MATERIALS AND METHODS

Study sites. *Ixodes ricinus* ticks were collected at two study sites: Ostrava city (49°47'N 18°14'E) and Proskovice (Ostrava surroundings, 49°44'N 18°12'E). The first study site is an urban park. Local fauna is represented by small mammals and birds, and vegetation by broadleaved deciduous trees and grass. The wood is surrounded by housing estates and used for leisure activities and dog-walking. The second study site is a natural ecosystem outside the town. This mixed forest with dominant broadleaved trees is rarely visited by people. Its fauna consists of small and medium-sized mammals, roe deer, birds, and occasionally wild boars.

Ticks were collected by flagging low vegetation between April and September (a period of seasonal activity of *Ixodes ricinus* in Central Europe) 2010. The sampled ticks were divided into test tubes according to gender and developmental stage and pooled (5 nymphs per tube, 3 adults per tube) before being frozen and maintained at –60 °C.

Homogenization of ticks and genomic DNA isolation. All *I. ricinus* ticks were surface sterilized with 70% ethanol (PCR quality) and mechanically disrupted using a TissueLyser apparatus (Qiagen, Hilden, Germany) in 245 µl of PBS (Oxoid, England). The total genomic DNA was extracted

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from 100 µl of the tick homogenate with a QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

***Babesia* spp. detection.** Single-step PCR was performed according to protocol published earlier [4]. The primers used for *Babesia* spp. detection were BJ1 (5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (5'-TAG TTT ATG GTT AGG ACT ACG-3'), amplifying a fragment of the 18S rRNA (411–452 bp). The total volume of DNA reaction mixture for *Babesia* spp. DNA detection was 25 µl (5µl of extracted DNA as a template) and 50 µl (10µl of extracted DNA as a template) for sequencing. The reaction was performed in a thermal Mastercycler epgradient (Eppendorf, Germany). The PCR assay consisted of an initial denaturation step (10 min at 94 °C), 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), and elongation (2 min at 72 °C). The final amplification lasted for 2 min at 72 °C. The PCR products were separated electrophoretically in 1.5% agarose gel under standard conditions. The products were treated with non-toxic GelRed stain (Biotium Inc., USA) and visualized using standard UV transillumination. Positive (*Babesia microti*, *Babesia venatorum* and *Babesia capreoli* DNA) as well as negative (ultra pure PCR H_2O) controls were included. PCR positive samples were subjected to sequence analysis.

Sequence analysis of PCR product. The PCR product was purified by precipitation with PEG/Mg/NaAc (26% polyethylene glycol, 6.5 mM $MgCl_2.6H_2O$, 0.6 M NaAc.$3H_2O$). Direct sequencing of the purified PCR product was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, U.S.A) according to the manufacturer's instructions, and purified with EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The PCR amplicons were bi-directionally sequenced once to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR Inc., USA) and also checked manually. The FASTA format and BLAST program (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches. Representative sequences were submitted to the GenBank database.

RESULTS

A total of 1,473 *I. ricinus* ticks (1,294 nymphs, 99 males and 80 females) (Tab.1) were examined. From 320 tick pools in total, 251 contained ticks from the study site representing a natural ecosystem and 69 pools contained ticks from the city park. No positive samples were detected in ticks collected in the city park. This can be explained by the absence of wild ungulates, the main reservoir hosts of *Babesia* spp. parasites. On the contrary, there were 7 samples positive from natural ecosystem, all of them were nymphs. MIR for the study site representing the natural ecosystem was 0.5% in total, and 0.6% calculated only for nymphs (Tab. 2). *Babesia* species identified by sequencing were *B. venatorum* (2 positive amplicons were deposited in GenBank under Accession Nos. KJ465867 and KJ465868, respectively,) and *B. capreoli* (one amplicon was deposited in GenBank under Accession No. KJ465869). Another 4 positive *Babesia* spp. specimens were assigned only to the genus *Babesia*.

Table 1. Number of tested *I. ricinus* ticks

	Males	Females	Nymphs	Ticks in total
Bělský les (urban ecosystem)	17/51[a]	16/45	36/180	69/276
Proskovice (natural ecosystem)	16/48	12/35	223/1,114	251/1,197
Total	**33/99**	**28/80**	**259/1,294**	**320/1,473**

[a]No. of pools / No. of individual ticks

Table 2. Prevalence (MIR, %) of *Babesia* spp. on study sites Bělský les and Proskovice

	City park (Bělský les)				Natural ecosystem (Proskovice)			
	Males	Females	Nymphs	Total	Males	Females	Nymphs	Total
Babesia spp.	0	0	0	0	0	0	0.6	0.5

Minimum infection rate (MIR) was calculated from the number of total ticks examined under the assumption that every positive pool contained only one infected tick.

DISCUSSION

For the purposes of epidemiology and phylogeny, PCR and sequence analyses of the amplicons has proved powerful in more exact species identification, especially in newly-recognized organisms [3]. The overall prevalence of babesiae in *I. ricinus* was detected as 0.5% in this study. The rates of *Babesia* spp. in *I. ricinus* are usually low and stay under 10%. In the Czech Republic, Rudolf et al. 2005 [5] tested pooled *I. ricinus* ticks for the presence of *B. microti* using the molecular approach. MIR was determined to be 1.5%. Infection rates of Babesia spp. in ticks in Europe are usually rather low, *Babesia* spp. prevalence rates under 1% in *I. ricinus* ticks were reported from several European countries: Norway 0.9% (*B. venatorum*, *B. divergens*, *B. capreoli* and previously undescribed *Babesia* species were identified in individually examined *I. ricinus* ticks [6]), Hungary – 0.5% and 0.3% (*B. divergens* and *B. microti*, respectively [7]) and Italy – 0.85% (*B. venatorum*) [8]. The prevalence rates might be underestimated in the study from Hungary, where only pooled nymphs were tested, and the Italian study, where only adults or pooled samples were examined, respectively. In Germany, the prevalence of *Babesia* spp. in individually examined ticks was reported to be 0.4% in 2009 and 0.5–0.7% in 2010 (*B. venatorum*, *B. divergens* and *B. gibsoni* [9]). In Switzerland, 0–1.3% of individually examined ticks were infected with *B. venatorum* and *B. microti*, depending on the study area [4]. A few studies from Germany, Austria and Poland report the prevalence of *Babesia* spp. to be higher. In Germany, 4.1%, 5.5% and 6.1% of individually tested *I. ricinus* ticks from recreational areas were infected, depending on the study area. *Babesia* species identified were *B. venatorum*, *B. microti*, *B. divergens* and *B. capreoli* [10].

Interestingly, *B. capreoli* is closely related to *B. divergens* and differs marginally in the 18S rDNA region [11]. This could be the reason for incorrect species identification in several old studies. Results from several studies from Poland also demonstrate the variation in prevalence rates: 16.3% in 2001 (*B. microti* and *B. divergens*: North-West Poland [12]), 5.4% in 2006 (*B. microti*: East Poland [13]), 3.1% in 2012 (*B. microti*: Central-Eastern Poland [14]). Ticks were tested either

separately [12, 14] or in combination of individual adults and pooled nymphs [13]. Different molecular approaches used in the discussed prevalence studies also had to be taken in account.

Despite the detection of tick-borne pathogenic babesiae in tick vectors, no autochthonous clinical cases (except of one imported infection) have been documented in the Czech Republic [15]. Unfortunately, the current incidence of neglected tick-borne diseases in the human population in Europe is not known. Whereas Lyme borreliosis, tick-borne encephalitis or tularaemia, are notifiable diseases in a number of European countries, other tick-borne infections, such as babesiosis or anaplasmosis, are not reportable. Moreover, the absence of seroepidemiological data from many European countries indicates that some neglected tick-borne human infections may go unrecognized.

CONCLUSIONS

This is the first report on the detection of *B. capreoli* and *B. venatorum* in host-seeking *I. ricinus* ticks in the Czech Republic. Finding of *Babesia venatorum* in *I. ricinus* ticks in the region poses a potential risk for acquiring human zoonotic babesiosis. Increased medical awareness, including information on the specific eco-epidemiology, risk factors, and improved diagnostic and preventive measures, are needed to provide a better insight of this rare but sometimes life-threatening zoonosis.

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PRÁCE 13

Venclíková K., Mendel J., Betášová L., Blažejová H., Jedličková P., Straková P., Hubálek Z., **Rudolf I.** 2016. Neglected tick-borne pathogens in the Czech Republic, 2011-2014. *Ticks and Tick-borne Dis.* 7: 107–112.

Stručná charakteristika: v rámci mezinárodního projektu EDENext byla dlouhodobě sledována prevalence tzv. opomíjených patogenů přenášených klíšťaty *I. ricinus* (*A. phagocytophilum*, *Babesia* spp., *Rickettsia* spp., '*Candidatus* Neoehrlichia mikurensis') v různých biotopech (urbánní, přírodní a pastvinný).

Hlavní přínos práce: podařilo se detegovat opomíjené patogeny ve všech typech biotopů**,** které tak představují významné riziko pro možnost nákazy člověka. Práce zabývající se surveillance klíšťaty přenášených patogenů je v českém prostředí spíše ojedinělá.

Příspěvek autora k dané práci: autor se podílel na designu studie, molekulárních analýzách, hodnocení i přípravě rukopisu.

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Original article

Neglected tick-borne pathogens in the Czech Republic, 2011–2014

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ABSTRACT

In this study, we screened a total of 2473 questing (years 2011–2014) and 199 engorged (years 2013 and 2014) *Ixodes ricinus* ticks for the presence of *Rickettsia* spp., "*Candidatus* Neoehrlichia mikurensis", *Anaplasma phagocytophilum*, and *Babesia* spp. Host-seeking ticks were collected at three study sites corresponding to natural woodland, urban park and pastureland ecosystem, and analyzed using molecular techniques. All pathogens tested were present at all study sites. The prevalence rates for *Rickettsia* spp., '*Candidatus* Neoehrlichia mikurensis', *Anaplasma phagocytophilum*, and *Babesia* spp. ranged from 2.6% to 9.2%, 0.8% to 11.6%, 0% to 12.1%, and 0% to 5.2%, respectively. Engorged *I. ricinus* ticks collected from sheep on pastureland in the years 2013 and 2014 yielded prevalence rates 7.4% and 6.3%, respectively, for *Rickettsia* spp., 38.5% and 14.1% for '*Candidatus* N. mikurensis', 18.5% and 12.5% for *A. phagocytophilum*, and 4.4% and 0.0% for *Babesia* spp. Monitoring of neglected tick-borne pathogens within the scope of epidemiological surveillance is an important tool for prevention and control of human tick-borne infections.

Introduction

Ixodid ticks are vectors of multiple pathogens, several of which can cause human infection (e.g., tick-borne encephalitis, Lyme borreliosis, anaplasmosis, rickettsioses). *Rickettsia* spp., '*Candidatus* Neoehrlichia mikurensis' and *Anaplasma phagocytophilum* are bacteria from the Order *Rickettsiales*. They are intracellular parasites depending on eukaryotic cell (Kawahara et al., 2004; Dumler et al., 2001), transmitted by ixodid ticks and causing a febrile disease with headache, muscle pain and rash (Parola et al., 2005; Welinder-Olsson et al., 2010; Bakken and Dumler, 2006). Their importance has been increasingly recognized during last years, and new *Rickettsia* organisms are still being described. In addition, some species of rickettsiae previously considered to be non-pathogenic have been associated with clinical human disease (*Rickettsia helvetica*).

'*Candidatus* N. mikurensis' is a recently recognized bacterium related to *A. phagocytophilum*. Its importance was recognized in 2010 by describing first human infection (Welinder-Olsson et al., 2010). Patients are usually immunocompromised and/or splenectomised, and fatal infection in such cases can occur.

A. phagocytophilum is a blood cell parasite distributed over Europe, Asia, America and North Africa. There are several variants circulating between vertebrate hosts and ixodid ticks but not all of them are pathogenic for human (Overzier et al., 2013).

Babesia spp. is a protozoan microorganism. It is the second most common blood-borne parasite of mammals after trypanosomes (Telford et al., 1993). The number of cases is rising and newly recognized species are associated with human disease (Hildebrandt et al., 2007).

Pathogens mentioned above are often 'neglected' by general practitioners. When unspecific clinical symptoms (fever, fatigue) appear after tick bite, Lyme borreliosis or tick-borne encephalitis are in the first line of suspicion. However, these often non-notifiable infections (human anaplasmosis, neoehrlichiosis, rickettsiosis and babesiosis) are usually diagnosed with delay or even remain unrecognized.

The aim of this study was to determine prevalence of selected pathogens in *Ixodes ricinus* ticks in different habitats (natural, urban and agricultural) in Moravia – the eastern part of the Czech Republic.

Materials and methods

Tick collections

I. ricinus ticks were collected by flagging (with white 0.5 m × 1 m cloth) vegetation at three study sites representing different ecosystems: Valtice – urban park, Pohansko – natural woodland ecosystem, and Suchov (Suchovské Mlýny) pastureland ecosystem.

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Valtice is an urban park (48°44′ N, 16°45′ E). It is a well-attended, regularly maintained locality used for leisure activities and dog walking. Local vertebrate fauna is represented by lizards, small rodents, insectivores, medium-sized mammals and birds. Regularly cut grass areas are separated by paths and tree lines. Pohansko (48°43′ N, 16°53′ E) represents a natural ecosystem of mixed floodplain forest and meadows. The vertebrate fauna consists of small rodents, birds, red deer, roe deer and wild boar and sporadically foxes. Suchov (48°53′ N, 17°34′ E) is a pastureland (for sheep) with solitary trees and bushes restricted by fencing. Other wild large animals are therefore excluded from the area. Engorged *I. ricinus* female ticks were also collected from sheep in 2013 and 2014 at the same study site. Collection of engorged ticks from sheep was performed in September (during sheep shearing) while host-seeking ticks were collected from vegetation from May to June.

The climatic region is characterized by annual average temperature of 8–10 °C, and the average precipitation is 500–600 mm (data from the Czech Hydrometeorological Institute).

Nucleic acid extraction

I. ricinus ticks were analyzed individually. All specimens were mechanically disrupted using TissueLyser apparatus (Qiagen, Hilden, Germany) in 105 μl of PBS (Oxoid, England). The total genomic DNA was extracted with a QIAamp DNA minikit (Qiagen, Hilden, Germany) from 100 μl of the tick homogenate according to the manufacturer's instructions.

Ticks were examined for the presence of the following bacterial species from the Order *Rickettsiales*: *Rickettsia* spp., '*Candidatus* N. mikurensis', *A. phagocytophilum*; moreover, for the protozoans *Babesia* spp.

PCR procedures

Single-step PCR was used for *Rickettsia* spp., '*Candidatus* N. mikurensis', and *Babesia* spp. detection. PCR protocols used were adapted from previously published studies (Table 1). The PCR products were separated electrophoretically in 1.5% agarose gel under standard conditions. The products were visualized by GelRed (Biotium Inc., USA) staining and UV transillumination. Selected samples (samples with sufficient DNA concentration) were sequenced.

Real-time PCR detection of *A. phagocytophilum* was performed according to Courtney et al. (2004) including specific primers and probe (Table 1). The PCR reaction was carried out on the 7500 Real-Time PCR system (Applied Biosystems, USA) by using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany).

Sequence analysis of PCR product

The PCR product was purified by precipitation with PEG/Mg/NaAc (26% polyethylene glycol, 6.5 mM $MgCl_2.6H_2O$, 0.6 M $NaAc.3H_2O$). Direct sequencing of the purified PCR product was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) according to the manufacturer's instructions, and purified with EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). PCR amplicons were bidirectionally sequenced once to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR Inc., USA) and also checked manually. The FASTA format and BLAST program (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches. Selected samples positive by PCR for *Rickettsia* spp. and '*Candidatus* N. mikurensis' were subjected to sequence analysis (30 amplicons for *Rickettsia* spp. and 20 amplicons for '*Candidatus* N. mikurensis').

Statistical evaluation

Prevalence rates of particular pathogens were calculated for every study site, agent and year, and differences among them were evaluated using contingency tables with chi-square (wherever possible: Siegel, 1956), otherwise with Fisher's 2×2 exact test or $2 \times r$ exact test.

Results

A total of 2473 questing *I. ricinus* ticks were collected and tested for the presence of pathogen DNA. Numbers of ticks and prevalence rates according to collection year, state of development and study sites are shown in Tables 2 and 3.

A total of 199 engorged *I. ricinus* female ticks collected from sheep were tested individually for afore mentioned pathogens presence. The prevalence rates are shown in Table 4.

Male and female ticks were merged for statistical calculations of infection rates and further assessed as "adults" group. For *Rickettsia* spp., the prevalence did not vary significantly among tick stages and study sites (Table 3). Amplicons were 100% identical with *Rickettsia monacensis* (GenBank accession no. JX003686) and *Rickettsia helvetica* (GenBank accession no. KF447530), respectively. The two species were distributed equally across all three study sites.

For '*Candidatus* N. mikurensis', the prevalence varied significantly between study sites in both adults and nymphs, between years in nymphs at Pohansko and in adults at Valtice and Pohansko, while no significant difference was found between developmental stages except for Suchov. In total 20 amplicons have shown 100% identity with the rickettsia 'Candidatus N. mikurensis' (GenBank accession no. GQ501090) which was detected in the blood of a 61-year-old man with signs of septicemia (Fehr et al., 2010).

A. phagocytophilum prevalence among the years varied significantly in both adults and nymphs at Valtice, among the study sites in nymphal ticks, and it differed between tick stages at all three study sites.

Table 1
PCR protocols and primers used for pathogen detection.

Organism	Primer sequences	Reference
Rickettsia spp.	Rp877p: 5′-GGG GAC CTG CTC ACG GCG G-3′ Rp1258n: 5′-ATT GCA AAA AGT ACA GTG AAC A-3′	Regnery et al. (1991)
"*Candidatus* N. mikurensis"	Mikurensis_R: 5′-GCC AAA CTG ACT CTT CCG-3′ Mikurensis_F: 5′-GGC GAC TAT CTG GCT CAG-3′	Fertner et al. (2012)
Anaplasma phagocytophilum	ApMSP2f: 5′-ATG GAA GGT AGT GTT GGT TAT GGT ATT-3′ ApMSP2r: 5′-TTG GTC TTG AAG CGC TCG TA-3′ ApMSP2p BHQ1 5′-TGGTGCCAGGGTTGAGCTTGAGATTG -3 FAM	Courtney et al. (2004)
Babesia spp.	BJ1: 5′-GTC TTG TAA TTG GAA TGA TGG-3′ BN2: 5′-TAG TTT ATG GTT AGG ACT ACG-3′	Casati et al. (2006)

Table 2
Prevalence of pathogens in host-seeking *Ixodes ricinus* ticks at three study sites, 2011–2014.

Year		Valtice adults	Valtice nymphs	Pohansko adults	Pohansko nymphs	Suchov adults	Suchov nymphs
2011	*Rickettsia*	5[a]	5	1	4	2	29
	Neoehrlichia	1	1	0	3	4	25
	Anaplasma	6	2	3	0	3	6
	Babesia	0	0	0	0	1	1
		131[b]	133	29	98	47	376
2012	*Rickettsia*	6	8	2	1	5	5
	Neoehrlichia	2	2	2	0	1	1
	Anaplasma	5	4	0	0	2	1
	Babesia	0	0	0	0	0	0
		47	105	8	72	39	80
2013	*Rickettsia*	7	8	1	12	1	9
	Neoehrlichia	6	2	1	26	1	8
	Anaplasma	7	3	3	2	0	1
	Babesia	1	1	0	2	3	6
		118	187	28	205	29	186
2014	*Rickettsia*	4	13	2	2	3	3
	Neoehrlichia	7	8	1	10	12	3
	Anaplasma	18	12	4	2	7	2
	Babesia	1	1	2	5	3	5
		86	161	25	128	69	86
2011–14	*Rickettsia*	22	34	6	19	11	46
	Neoehrlichia	16	13	4	39	18	37
	Anaplasma	36	21	10	4	12	10
	Babesia	2	2	2	7	7	12
		382	586	90	503	184	728
Prevalence rate (%)							
	Rickettsia	**5.8**	**5.8**	**6.7**	**3.8**	**6.0**	**6.3**
	95% CI	2.8–8.8	3.4–8.2	0.1–12.3	1.7–5.9	1.6–10.4	4.0–8.6
	Neoehrlichia	**4.2**	**2.2**	**4.4**	**7.8**	**9.8**	**5.1**
	95% CI	1.6–6.8	0.7–7.3	0.0–9.8	4.8–10.8	4.3–14.8	3.1–7.1
	Anaplasma	**9.4**	**3.6**	**11.1**	**0.8**	**6.5**	**1.4**
	95% CI	5.7–13.1	1.7–5.5	2.8–19.4	0.0–1.8	2.0–11.0	0.3–2.5
	Babesia	**0.5**	**0.3**	**2.2**	**1.4**	**3.8**	**1.6**
	95% CI	0.0–1.4	0.0–0.9	0.0–6.0	0.1–2.7	0.3–3.7	0.4–2.8

[a] No. ticks positive.
[b] No. ticks examined.
CI: confidence intervals.

Table 3
Significance of differences in the prevalence of pathogens: contingency tables, chi-square test (wherever applicable: Siegel, 1956), in other cases Fisher 2 × 2 exact test or 2 × *r* exact test were used.

	Rickettsia spp.	'*Candid*. N. mikurensis'	*Anaplasma phagocytophilum*	*Babesia* spp.
Between tick stages (adults vs. nymphs, 2011–2014 total)				
Valtice	NS	NS	***	NS
Pohansko	NS	NS	***	NS
Suchov	NS	*	***	NS
Between study sites (2011–2014 total)				
Adult ticks	NS	*	NS	**
Nymphal ticks	NS	***	**	NS
Between years (2011–2014)				
Valtice adults	NS	*	***	NS
Valtice nymphs	NS	NS	*	NS
Pohansko adults	NS	*	NS	NS
Pohansko nymphs	NS	**	NS	NS
Suchov adults	NS	NS	NS	NS
Suchov nymphs	NS	NS	NS	***

NS: the difference is not significant ($P > 0.05$).
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.

Table 4
Prevalence of particular pathogens in engorged *I. ricinus* females collected from sheep (S) vs. host-seeking females collected from vegetation (V) in Suchov. Significance of the differences between "S" and "V" ticks was tested by Fisher's exact 2 × 2 test.

Collection year		*Rickettsia* spp.	'*Candidatus* N. mikurensis'	*Anaplasma phagocytophilum*	*Babesia* spp.	No. ticks tested
2013	S	10 (7.4%)[a]	52 (38.5%)[a]	25 (18.5%)[a]	6 (4.4%)[a]	135
	V	0 (0.0%)	1 (5.6%)	0 (0.0%)	1 (5.6%)	18
2014	S	4 (6.3%)[a]	9 (14.1%)[a]	8 (12.5%)[a]	0 (0.0%)[a]	64
	V	0 (0.0%)	7 (19.4%)	3 (8.3%)	3 (8.3%)	36
Total	S	14 (7.0%) (3.4–10.6%)[b]	61 (30.7%) (24.2–37.2%)[b]	33 (16.6%) (11.3–21.9%)[b]	6 (3.0%) (0.6–5.4%)[b]	199
	V	0 (/0.0%) –	8 (14.8%) (5.1–24.5%)[b]	3 (5.6%) (0.0–11.9%)[b]	4 (7.4%) (0.3–14.5%)[b]	54
Significance		*	*	*	NS	

[a] Number of positive ticks (prevalence %).
[b] Confidence interval, 95%.
NS: the difference is not significant ($P > 0.05$)
* Significant at $P < 0.05$.

Prevalence of *Babesia* spp. in adult ticks was significantly higher at Suchov than in the two other study sites, and in nymphal ticks in the years 2014 and 2013 as opposed to 2011 and 2012. In addition, low DNA content in positive specimens did not allow us to discriminate between *Babesia* spp.

We compared the prevalence of pathogens in questing and engorged ticks on the "Suchov" study site in the years 2013 and 2014. *Ricketssia* spp., '*Candidatus* N. mikurensis' and *A. phagocytophilum* showed significantly higher prevalence rate in engorged ticks collected from sheep compared to questing ticks, while the difference was insignificant for *Babesia* (Table 4).

Discussion

This paper presents unique results of a four-year surveillance study of tick-borne pathogens in the Czech Republic. We confirmed a long-term presence of several agents (*Rickettsia* spp., '*Candidatus* N. mikurensis', *A. phagocytophilum* and *Babesia* spp.) in questing and engorged *I. ricinus* ticks in different ecosystems.

The importance of *Rickettsia* spp. has been increasingly recognized worldwide. New species are being described and/or connected to a human disease (Pérez-Pérez et al., 2010; Mediannikov et al., 2008; Jado et al., 2007; Paddock et al., 2004). The prevalence of *Rickettsia* spp. in Czech *I. ricinus* ticks is continuous without a significant variation among years or ecosystems. The prevalence under 10% is supported by our previous study from 2010 (Venclíková et al., 2014) and by Špitalská et al. (2014) who reported *Rickettsia* spp. prevalence 9% in Slovakia. The prevalence of rickettsiae in *I. ricinus* ticks in Croatia reached up to 7.9% (Tijsse-Klasen et al., 2013) and 11.7% in Belarus (Reye et al., 2013). Michelet et al. (2014) published an extensive study monitoring 37 bacterial and protozoan agents in three European countries (France, Denmark and the Netherlands), including all agents followed in this study. The *Rickettsia* spp. prevalence rates were 14.3%, 10.4–14.3%, 4.5–11.9%, respectively.

There is a number of rickettsial species circulating in Europe that are pathogenic for human. Clinicians are mostly unaware of the risks and the diagnosis is delayed or incorrect which can lead to a treatment failure. New symptoms are being assigned to known agents and the infections are becoming more severe. The prognosis is worsened with advanced age, immunodeficiency, or alcoholism (Parola et al., 2013).

First '*Candidatus* N. mikurensis' detection reported by Schouls et al. (1999). The connection of this bacterium to a human infection (Welinder-Olsson et al., 2010) drew an attention. The prevalence reported in this study was 0.8–11.6%, depending significantly on study site and year. Our previous study showed the prevalence of 0.4% (urban park) and 4.4% (woodland ecosystem) (Venclíková et al., 2014). Our results correspond to prevalence reports of the bacterium in Europe: 4.2% in Austria (Glatz et al., 2014), 0.2% in Poland (Welc-Falęciak et al., 2014), 1.9% in Hungary (Szekeres et al., 2015), 1.1–11.6% in Slovakia (Pangrácová et al., 2013; Derdáková et al., 2014), and 7% in the Netherlands (Jahfari et al., 2012). Extensive prevalence studies are still missing in Europe although an increasing number of human cases show the need for more data concerning this pathogen (Welinder-Olsson et al., 2010; Fehr et al., 2010; Maurer et al., 2013; Pekova et al., 2011). Patients are usually immunocompromised but a fatal case of a patient with no immune deficiency was also reported (Von Loewenich et al., 2010). Also Li et al. (2012) reported a series of seven previously healthy patients with neoehrlichial infection. To the best of authors' knowledge, this is the first observation of '*Candidatus* N. mikurensis' in engorged ticks removed from sheep. Interestingly, high prevalence of '*Candidatus* N. mikurensis' in these ticks highlights the need for further experimental studies (including xenodiagnostic experiments) to assess possible reservoir role of sheep in maintenance of this pathogen in specific habitat, where wild rodents-proven vertebrate reservoirs of '*Candidatus* N. mikurensis' (Andersson and Raberg, 2011; Jahfari et al., 2012; Burri et al., 2014) are not prevalent. However, another hypothesis for high '*Candidatus* N. mikurensis' prevalence in engorged ticks should be taken in account, e.g., possible multiplication of the agent in feeding ticks, and, consequently, a shift in the agent's detection threshold in the molecular screening. This phenomenon was simultaneously documented in ticks removed from humans and tested for tick-borne encephalitis virus (Suess et al., 2006). Another explanation could be transmission of these rickettsiae by co-feeding of *I. ricinus* female ticks on sheep.

A. phagocytophilum is well established in European ixodid ticks. The agent has been detected in most countries (Stuen et al., 2013). The prevalence varies greatly, depending on year and study site: 0.3–25.4% (Cotté et al., 2010; Karbowiak et al., 2014). A year-to-year variation in prevalence, typical for *A. phagocytophilum*, might be affected by global factors like weather condition and appearance of vector and hosts at the site (Overzier et al., 2013). According to a recent study (Szekeres et al., 2015), different prevalences of pathogens between urban and natural sites might be explained by different type of habitats, e.g., natural habitats are open with a broad range of vertebrate hosts (rodents, game animals), while urban habitats are closed with limited numbers of hosts (hedgehogs, dogs, cats, synanthropic birds). The prevalence of *A. phagocytophilum* found in this study (0–12.1%) falls within range of the prevalence rates published in our previous study (9.4%

for urban park and 1.9% for natural ecosystem: Venclíková et al., 2014) or those reported recently in other European countries: 3.8% (Derdáková et al., 2014) and 2.7% (Pangrácová et al., 2013) in Slovakia, 3.1% in Hungary (Szekeres et al., 2015), l1.2% in France, 11.9% (Grib Skov locality) and 0.4% (Vestskoven locality) in Denmark and 2% in the Netherlands (Michelet et al., 2014).

A. phagocytophilum is the only pathogen in this study with significant difference in prevalence between tick stages at all three study sites (adults infected more often than nymphs). But only nymphs revealed difference in prevalence according to study sites.

Human anaplasmosis is not a reportable disease in most countries and thus it is impossible to assess its risk for public health (Edouard et al., 2012). Moreover, the coinfections with *Borrelia burgdorferi* or tick-borne encephalitis virus are quite common. In addition, there are recognized several genetic variants of *A. phagocytophilum*. Overzier et al. (2013) identified 9 variants according to 16S rRNA gene sequences. Different host species were susceptible to different genetic variants and so far only three variants have been associated with human cases. The *A. phagocytophilum* prevalence and genetic variants depend on study site structure and reservoir host availability.

According to Jahfari et al. (2014), sequences from *gro*EL operon delineate ecotypes of *A. phagocytophilum* more clearly than sequences of 16S rRNA. All human associated sequences used in the study clustered in Ecotype I. that also shows the largest range of hosts. Ecotypes II, III and IV have roe deer, rodents and birds, respectively, as dominant hosts.

A multilocus sequence typing scheme was also suggested by Huhn et al. (2014). Information concerning host species, geographic distribution and zoonotic potential of certain variant can be assessed by this method.

Babesiae were present at all study sites in 2013 and 2014, at Suchov in 2011. Interestingly, all samples tested negative in 2012. The presence of *Babesia* spp. in ticks in different ecosystems is not continuous spatially. We can hypothesize whether the infection disappears and is later re-introduced or whether it is maintained in reservoir hosts at a very low level. The lowest prevalence reported here was detected in urban park in Valtice and the highest in Suchov, a pastureland where sheep are bred. *Babesia* prevalence in European ticks is generally low: 0.9% in Norway (Øines et al., 2012), 1.7% in the Netherlands (Coipan et al., 2013), 0.2–0.3% in France, 0.1–1.4% in Denmark and 0.2–0.8% in the Netherlands (Michelet et al., 2014).

All three study sites represent diverse ecosystems with different host availability and human influence. Small mammals and birds are represented at all sites but probably with different densities. Well-maintained urban park Valtice does not provide habitat for larger wild animals (hares, deer) but local ticks can feed on dogs, cats and birds (occasional hosts). However, hedgehogs might play an important role for the maintenance of the agents in an urban area (Foldvari et al., 2014). Pohansko as a natural woodland ecosystem with small and larger mammals and birds presents a good offer of hosts for ticks. On the other hand, vertebrate hosts are here more dispersed and it might be more difficult to find a suitable competent host. In Suchov, sheep as the most common hosts are restricted to defined areas and live in herds. The close proximity of hosts facilitates the tick's chance to find a new host and transmit the infection.

Conclusion

We proved a continuous presence of four tick-borne pathogens in *I. ricinus* ticks in the Czech Republic. Although all pathogens occur in ticks at relatively low frequency, they present the health-risk for Czech human population. The physicians should be aware of the infection possibility and consider a tick-borne infection when nonspecific febrile and other symptoms are present.

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PRÁCE 14

Duscher G., Hodžić A., Weiler M., Vaux A.G.C., **Rudolf I.**, Sixl W., Medlock J.M., Versteirt V., Hubálek Z. 2016. First report of *Rickettsia raoultii* in field collected *Dermacentor reticulatus* ticks from Austria. *Ticks and Tick-borne Dis.* 7: 720–722.

Stručná charakteristika: v rámci mezinárodního projektu VECTORNET jsme se pokusili doplnit chybějící data ('knowledge gaps') o výskytu klíšťete *D. reticulatus* v oblasti Podunají v Rakousku. Toto klíště totiž přenáší řadu humánně i veterinárně významných patogenů.

Hlavní přínos práce: podařilo se nám najít některé nové lokality výskytu *D. reticulatus* a také poprvé v Rakousku detegovat emergentní patogen *Rickettsia raoultii* v nenasátých klíšťatech.

Příspěvek autora k dané práci: autor se podílel na vyhledávání a sběru klíšťat ve vytipovaných biotopech, molekulárních analýzách i přípravě rukopisu.

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Letter to the editor

First report of *Rickettsia raoultii* in field collected *Dermacentor reticulatus* ticks from Austria

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ABSTRACT

In a set of pooled field collected *Dermacentor reticulatus* ticks, *Rickettsia raoultii*, the causative agent of Tick-borne lymphadenopathy/*Dermacentor*-borne necrosis erythema and lymphadenopathy, was found for the first time in Austria. The coordinates of the positive locations for tick and pathogen abundance are given and shown in a map.

Q3 Tick-borne lymphadenopathy (TIBOLA), *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL) in humans have been associated with two rickettsial species of the spotted fever group, namely *Rickettsia slovaca* and *Rickettsia raoultii*. The latter species is more likely to be found in ticks only, whereas *R. slovaca* is more often associated with human cases, suggesting this species to be more pathogenic than *R. raoultii* (Parola et al., 2009). But studies on ticks derived from TIBOLA patients in Hungary suggest a similar role of importance for both pathogen species (Földvári et al., 2013).

Although data on the occurrence of these bacterial species and their two main tick vectors, namely *Dermacentor marginatus* and *Dermacentor reticulatus*, exists from many European countries such as Croatia, France, Germany, Hungary, the Netherlands, Poland, Portugal, Slovakia, Spain and United Kingdom (Földvári et al., 2013; Lakos, 1997; Špitalská et al., 2012; Szekeres et al., 2016; Tijsse-Klasen et al., 2010), there is little information on this topic available for Austria. Until now the only confirmed *Rickettsia* species in Austria is *Rickettsia helvetica* from *Ixodes ricinus* (Blaschitz et al., 2008). However, during a screening of ticks originating from dogs, indications for the occurrence of *R. raoultii* in Austria were found (Wijnveld et al., 2015). There are sporadic reports of *D. reticulatus* ticks (Rubel et al., 2016), however there is a lack of clear information on the distribution of the tick species and the pathogen in this region. Even though Austria is a small country, the presence of these ticks and bacteria could be of great importance due to its location in the centre of Europe and the potential impact on neighbouring countries.

In order to obtain data on the presence of *Dermacentor* ticks and their pathogens, field surveillance was conducted during April and May 2015. Host-seeking ticks were sampled in several suitable habitats along the Danube and March Rivers, at Neusiedler See and in southern Austria by using the flagging method (Fig. 1). The ticks were identified and only *D. reticulatus* specimens were further processed. A total of 153 *D. reticulatus* adults, comprising 80 females and 73 males, were identified. They were merged in 32 pools (average: 4.8 individuals per pool; maximum: 8 individuals per pool), depending on collection date, location and sex, then homogenised in phosphate buffered saline without antibiotics, and split into two parts. The main aim of the study was to test for *Rickettsia* spp. but also other pathogens were considered. Therefore one part of the homogenised ticks was resuspended in PBS and was inoculated subcutaneously in adult ICR SPF mice to detect *Francisella tularensis*: (Hubálek et al., 1998), and the other used for DNA extraction and subsequent molecular analyses. The DNA was extracted as previously described (Venclikova et al., 2014) and the pools were screened for bacteria such as *Anaplasma* sp., *Ehrlichia* sp., '*Candidatus* Neoehrlichia mikurensis', *Rickettsia* spp. and protozoa such as *Babesia* spp., *Hepatozoon* sp. and *Theileria* sp. by using molecular tools published elsewhere (Hodžić et al., 2015). Out of the 32 tick pools, 21 (65%) delivered positive results with the *Rickettsia* specific PCR, but negative results were obtained with all other pathogens tested (including *F. tularensis*). DNA of *Rickettsia* sp. was identified on each site *D. reticulatus* was found. Due to the fact that the pool size varied, the minimum infection rate was calculated under the assumption that per positive pool at least one individual was positive. The sum of the average mean ratio of each pool divided by the number of pools was used to give a rough estimation of the minimal infection rate in the samples and was used to calculate a 95% confidence interval by using Excel® 2002 (Microsoft, Washington): 14.9% [10.3–19.5%]. In order to determine the *Rickettsia* species, two PCRs were performed, one targeting the *gltA* gene (382 bp amplicon) and one the *ompA* gene (632 bp amplicon) (Roux et al., 1996). All pools were screened with *ompA* whereas for *gltA* two were randomly chosen (Nr. 3 and Nr. 26). The sequences obtained were 100% identical to each other and during Blast® search, a 100% [e.g. Genbank® HM161792] and 99% [e.g. Genbank® KT261764] identity to *R. raoultii* was confirmed for *ompA* and *gltA*, respectively. The sequences for *ompA* and *gltA* are deposited on Genbank® [for *gltA*: KT895941, for *ompA*: KT895942].

Both *D. reticulatus* and *R. raoultii* are abundant in Austria representing a public health threat. The minimum estimated prevalence of 14.9% in the ticks is rather low in comparison to other studies on individual samples from Poland and Germany, where in both cases

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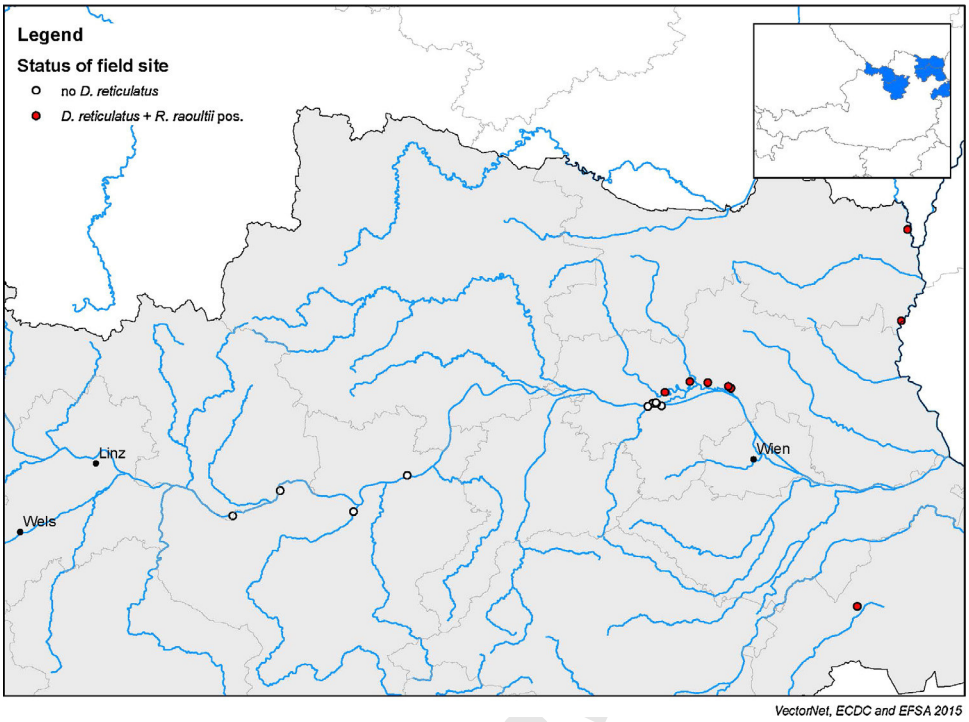

Fig. 1. Sampling locations in Austria where *D. reticulatus* is not abundant (white dots) as well as positive sites for *D. reticulatus* and *R. raoultii* (red dots). [WGS84 coordinates of the positive sites: 16.307638/48.356777; 16.299482/48.361061; 16.235937/48.372559; 16.179079/48.377647; 16.097589/48.359829; 16.865215/48.466549; 16.912508/48.657692; 16.644266/47.873352]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

56.7% of *D. reticulatus* were positive for *R. raoultii* (Chmielewski et al., 2009; Silaghi et al., 2011). But it is almost similar to Slovakia where 22.3% of the *D. reticulatus* collected from vegetation were positive for *R. raoultii* (Špitalská et al., 2012) or the United Kingdom with 27% *Rickettsia* sp. (mainly *R. raoultii*) positive *D. reticulatus* (Tijsse-Klasen et al., 2010). Anyhow, a higher focal prevalence from 57.8% vs. 15.5% in the whole country already has been reported from Hungary (Hornok et al., 2010; Szekeres et al., 2016). Additionally, variations between the studies based on different molecular methods and sensitivities have to be considered. However the value of the pooled samples in this study needs to be investigated by testing individual analysis in the future to determine the actual prevalence and not only the minimum infection rate.

Additionally, further efforts are needed to identify more sites of *D. reticulatus* occurrence as well as their pathogen load in Austria. Anyhow, this observation increased our knowledge on the distribution of the pathogens in the *D. reticulatus* and it is clearly demonstrated that this pathogen is circulating in ticks populations in Austria.

Ethical statement

All experiments with laboratory mice were conducted in accordance with the Czech Animal Protection Act no. 246/1992, and the protocols were approved by the Institutional and Central Care and Use Committees at the Academy of Sciences of the Czech Republic in Prague and by the Veterinary Service in Brno. The facility is accredited by the Czech National Committee on Care and Use of Laboratory Animals (6630/2008-10001).

Conflict of interest statement

The authors declare that they have no competing interests.

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PRÁCE 15

Hubálek Z., Zeman P., Halouzka J., Juřicová Z., Šťovíčková E., Bálková H., Šikutová S., **Rudolf I.** 2004. Protilátky k virům přenosným komáry u středočeské populace z oblasti zasažené povodní v roce 2002. *Epidemiol. Mikrobiol. Imunol.* 53: 112−120.

Stručná charakteristika: katastrofální povodně v roce 2002 v Čechách byly spouštěcím faktorem pro vznik studie (podpořené i speciálním povodňovým grantem GAČR), která si kladla za cíl vyšetřit středočeskou populaci (497 místních obyvatel) v oblasti Polabí na přítomnost protilátek na viry přenosné komáry (Ťahyňa, West Nile, Sindbis, Batai).

Hlavní přínos práce: práce je sérologickou surveillance arbovirů v místech zasažených povodněmi a dokládá, že při zvýšení populační denzity komárů v důsledku silných dešťů a rozvodnění toků může docházet i k lidským infekcím způsobených např. virem Ťahyňa, Batai či Sindbis. Práce také prokázala aktivní přírodní ohnisko viru Ťahyňa v regionu Polabí.

Příspěvek autora k dané práci: autor se podílel na inaktivaci sér, hodnocení neutralizačního testu a přípravě rukopisu.

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Protilátky k virům přenosným komáry u středočeské populace z oblasti zasažené povodní v roce 2002

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Souhrn

Ve středočeském zátopovém území bylo pomocí hemaglutinačně-inhibičního testu (HIT) a plak-redukčního neutralizačního testu (PRNT) vyšetřeno 497 místních obyvatel na protilátky proti komáry přenášeným virům Ťahyňa (TAHV), West Nile (WNV), Sindbis (SINV) a Batai (BATV; synonymum Čalovo). Krevní vzorky byly odebrány v září 2002 po kulminaci povodně v době kalamitního výskytu komárů. Séra 16,5 % vyšetřených osob obsahovala protilátky neutralizující TAHV (v HIT reagovalo 14,9 % osob). Proti WNV v HIT sice reagovalo 6,8 % a v PRNT 1,2 % osob, ovšem za současné reakce s virem klíšťové encefalitidy (KE); výsledek byl interpretován jako zkřížená reakce, a agens vyvolávajícím imunitní odpověď byl zřejmě virus KE. Protilátky k SINV i BATV byly prokázány v HIT u 1,4 % osob; v PRNT se však podařilo prokázat protilátky k BATV pouze u 1 osoby (0,2 %). Rozbor séroprevalence k TAHV v PRNT (obdobně i v HIT) ukázal její nezávislost na pohlaví (15,8 % u mužů, 16,9 % u žen), průkazně stoupající míru s věkem (1,4 % u osob mladších 20 let, 11,2 % u osob mezi 20 a 50 lety, 26,2 % u osob starších 50 let) a s intenzitou postižení místa bydliště komáří kalamitou (v kontrolní zóně D – převážně Praha – bylo séropozitivních pouze 5,0 % osob, v zóně C 14,7 %, v zóně B 20,5 %, a v nejrizikovější záplavové zóně A s maximem výskytu kalamitních komárů 28,0 % osob); nejvyšší séropozitivita (> 25 %) byla zaznamenána u obyvatel obcí Obříství, Kozly, Tuhaň, Chrást, Chlumín a Hostín. Druhé (párové) krevní vzorky byly odebrány od 150 osob z téhož souboru s odstupem 6 měsíců: k infekci virem Ťahyňa během povodně anebo na podzim po ní došlo u jedné obyvatelky Obříství (sérokonverze v HIT i PRNT), méně přesvědčivé výsledky byly zaznamenány u dalších 3 osob, obyvatel Chlumína a Obříství (sérokonverze či průkazné zvýšení titru protilátek se projevilo pouze v HIT). Náš výzkum tak indikoval existenci přírodního ohniska valtické horečky (infekce TAHV) podél toku Labe v okolí města Neratovice (Obříství, Chlumín, Tuhaň; Kozly, Tišice, Chrást). Nízká aktivita TAHV byla zjištěna na dolním toku Vltavy od Zlončic až po Bukol/Zálezlice. Zvýšení populační denzity komárů po záplavách může vytvořit podmínky pro větší incidenci virových nákaz jimi přenosných, v podmínkách středních Čech, konkrétně valtické horečky. Optimální systémovou strategií pro kontrolu těchto nákaz je epidemiologická surveillance (zahrnující monitorování početnosti komárů a jejich promořenosti viry v přírodních ohniscích), na jejímž základě je možno navrhovat a uskutečňovat preventivní protiepidemická opatření, např. integrovanou kontrolu populací komárů.

Klíčová slova: komáři – *Culicidae* – Ťahyňa virus – West Nile virus – Sindbis virus – Batai virus – Čalovo virus – povodeň.

Summary

Hubálek Z., Zeman P., Halouzka J., Juřicová Z., Šikutová S., Rudolf I.: Antibodies Against Mosquito-born Viruses in Human Population of an Area of Central Bohemia Affected by the Flood of 2002

In the Central-Bohemian area affected by the flood of 2002, 497 residents were screened for antibodies against the mosquito-borne viruses Ťahyňa (TAHV), West Nile (WNV), Sindbis (SINV) and Batai (BATV; syn. Čalovo) using the haemagglutination-inhibition (HIT) and plaque-reduction neutralization (PRNT) tests. Blood samples were collected in September 2002 when the mosquito populations showed the maximum density following the flood. Antibodies against TAHV (16.5 % persons in PRNT, 14.9 % in HIT), SINV (1.4 % in HIT) and BATV (1.4 % in HIT, 0.2 % in PRNT) were detected. Although 6.8 % and 1.2 % of the subjects tested reactive with WNV in HIT and PRNT, respectively, the results were interpreted as cross-reactivity with tick-borne encephalitis virus. The seroprevalence of TAHV (both in PRNT and HIT) showed no association with gender (15.8 % of males, 16.9 % of females), increased with age (1.4 % of persons younger than 20 years, 11.2 % of persons aged between 20 and 50 years, and 26.2 % of persons older than 50 years were positive), and correlated with the mosquito peri-residential challenge (5.0 % residents seropositive in a mosquito-free control

zone D – mostly Prague, 14.7 % in a mild-risk zone C, 20.5 % in a moderate-risk zone B, and 28.0 % in the most heavily mosquito-infested risk zone A). The highest TAHV seropositivity rate (> 25 %) was found amongst the inhabitants of the villages Obříství, Kozly, Tuhaň, Chrást, Chlumín and Hostín. Paired blood samples were obtained from 150 of the persons at a 6-month interval: an infection episode with TAHV during or after the flood was clearly evidenced in one person living in Obříství, and less convincing findings of recent TAHV infections were found in other three residents of Chlumín and Obříství (seroconversion and/or significant antibody titres increase detected in HIT only). This serosurvey indicated the existence of an active natural focus of Valtice fever (TAHV infection) stretched along the river Labe nearby Neratovice (Obříství, Chlumín, Tuhaň; Kozly, Tišice, Chrást), and a low TAHV activity area along the lower reaches of the river Vltava between Zlončice and Bukol/Zálezlice. An increased population density of mosquitoes after the flood may have boosted the incidence of mosquito-borne virus diseases, particularly Valtice fever, in Central Bohemia. An optimum prophylactic strategy to control these diseases would be epidemiological surveillance (including monitoring of both the density of mosquitoes and their rate of infection with viruses in natural foci) on the basis of which antiepidemic measures such as integrated mosquito control can be taken.

Key words: mosquitoes – *Culicidae* – Ťahyňa virus – West Nile virus – Sindbis virus – Batai virus – Čalovo virus – flood.

V novodobé historii České republiky došlo roku 2002 v poměrně velmi krátkém časovém rozpětí (po povodni v červenci 1997, jež postihla převážně Moravu) již podruhé ke katastrofálním záplavám, tentokrát především v Čechách, které po neobyčejně silných dešťových srážkách devastovaly od poloviny srpna mj. také dolní tok Vltavy včetně širšího území jejího soutoku s Labem: např. na Mělnicku bylo počínaje 15. srpnem zatopeno přes 30 obcí, a následně zničeno více než 300 domů (29). V této oblasti, která se i za normálních okolností vyznačuje poměrně pravidelným sezónním výskytem komárů, zejména v lužních lesích na soutoku obou řek, došlo od 20. srpna místy až k masovému líhnutí komárů především „kalamitních" druhů *Ochlerotatus sticticus*, *Aedes vexans* a *Ae. cinereus*, jež kulminovalo 3.–9. září intenzitou až 70 náletů samic na člověka za minutu; abundance komárů, poklesla pak dosti prudce v druhé polovině září, ale poslední jedinci vymizeli teprve v polovině listopadu (35–37). Od 24. srpna muselo být na Mělnicku provedeno několik leteckých a pozemních postřiků nejohroženějších lokalit adulticidním insekticidem Aqua Reslin Super (s aktivní látkou permethrinem).

Kalamitně přemnožení komáři jsou nejen trapiči člověka, negativně ovlivňujícími jeho aktivitu a zdravotní stav, ale mohou přenášet i některá nakažlivá onemocnění. Na Mělnicku se vyskytují komáři 18 druhů (33, 35–37) s dominujícími kalamitními druhy rodů *Aedes* a *Ochlerotatus*, vázanými na lužní ekosystém s periodickou inundací. Jejich larvy se líhnou z vajíček nakladených na půdě (kde mohou přežít řadu let) až po jejím vysušení a opětovném zaplavení. Na většině plochy tohoto území jsou navíc poměrně silné populace obratlovčích hostitelů – volně žijících savců a ptáků, představujících v některých případech současně amplifikátory komáry přenášených virů. Tím jsou vytvořeny potenciálně příznivé ekologické podmínky pro existenci a perzistenci přírodních ohnisek nákaz viry přenosnými komáry.

Náš odhad při formulaci předkládané studie byl založen na znalosti skladby středočeské fauny komárů čítající 26 druhů (tab. 1), a předpokládal možnost potenciálního výskytu nákaz člověka čtyřmi viry, které byly v Evropě z těchto druhů komárů již izolovány: Ťahyňa, a s nižší pravděpodobností West Nile, Batai (Čalovo) a Sindbis (11, 14). K posouzení míry rizika nákazy člověka viry přenosnými komáry ve středočeském zátopovém území bylo provedeno vyšetření místních obyvatel na přítomnost protilátek k uvedeným agens. Taková vyšetření v tomto regionu nebyla dosud kupodivu realizována. Pouze ve středním Polabí mezi Přerovem n.L. a Starou Boleslaví bylo před 30 lety jednorázově vyšetřeno hemaglutinačně-inhibičním testem 84 osob, přičemž byly prokázány protilátky k viru Ťahyňa u 9,5 % z nich; nejvyšší séroprevalence byla zaznamenána u Čelákovic a Sedlčánek: 15,5 % (21). Hlavním účelem našeho projektu bylo stanovení frekvence výskytu protilátek k arboviru Ťahyňa a dalším třem výše uvedeným virům přenášeným komáry u obyvatel obcí povodňového území.

Materiál a metodika

Vzorky krve a lokality jejich odběru

Záplavou postižené území bylo rozčleněno do dvou oblastí (I, II), a oblast I navíc do tří zón (A, B, C) podle četnosti výskytu komárů.

I. Riziková zátopová oblast

Zóna A: oblast lužních lesů táhnoucí se v šířce asi 1–1,5 km po obou březích Labe v úseku Obříství/Kly po Lobkovice/Kozly (výše proti proudu se vzedmutí hladiny neprojevilo), charakteristická maximem výskytu kalamitních komárů rodů *Aedes* a *Ochlerotatus*. Obce zóny A: Dušníky, Chrást, Kly, Kozly, Libiš, Lobkovice, Mlékojedy, Obříství, Tišice, Tuhaň a Větrušice.

Tab. 1. Fauna komárů středních Čech (ref.: 25, 32–37, 41, 42), a viry izolované z těchto druhů v České republice nebo (*) v jiných zemích Evropy (ref.: 14)

Table 1. The fauna of mosquitoes of Central Bohemia (ref.: 25, 32–37, 41, 42), and viruses isolated from these species in the Czech Republic or (*) other European countries (ref.: 14)

Druh komára	Četnost výskytu	Izolované viry
Anopheles claviger	dosti vzácný	*Batai
An. maculipennis	nehojný	Batai, *West Nile
An. messeae	nehojný	–
An. plumbeus	vzácný	–
Aedes cinereus	hojný	Ťahyňa, *Sindbis
Ae. vexans	velmi hojný	Ťahyňa
Ochlerotatus annulipes	hojný	–
Oc. cantans	hojný	Ťahyňa, *West Nile
Oc. cataphylla	dosti hojný	–
Oc. communis	nehojný	*Sindbis, *Ťahyňa
Oc. dorsalis	nehojný	Ťahyňa
Oc. excrucians	dosti hojný	*Ťahyňa (vzácně)
Oc. flavescens	nehojný	*Ťahyňa
Oc. geniculatus	nehojný	–
Oc. leucomelas	vzácný	–
Oc. pulchritarsis	vzácný	–
Oc. punctor	vzácný	–
Oc. sticticus	velmi hojný	Ťahyňa (vzácně)
Culex pipiens	hojný	West Nile, *Sindbis
Cx. territans	vzácný	–
Culiseta alaskaensis	vzácný	–
Cs. annulata	nehojný	Ťahyňa
Cs. glaphyroptera	vzácný	–
Cs. morsitans	vzácný	–
Cs. subochrea	vzácný	–
Mansonia richiardii	dosti vzácný	*Batai, *West Nile, *Sindbis

Zóna B: přechodné území mezi zónou A a C, s méně významnými lokálními líhništi a/nebo možností záletu komárů z lužních lesů (tj. ze zóny A). Obce zóny B: Chlumín, Kostelec n.L., Kozárovice, Lužec n. Vlt., Neratovice, Zálezlice.

Zóna C: oblast podél Vltavy a Labe mezi Kralupy n. Vlt. a Horními Počaply bez lužních lesů, kde došlo v lagunách jen lokálně k významnějšímu vývoji komárů rodu *Culex*. Obce a města zóny C: Bukol, Dědibaby, Dolany, Dolní Beřkovice, Horní Počaply, Hostín, Kralupy n. Vlt., Křivenice, Křivousy, Lobeček, Mělník, Nová Ves, Nelahozeves, Nové Ouholice, Odolena Voda, Spomyšl, Staré Ouholice, Veltrusy, Velvary, Vlinéves, Vodochody, Vojkovice, Všestudy, Zlončice.

II. Kontrolní oblast

Zóna D, s velmi omezeným anebo nulovým výskytem komárů (především město Praha, dále Štětí, Mladá Boleslav, Hradišťko, Kněžičky).

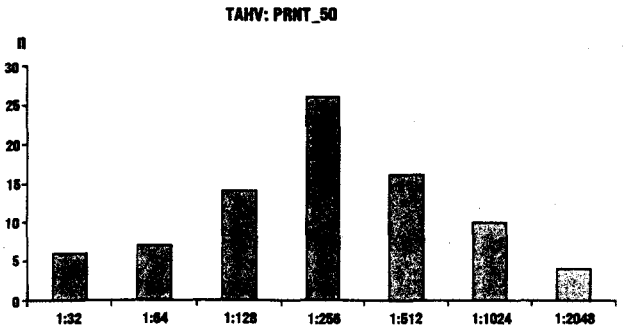
Krevní vzorky byly odebrány ve dnech 6.–13. září 2002 (tedy asi 3 týdny po kulminaci povodně na Mělnicku a 2 týdny po začátku líhnutí komárů) od 497 osob různých věkových skupin z výše uvedených lokalit, jež navštívily lékaře v ambulancích nebo v pojízdných laboratořích určených pro bezplatné vyšetření osob na virovou hepatitidu. Vzorky byly získány s informovaným písemným souhlasem pacienta (zahrnujícím mj. pasáž, že „poskytnutý vzorek může být použit také pro epidemiologický průzkum možného výskytu jiných nákaz v důsledku povodní, jako jsou onemocnění přenosná komáry"). Krevní séra byla pak uchovávána při -20 °C a transportována zmražená k vyšetření do laboratoře ÚBO AVČR ve Valticích. Druhé (párové) vzorky krevních sér se podařilo odebrat s odstupem 6 měsíců od 150 osob z prvého souboru ve dnech 9. dubna až 15. května 2003 (34 osob ze zóny A, 43 osob zóny B a 73 osob zóny C). Tyto druhé vzorky sér byly rovněž uchovávány až do vyšetření při -20 °C.

Hemaglutinačně-inhibiční test (HIT)

Nativní séra byla extrahována acetonem a vysycena houseřími erytrocyty (18). Antigeny virů byly připraveny standardní metodou sacharózo-acetonové extrakce (5). Kmeny a pasáže použité pro přípravu jednotlivých antigenů zahrnovaly: 92 (SM_{45}) viru Ťahyňa (TAH: 3); Eg-101 (SM_{19}) viru West Nile (WN: 28); 184 (SM_9) viru Batai (BAT: 2); EgAr 339 (SM_{19}) viru Sindbis (SIN: 43); a komerční antigen viru klíšťové encefalitidy (KE, Test-Line s.r.o.). Vlastní test byl proveden podle standardní metody (5) s použitím 8 jednotek hemaglutininu. Do jamek v mikrodestičkách s 96 V-jamkami byla naředěna v 0,4 % bovalbuminu v borátovém pufru (pH 9) dvojkovou řadou séra v objemu 25 µl a ponechána s antigenem (25 µl) v chladničce přes noc; poté bylo přidáno 50 µl suspenze erytrocytů, a výsledek byl odečten po 1 h. v chladničce (19). Do testů byla zařazena pozitivní a negativní kontrolní séra (Imuna Šarišské Michalany, Test-Line); za pozitivní byly považovány titry vyšší než 1:20.

Plak-redukční neutralizační test (PRNT)

Nativní séra byla před vyšetřením inaktivována 30 min/56 °C. V testech byly použity následující kmeny (a jejich pasáže): T-16 (SM_6) viru TAH (4); Eg-101 (SM_{17}) viru WN; Hypr ($HeLa_{55}M_{10}SM_1$) viru KE; a 184 (SM_{10}) viru BAT. Test byl proveden v mikrodestičkách fy. Sarstedt s 96 jamkami s plochým dnem: po inkubaci (60 min/37 °C) směsi 30 µl viru (20–50 plakových jednotek, PFU) v médiu L-15 s 30 µl ředěného séra bylo přidáno 60 µl suspenze buněk Vero (SPEV u viru KE)



Obr. 1. Rozdělení titrů protilátek neutralizujících virus Ťahyňa v PRNT (n = počet osob)
Fig. 1. Distribution of titres of antibodies to Ťahyňa virus in PRNT (n = number of seroreactors)

v médiu L-15 s 3 % inaktivovaného fetálního telecího séra (Flow Laboratories), po inkubaci 4 h/37 °C pak 120 µl karboxymetylcelulózového přelivu s L-15 a 3 % fetálního séra, a závěrečná inkubace probíhala podle druhu viru 4–6 d./37 °C (13, 26). Do testů byla zařazena pozitivní a negativní kontrolní séra, a jako pozitivní byla hodnocena ta vyšetřovaná séra, která vyvolala ve screeningu při ředění 1:8 minimálně 90% snížení počtu PFU. Všechna takto reagující séra byla dodatečně titrována v sériovém ředění dvojkovou řadou, a zvlášť byly hodnoceny titry snižující počet PFU o 50 % ($PRNT_{50}$) a 90 % ($PRNT_{90}$). Pro vyloučení zkřížené flavivirové reaktivity byla všechna séra reagující s WNV dodatečně testována v PRNT také s virem KE na embryonálních prasečích buňkách SPEV. Zatímco s viry TAH a WN byla v PRNT vyšetřena séra všech osob, s BATV byl PRNT použit pouze jako test konfirmační u osob reagujících s antigenem tohoto viru v HIT.

Výsledky

Prevalence protilátek k virům přenosným komáry

Virus Ťahyňa

Séra 82 osob z 497 vyšetřených (16,5 %) obsahovala protilátky neutralizující virus Ťahyňa, a v HIT reagovalo 74 osob (14,9 %). Titry TAHV protilátek kolísaly v $PRNT_{50}$ od 1:32 do 1:2048 (geometrický průměr 1:260), v $PRNT_{90}$ od 1:16 do 1:1024 (geom. průměr 1:119), a v HIT od 1:20–40 do 1:160 (geom. průměr 1:40); rozložení titrů neutralizačních protilátek ukazuje obr. 1. Byla zjištěna dobrá shoda séropozitivity mezi PRNT a HIT: 64 osob reagovalo v obou testech, 18 pouze v PRNT, 10 pouze v HIT, a 405 v žádném z obou testů.

Rozdíl mezi pohlavími v prevalenci protilátek neutralizujících TAHV byl statisticky neprůkazný ($\chi^2 = 0{,}107$; $P = 0{,}744$): séropozitivních v PRNT bylo 15,8 % z 202 vyšetřených mužů a 16,9 % z 295 vyšetřených žen.

Analýza protilátek k TAHV podle věkových skupin (tab. 2) prokázala výrazně heterogenní distribuci séropozitivity, s rostoucí mírou prevalence ve vyšších věkových kategoriích ($\chi^2 = 39{,}809$; $P < 0{,}001$). U osob mladších 19 let byly zjištěny protilátky k TAHV jen ojediněle (šlo o 6letou dívku z Dědibab), což indikuje nízkou aktivitu TAHV ve středních Čechách v období posledních 20 let.

Při rozboru distribuce protilátek neutralizujících virus Ťahyňa podle místa bydliště (tab. 3, obr. 2) byla zjištěna nejvyšší prevalence v záplavové zóně A s nejvyšším výskytem komárů (28,0 % pozitivních osob), poněkud nižší v zóně B (20,5 %), ještě nižší v zóně C (14,7 %), zatímco v kontrolní zóně D bylo séropozitivních pouze 5,0 % osob. Rozdíl v séroprevalenci mezi zónami je signifikantní ($\chi^2 = 14{,}574$; $P = 0{,}002$): průkazně nejnižší je prevalence v zóně D, a významně se liší také séroprevalence v zóně A oproti C ($\chi^2 = 7{,}243$; $P = 0{,}007$), ne však zóna A od B nebo B od C. Obdobné výsledky poskytla analýza séropozitivity v HIT. Detailní rozbor podle obcí v rámci zóny A (počet v PRNT séropozitivních/vyšetřených osob): Obříství 10/38, Kozly

Tab. 2. Srovnání prevalence protilátek neutralizujících virus Ťahyňa v závislosti na věku osob po povodních ve středních Čechách roku 2002 (SČ 2002) a na jižní Moravě roku 1997 (JM 1997 – ref.: 11, 15, 17); n, počet vyšetřených osob

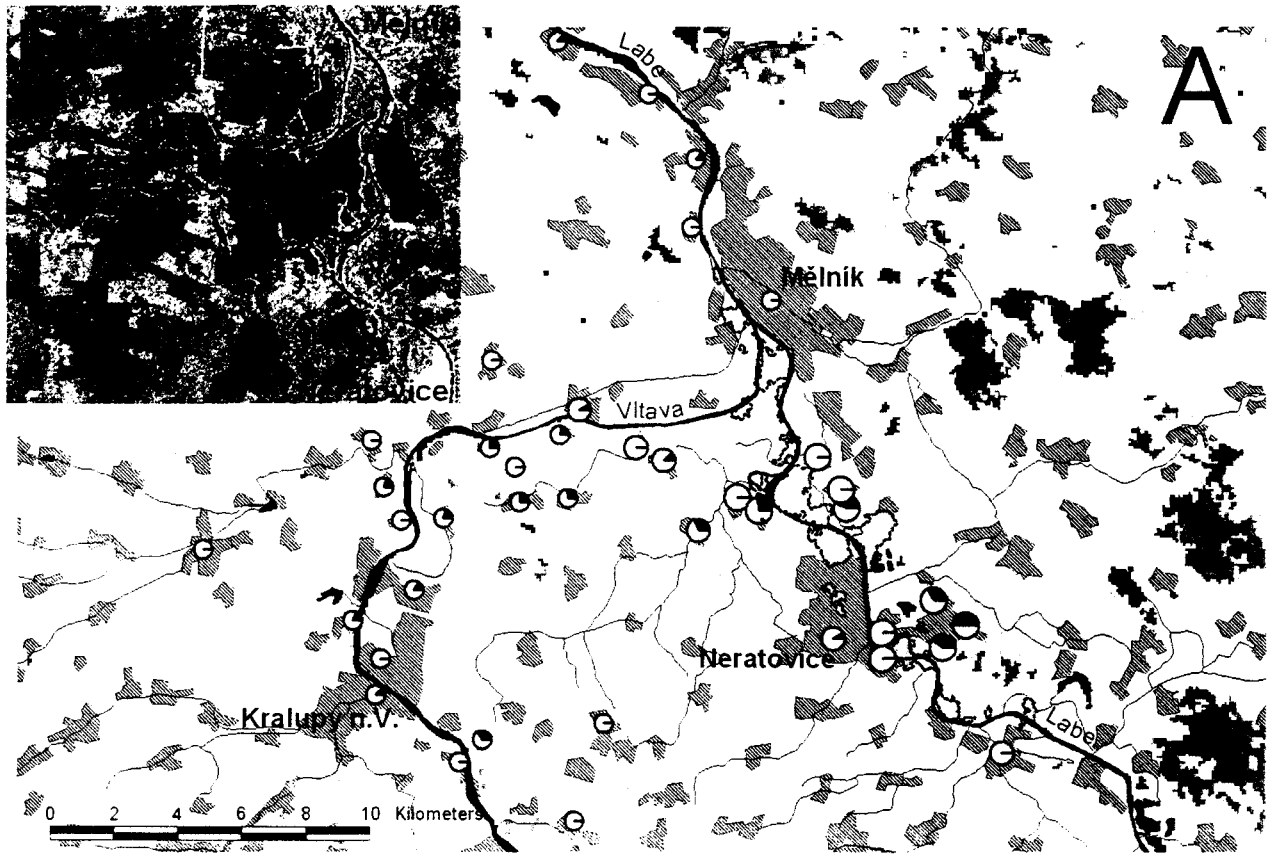
Table 2. Comparison of the prevalence rates of human Ťahyňa virus neutralizing antibodies between age categories as recorded after the floods in Central Bohemia, 2002 (SČ 2002) and southern Moravia, 1997 (JM 1997 – ref.: 11, 15, 17); *n*, no of persons tested

Věk (roků)	<10	10–19	20–29	30–39	40–49	50–59	60–69	70+
SČ 2002								
n	18	53	74	69	62	86	78	57
Podíl pozitivních	5,6 %	0,0 %	5,4 %	17,4 %	11,3 %	19,8 %	32,1 %	28,1 %
JM 1997								
n	39	49	128	79	80	90	59	95
Podíl pozitivních	0,0 %	8,2 %	19,5 %	63,3 %	62,5 %	81,1 %	79,7 %	88,4 %

Tab. 3. Prevalence protilátek neutralizujících virus Ťahyňa v závislosti na místě trvalého bydliště osob podle zón A až D (viz Materiál a Metody) po povodních ve středních Čechách roku 2002

Table 3. Prevalence rates of Ťahyňa virus neutralizing antibodies after the flood of 2002 in Central Bohemia in relation to the place of residence (risk zones A to D) of the persons tested

Záplavová zóna	A	B	C	D
n	75	83	279	60
Podíl pozitivních	28,0 %	20,5 %	14,7 %	5,0 %



Obr. 2. Potenciální ohniska komáry přenosných viróz na Mělnicku – lužní lesy identifikované na satelitních snímcích Landsat MSS (přerušovaná čára) – na pozadí říční sítě a sídel (DMU-200, VTOPÚ Dobruška) a proporce TAHV – séropozitivních osob ve vyšetřovaných lokalitách; zóna A je vyznačena velkými, zóna B středními a zóna C malými kroužky. Insert B: satelitní radarový snímek soutoku Labe a Vltavy ze 17. 8. 2002 ukazuje rozliv vody (černé plochy) dva dny po kulminaci povodně; zatopené lužní lesy s následným kalamitním výskytem komárů r. *Aedes* lze identifikovat jako světlé plochy podél Labe a při soutoku Vltavy vystupující nad vodní hladinu, laguny v polích výše proti toku Vltavy (vlevo) a dále od Labe (vpravo) se staly líhništi komárů převážně r. *Culex*

Fig. 2. Potential foci of mosquito-borne viruses in the Mělník area – floodplain forests identified on Landsat MSS satellite images (dotted line) – with hydrology and settlement in background (DMU-200, VTOPÚ Dobruška), and the proportion of TAHV – seropositive residents at particular localities (large, medium and small circles indicate sites placed to the risk zone A, B and C, respectively). Insert B: a radar satellite image of the conflux of the rivers Labe and Vltava on August 17, 2002 shows the extent of floodwater (dark areas) two days after the flood culmination; inundated forests with subsequent massive occurence of *Aedes* mosquitoes are visible as light areas surrounding the river Labe upstream of the conflux, and scattered lagoons seen in fields along both rivers far left and right turned to breeding sites of predominantly *Culex* mosquitoes

4/10, Tuhaň 3/7, Chrást 3/6, Tišice 1/3, ostatní negativní – ovšem s nízkým počtem vyšetřených osob (Dušníky 0/4, Větrušice 0/1, Kly 0/3, Mlékojedy 0/1, Lobkovice 0/2). V zóně B byla zaznamenána séropozitivita u obyvatel obcí Chlumín 13/42, Zálezlice 2/15, Lužec n. Vlt. 1/13 a Neratovice 1/12, ostatní obce byly „negativní" (Kostelec n.L. 0/1, Kozárovice 0/1). V zóně C byly „pozitivní" obce Dolní Beřkovice 4/29, Horní Počaply 10/71, Nelahozeves 3/16, Hostín 5/19, Kralupy n. Vlt. 6/49, Veltrusy 2/11, Dědibaby 3/12, Všestudy 3/14, Bukol 2/9, Nové Ouholice 1/5, Vojkovice 1/4, Zlončice 1/3; „negativní" byly ostatní obce či města (Dolany 0/1, Křivenice 0/16, Mělník 0/2, Staré Ouholice 0/3, Spomyšl 0/2, Odolena Voda 0/2, Vliněves 0/2, Lobeček 0/1, Nová Ves 0/1, Křivousy 0/4, Vodochody 0/1, Velvary 0/1). V kotrolní zóně D byla pozitivita zaznamenána jen ojediněle (např. u obyvatel Prahy 2/54). Jestliže jsme analyzovali prevalenci séropozitivních osob v zónách A až C podle vzdálenosti místa jejich bydliště od okraje lužního lesa, obdrželi jsme následující podíly (v závorce počet vyšetřených osob): < 1 km 28,2 % (78); 1,0–2,9 km 21,3 % (75); 3,0–5,9 km 17,1 % (70); ≥ 6,0 km 13,6 % (214). Heterogenita tohoto rozdělení je statisticky průkazná (χ^2 = 8,515; P = 0,003) a potvrzuje klesající podíl séropozitivních osob s rostoucí vzdáleností jejich bydliště od lužního lesa.

Virus West Nile

Proti WNV nebyla zjištěna žádná specifická reakce: v HIT sice reagovalo celkem 34 osob (6,8 %) v titrech 1:40 až 1:80, ale všechna tato séra reagovala v HIT rovněž s antigenem viru KE v titrech

Tab. 4. Jedna prokázaná (č. 578/23) a tři možné recentní infekce osob virem Ťahyňa po povodni ve středních Čechách v roce 2002. První vzorky jsou ze září 2002, druhé z dubna 2003; uvedeny reciproké titry protilátek

Table 4. One confirmed (no 578/23) and three possible cases of recent infection with Ťahyňa virus after the flood of 2002 in Central Bohemia. The first and second blood samples were taken in September 2002 and April 2003, respectively (shown are reciprocal titres of antibodies).

Č. prot.	Sex, věk	HIT		PRNT	
		1. vzorek	2. vzorek	1. vzorek	2. vzorek
479/21	M, 40	20–40	80	128	128
503/35	M, 32	<20	20–40	128	64–128
538/37	F, 80	20–40	80	64	32
578/23	F, 55	<20	20–40	<8	512

přibližně stejných anebo vyšších (až 1:160), takže tyto výsledky lze interpretovat jako zkřížené reakce s antigenně příbuzným flavivirem KE, který se v této oblasti může vyskytovat anebo proti kterému mohly být některé osoby očkovány. V $PRNT_{90}$ reagovalo s WNV jen 6 osob (1,2 %) ovšem ve velmi nízkém titru 1:8 až 1:16, a současně tato séra reagovala v PRNT nebo v HIT s virem KE, takže reakce byla opět interpretována jako zkřížená; agens vyvolávajícím imunitní odpověď byl zřejmě virus KE. V $PRNT_{50}$ reagovalo sice s WNV více osob, 42 (tj. 8,5 %), ale opět šlo vesměs o nízké titry 1:8 až 1:32 a současně zkřížené reakce s virem KE, jako bylo ověřeno v HIT i PRNT.

Virus Sindbis

Protilátky k viru Sindbis byly zjišťovány pouze pomocí HIT, a prokázány u 7 osob (1,4 %), titry byly poměrně nízké – 1:20 až 1:40.

Virus Batai (Čalovo)

V HIT byly prokázány protilátky u 7 osob (1,4 %) v nízkém titru 1:20, ale při ověřování těchto pozitivních sér v PRNT se podařilo prokázat specifické protilátky pouze u jednoho séra (0,2 %), titr v $PRNT_{50}$ činil 1:64.

Recentní infekce viry přenášenými komáry

Párové krevní vzorky byly odebrány 150 osobám s odstupem 6 měsíců. Sérokonverze anebo průkazný vzestup titru protilátek mezi prvním a druhým vzorkem séra byl zjištěn jen s virem Ťahyňa v několika málo případech (tab. 4). K infekci během povodně anebo na podzim po ní došlo jistě u osoby č. 578/23 (obyvatelka Obříství, zóna A), jak potvrdila sérokonverze v PRNT i HIT. Méně přesvědčivé výsledky byly zaznamenány u dalších 3 osob, u kterých se projevila sérokonverze či průkazné zvýšení titru protilátek pouze v jednom testu – HIT: č. 479/21 (bydliště Chlumín, zóna B); č. 503/35 (bydliště Chlumín); č. 538/37 (bydliště Obříství). Je nutno poznamenat, že protilátky virus neutralizující se objevují po infekci TAHV dříve než hemaglutinaci-inhibující. Klinické anamnézy těchto osob s předpokládanou recentní infekcí virem Ťahyňa se nepodařilo získat. Sérokonverze proti sledovaným arbovirům nebyla zjištěna u žádné ze 73 vyšetřených osob ze zóny C.

Diskuse

Sérologický přehled obyvatel středních Čech po srpnových povodních roku 2002 nezjistil aktuální cirkulaci komáry přenosných virů West Nile, Sindbis a Batai (protilátky k těmto virům totiž buď nebyly zjištěny vůbec anebo jen v nevýznamné frekvenci), prokázal však aktivitu viru Ťahyňa. Přírodní ohnisko valtické horečky bylo indikováno pomocí séroepidemiologické analýzy na lokalitách Obříství-Chlumín-Tuhaň, a Kozly-Tišice-Chrást, situovaných na obou březích Labe před soutokem s Vltavou v okolí města Neratovic. V nižší frekvenci byly prokazovány protilátky k TAHV na dolním toku Vltavy od Zlončic až po Bukol/Zálezlice, případně na jiných lokalitách Mělnicka. Prevalence protilátek k TAHV u vyšetřovaných osob rostla s rizikovostí lokality jejich bydliště (od kontrolní zóny D přes zóny C a B až k nejrizikovější zóně A), přičemž riziko bylo výsledkem kombinace míry zátopy a velikosti místních líhnišť komárů. Je známo, že vektory TAHV jsou především komáři luhu. Proto se současně projevila také závislost míry séroprevalence obyvatel na vzdálenosti místa jejich bydliště od okraje lužního lesa.

Tato studie informuje jednak o stavu protilátek před záplavami, neboť první vzorky sér byly odebrány pouhé 2 týdny po masovém líhnutí komárů, avšak vyšetření druhých vzorků sér umožnilo vyhodnotit dopad povodně na aktivaci komáry přenosných virových nákaz. V rámci zón A, B a C jsme analýzou párových vzorků krevního séra zachytili sérokonverzi protilátek k viru Ťahyňa minimálně u jedné osoby ze 150 (tj. 'attack rate' ~ 0,7 %). Při předpokládaném úhrnu 100 tisíc obyvatel žijících v těchto ohrožených zónách (odhad podle posledního censu obyvatel) mohlo zde tedy hypoteticky po povodni prodělat infekci virem Ťahyňa celkem 650 osob (95 % – interval spolehlivosti je 20–3719).

V České republice byl izolován TAHV z komárů poprvé na jižní Moravě v roce 1963 (24). Jak následně prokázali pracovníci Parazitologického ústavu ČSAV při dlouhodobých výzkumech přírodního ohniska valtické horečky na Břeclavsku, podíl komárů s virem kolísá od 0,01 % do 0,4 % populace v závislosti na druhu komára, lokalitě, sezóně a roku (6–9, 27, 40). U komárů byl zjištěn i transovariální přenos viru Ťahyňa infikovanou samičkou na potomstvo (10). V kombinaci s amplifikací viru Ťahyňa v kompetentních hostitelích (zajíc) a navíc s možností sexuálního přenosu viru z komářího samce na samici, tyto mechanismy zaručují dlouhodobou perzistenci viru Ťahyňa v přírodním ohnisku. Komáři tak vlastně představují nejen přenašeče, ale i rezervoár nákazy. Mimo oblast jižní Moravy jsou v ČR známa přírodní ohniska valtické horečky také z nížin Ostravska, a v menší míře cirkuluje TAHV i v Čechách, např. v povodí Labe, Vltavy a Ohře (20–23). Valtická horečka byla kromě ČR a SR popsána v několika dalších zemích Evropy (1, 14).

Je známo, že podíl obyvatel s protilátkami k TAHV v endemických oblastech vzrůstá s věkem. Např. podle vyšetření z let 1973–76 mělo na Břeclavsku protilátky neutralizující TAHV jen 7 % dětí do věku 4 let, avšak 81 % osob starších 25 let; průměrná promořenost celého souboru byla 55 % (12). Po povodních v roce 1997 na Moravě bylo vyšetřeno na protilátky neutralizující TAHV z Břeclavska 619 osob, z nichž bylo 54 % pozitivních (15). Ve srovnání s obdobným vyšetřením v r. 1976 nebyly však v roce 1997 prokázány protilátky k TAHV u osob mladších 19 let, kdežto v souboru z roku 1976 bylo promořeno 46 % dětí již ve věku 5–6 let. Zatímco v r. 1976 měla věková skupina osob s 50% průměrnou prevalencí protilátek na Břeclavsku stáří 7 let, v roce 1997 to bylo 30 let (12, 15). Tyto údaje dokumentují výrazné utlumení aktivity přírodního ohniska valtické horečky v posledních 20 letech, koincidující s dokončením vodohospodářských úprav Dyje v polovině 70. let, jež vedly k zamezení periodických záplav, výraznému poklesu hladiny spodní vody, a podstatné redukci populací kalamitních komárů rodu *Aedes*, vektorů viru TAHV. Izolace 7 kmenů tohoto viru z komárů v letech 1997 a 1999 však ukázala, že přírodní ohnisko perzistuje (15–17). Poněkud podobnou situaci s věkovou distribucí protilátek k TAHV jsme nyní zjistili ve středních Čechách: podíl séropozitivních osob mladších 20 let byl velmi nízký. Přitom byl však po povodni zjištěn nejméně jeden případ recentní infekce virem Ťahyňa. To dokládá perzistující středočeskou aktivitu TAHV a jeho endemičnost, i když v měřítku nižším než v jihomoravském ohnisku valtické horečky. Virus Ťahyňa by proto neměl být opomíjen jako potenciální původce lokálních onemocnění valtickou horečkou ani v podmínkách středních Čech.

Valtická horečka a další virózy přenosné komáry nejsou v podmínkách České republiky zatím vnímány jako zdravotnický problém. Zpravidla neohrožují život pacienta, mohou však způsobit pracovní neschopnost, případně u dětí absenci ve škole. Je však nutno poznamenat, že většina komáry přenosných nákaz uniká pozornosti lékařů anebo může být nedostatečně diagnostikována, poněvadž jejich sérologická laboratorní diagnostika se u nás rutinně tč. vůbec neprovádí. Environmentální faktory, včetně antropogenních, vedoucí ke zvýšení populační denzity komárů (např. silné dešťové srážky následované záplavami nebo povodněmi, umělé zaplavování lesa, zavlažování, revitalizace vodních toků či mokřadů, anebo vyšší teploty) mohou vytvořit podmínky pro zvýšenou incidenci nemocí jimi přenosných. Pak i v mírném pásmu mohou tyto nákazy přerůstat ve významný zdravotnický problém (30, 38). Pokud by se ukázala prevence těchto nákaz žádoucí i u nás, optimální systémovou strategií by byla epidemiologická surveillance (31), zahrnující monitorování: 1) početnosti populací komárů (včetně sledování jejich líhnišť) v přírodních ohniscích; 2) přítomnosti virů v komárech; 3) nemocnosti lidské populace v období květen až září (klinická a sérologická sledování); 4) přítomnosti protilátek u domácích a volně žijících obratlovců. Na základě jejích výsledků by pak bylo možné navrhovat a uskutečňovat preventivní opatření, např. integrovanou kontrolu populací komárů (39), s preferencí aplikace selektivních larvicidních biopreparátů na bázi *Bacillus thuringiensis israelensis* („Vectobac") nebo *B. sphaericus*, popřípadě jiných selektivních protikomářích larvicidů na líhništích v lužních lesích při respektování mimořádné biologické a vodohospodářské hodnoty těchto ekosystémů. Aplikaci kontaktních insekticidů proti dospělým komárům (adulticidů) v lesním lužním ekosystému nelze totiž provádět jako plošné opatření, a bariérové postřiky okolo obydlených míst jsou jen nouzovým východiskem v kritických situacích.

Poděkování

Tato studie mohla být uskutečněna jen díky spolupráci se Sdružením zdravotnických zařízení – oddělením klinické biochemie Klaudiánovy nemocnice v Mladé Boleslavi, a s dalšími zdravotníky v terénu. Finanční podporu tomuto výzkumu poskytla GA ČR (projekty 310/03/Z033 a 206/03/0726).

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Mosquitoborne Viruses, Czech Republic, 2002

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Specimens from residents (N = 497) of an area in the Czech Republic affected by the 2002 flood were examined serologically for mosquitoborne viruses. Antibodies were detected against Tahyna (16%), Sindbis (1%), and Batai (0.2%) viruses, but not West Nile virus. An examination of paired serum samples showed 1 Tahyna bunyavirus (California group) infection.

The 2002 flood in Bohemia struck the Czech Republic just a few years after the 1997 flood (in Moravia and Silesia). Apart from Prague, extensive rural areas along the Vltava and Labe Rivers were flooded in August 2002. In the Melník area, which offers favorable habitats for mosquitoes under normal conditions (1), mass mosquito breeding (largely *Ochlerotatus sticticus*, *Oc. cantans, Aedes vexans,* and *Ae. cinereus*) occurred after August 20. This increased mosquito population peaked September 3–9, with a biting frequency of 70 bites per person per minute. The mosquito population declined during the second half of September and disappeared by November.

The Study

To estimate the risk for infections with mosquitoborne viruses, we screened the human population of the flooded area (Figure 1) for antibodies against the viruses known to occur in central Europe (2): Tahyna (TAHV), *Orthobunyavirus* of the California group, *Bunyaviridae*; West Nile (WNV), *Flavivirus* of the Japanese encephalitis group, *Flaviviridae*; Sindbis (SINV), *Alphavirus*, *Togaviridae*; and Batai (BATV), *Orthobunyavirus* of the Bunyamwera group, *Bunyaviridae*.

We subdivided the flooded area into 4 risk zones according to quantities of mosquitoes. Zone A was a forested floodplain along the Labe River between Obríství-Kly and Lobkovice-Kozly (11 villages), with large quantities of mosquitoes. Zone B was an intermediate area between zones A and C (5 villages, 1 small town), with fewer breeding sites but possibility for mosquito migration from zone A. Zone C was the area along the Vltava and Labe Rivers between Kralupy and Horní Pocaply (25 villages and small towns), with no floodplain forests and few breeding sites for mosquitoes. Zone D was a control zone, with only sporadic occurrences of mosquitoes (mainly in Prague).

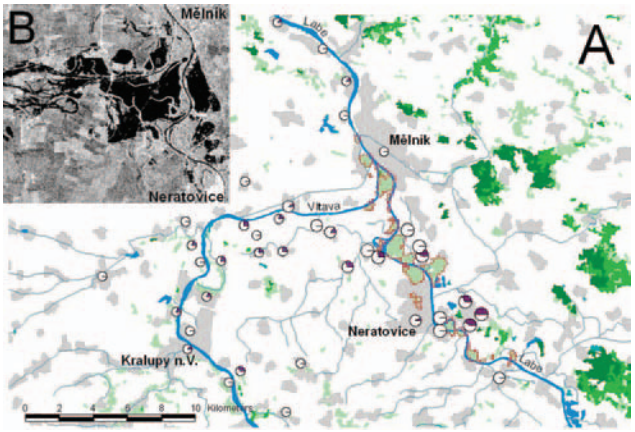

Figure 1. A), Potential foci of mosquitoborne viruses in the Melník area. Floodplain forests identified on the Landsat MSS satellite images (dotted red line), with hydrology and settlement in background (DMU-200, VTOPÚ Dobruška), and proportion of Tahyna virus seropositive residents at particular localities (large, medium, and small circles indicate the risk zones A, B, and C, respectively). B) [inset] radar satellite image of the conflux of the Labe and Vltava Rivers on August 17, 2002 (2 days after the flood culmination), showing extent of floodwater (dark areas). Inundated forests, with subsequent mass occurrences of *Ochlerotatus* and *Aedes* mosquitoes, are visible as lighter areas surrounding the Labe River upstream of the confluence; scattered lagoons (dark areas) in arable fields along both rivers far left and right turned into breeding sites of predominantly *Culex* mosquitoes.

Informed written consent and serum samples were obtained from 497 survey participants of various ages from September 6 to September 13, 2002 (3 weeks after the flood culmination and 2 weeks after the mosquito emergence). Paired serum samples were taken from 150 of the survey participants 7 months later, from April 9 to May 15, 2003 (34 in zone A, 43 in zone B, 73 in zone C).

Serologic examination was performed with the hemagglutination-inhibition (HIT) and plaque-reduction neutralization tests (PRNT) in microplates (3–5). The strains used for HIT were TAHV 92, WNV Eg101, BATV 184, and SINV Eg339; a commercial control antigen (Test-Line Ltd., Brno, Czech Republic) of Central European tick-borne encephalitis virus (CEEV) was used. All serum samples were acetone-extracted and tested with sucrose- and acetone-processed antigens by using 8 hemagglutinin units; titers >20 were considered positive. For PRNT, TAHV T16, WNV Eg101, CEEV Hypr, and BAT 184 viral strains were used. The test was conducted on Vero or SPEV (embryonic pig kidney: for CEEV) cells. All serum

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samples were heat inactivated and screened for antibodies at 1:8; those reducing the number of virus plaques by 90% were considered positive and titrated to estimate dilutions causing plaque–number reduction by 50% ($PRNT_{50}$) and 90% ($PRNT_{90}$). The serum samples reacting with WNV were examined for cross-reactivity with CEEV. PRNT with BATV was used only as a confirmatory test for the serum samples reacting with BATV in HIT.

Against TAHV, 82 (16.5%) of 497 study participants had neutralizing antibodies, and 74 (14.9%) were seropositive in HIT. In $PRNT_{50}$, the titers were 32–2048 (geometric mean titer [GMT] 260), in $PRNT_{90}$ 16–1024 (GMT 119), and in HIT 20–40 to 160 (GMT 40). Figure 2 illustrates the distribution of neutralizing antibody titers. No difference occurred in neutralizing antibody prevalence between sexes, 32 (15.8%) of 202 males and 50 (16.9%) of 295 females ($\chi^2 = 0.11$; $p = 0.744$). The prevalence rate increased significantly with age (Table 1: $\chi^2 = 39.809$; $p < 0.001$); TAHV antibodies were found infrequently in persons <19 years of age. Neutralizing antibody distribution, with respect to the residence location (Table 2, Figure 1), showed the highest seroprevalence in zone A (28%), lower seroprevalences in zones B and C, and 5% in the control zone D ($\chi^2 = 14.57$; $p = 0.002$). Significant differences were found between zone D and all other zones, and between zones A and C ($\chi^2 = 7.243$; $p = 0.007$), but not between zones A and B or B and C; HIT yielded similar results. The seroprevalence in relation to the proximity of study participants' locations to the nearest floodplain forest within zones A, B, and C demonstrated decreasing seroprevalence with increasing distance to the forest ($\chi^2 = 8.51$; $p = 0.003$) (Table 2).

Against WNV, no specific reactions were found. Although serum samples from 34 (6.8%) study participants reacted in HIT with the WNV at titers 40 to 80, all of them also reacted with CEEV at titers similar or higher (≤160). CEEV could have occurred in the area, and some study participants may have been vaccinated against tick-borne encephalitis. In $PRNT_{90}$, 6 study participants (1.2%) reacted with WNV but at low titers of 8 to 16; these serum samples also reacted in PRNT with CEEV; thus, the results

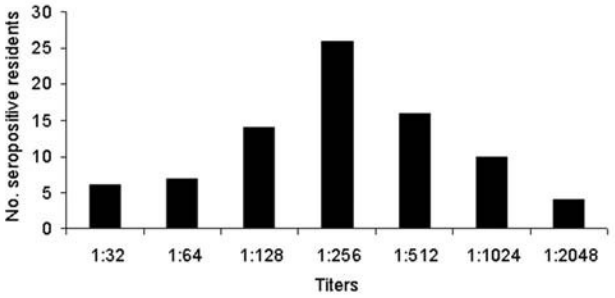

Figure 2. Distribution of 50% plaque-reduction neutralization titers of antibodies to Tahyna virus.

were considered to be crossreactions as well. Additionally, 42 (8.5%) seroreactors against WNV appeared in the less stringent $PRNT_{50}$, but all titers were low (8–32) and cross-reacted with CEEV.

Against SINV, antibodies were tested with HIT only and detected in specimens from 7 (1.4%) study participants, with low titers of 20 to 40. Of the BATV, specimens from 7 study participants reacted in HIT at a low titer of 20. By confirmatory testing of these serum samples in PRNT, only 1 (0.2%) showed specific antibodies to BATV; the titer was 64 in $PRNT_{50}$ and 32 in $PRNT_{90}$.

Seroconversion (≥4-fold rise in titer) was found with TAHV only. After the flood the infection episode occurred in one 55-year-old woman from Obříství (zone A), as shown by the seroconversion in both HIT (<20/40) and $PRNT_{50}$ (<8/512). Three other study participants seroconverted in 1 test only: a 40-year-old man from Chlumín, zone B (HIT 20/80; PRNT 128/128); a 32-year-old man from Chlumín (HIT <20/20–40; PRNT 128/64); and an 80-year-old woman from Obříství (HIT 20/80; PRNT 64/32). These results are less convincing. Upon our request, local general practitioners did not corroborate consistent signs of a disease reported by these 4 study participants from October 2002 to April 2003. In general, clinical symptoms of TAHV infection are milder in adults than in children (7). Seroconversion against mosquitoborne viruses was not detected in any of the 73 study participants in zone C.

Table 1. Comparison of the prevalence of neutralizing antibodies to Tahyna virus by age groups after the floods in central Bohemia in 2002 and southern Moravia in 1997*†

Age (y)	CB 2002, n	% positive	SM 1997, n	% positive
0–9	18	5.6	39	0.0
10–19	53	0.0	49	8.2
20–29	74	5.4	128	19.5
30–39	69	17.4	79	63.3
40–49	62	11.3	80	62.5
50–59	86	19.8	90	81.1
60–69	78	32.1	59	79.7
≥70	57	28.1	95	88.4

*CB, central Bohemia; SM, southern Moravia; n, number of residents examined.
†Source (6).

Table 2. Prevalence of neutralizing antibodies to Tahyna virus after the 2002 flood, Central Bohemia*

	n†	% positive
Risk zone		
A	75	28.0
B	83	20.5
C	279	14.7
D	60	5.0
Distance to FPF (km)		
<1.0	78	28.2
1.0–2.9	75	21.3
3.0–5.9	70	17.1
≥6.0	214	13.6

*As related to the residence location: risk zones A to D; and distance to floodplain forest (FPF, within zones A, B, and C only).
†n, number of residents examined.

Conclusions

On the basis of this serosurvey, recent infections with WNV (in contrast to South Moravia after the 1997 flood [5,6]), SINV, and BATV have not been found in Central Bohemia after the flood. However, activity of another mosquitoborne virus, TAHV, has been found in a natural focus along the Labe River at Neratovice. This focus has so far gone unnoticed (8). Lower frequency of TAHV antibodies has been detected along the lower reaches of the Vltava River. The prevalence of antibodies to TAHV increased with risk-zone ranking (from zone D to the highest risk zone A) and with decreasing distance to floodplain forests, the primary breeding habitat of vector mosquitoes (9–11).

In disease-endemic areas, the proportion of residents with antibodies against California group viruses increase with age (6,12). A similar situation occurred in the Central Bohemian flooded area, where antibodies to TAHV were detected in a low proportion of residents <20 years of age. Nevertheless, TAHV seems to be active in the area. At least 1 seroconversion among 150 residents (attack rate ≈0.67%) against TAHV has been proven. With ≈100,000 inhabitants in the risk zones (1992 census), ≈670 (95% confidence interval 20–3,719) persons could have been infected after the flood.

Environmental factors, such as heavy rains followed by a flood, artificial inundation of floodplain forests, or rehabilitation of wetlands that support mosquito-vector populations, could give rise to preconditions for an increased incidence of mosquitoborne infectious diseases, even in temperate climates. Under such circumstances, the optimum strategy is an epidemiologic surveillance that includes monitoring, especially of infection rate of mosquito populations and incidence of mosquitoborne diseases in humans. The surveillance results could then be used in integrated mosquito control.

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PRÁCE 17

Bakonyi T., Hubálek Z., **Rudolf I.**, Nowotny N. 2005. Novel Flavivirus or New Lineage of West Nile Virus, Central Europe. *Emerg. Infect. Dis.* 11: 225–231.

Stručná charakteristika: West Nile virus (WNV) (čel. *Flaviviridae*), patří mezi viry přenosné komáry. WNV v přírodním ekosystému cirkuluje mezi ptáky a ornitofilními komáry rodu *Culex* (nejčastěji *Culex pipiens* a *Culex modestus*), a způsobuje tzv. západonilskou horečku u lidí a koní. Po povodních v roce 1997 se naší laboratoři podařilo izolovat WNV kmen 97-103, o dva roky později kmen WNV 99-222. Genom viru (kmen 97-103) byl ve spolupráci s Vídeňskou veterinární univerzitou kompletně sekvenován.

Hlavní přínos práce: na základě celogenomové sekvenační analýzy WNV kmenu 97-103 bylo zjištěno, že kmen představuje novou 3. genomickou linii WNV (liší se významně na základě analýzy sekvence nukleotidů i aminokyselin od předešlých dvou linií) a byl nazván virus Rabensburg (dle místa nálezu).

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Novel Flavivirus or New Lineage of West Nile Virus, Central Europe

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A flavivirus (strain 97-103) was isolated from *Culex pipens* mosquitoes in 1997 following floods in South Moravia, Czech Republic. The strain exhibited close antigenic relationship to West Nile virus (WNV) prototype strain Eg-101 in a cross-neutralization test. In this study, mouse pathogenicity characteristics and the complete nucleotide and putative amino acid sequences of isolate 97-103, named Rabensburg virus (RabV) after a nearby Austrian city, were determined. RabV shares only 75%–77% nucleotide identity and 89%–90% amino acid identity with representative strains of WNV lineages 1 and 2. Another RabV strain (99-222) was isolated in the same location 2 years later; it showed >99% nucleotide identity to strain 97-103. Phylogenetic analyses of RabV, WNV strains, and other members of the Japanese encephalitis virus (JEV) complex clearly demonstrated that RabV is either a new (third) lineage of WNV or a novel flavivirus of the JEV group.

West Nile virus (WNV), a member of the Japanese encephalitis virus (JEV) group within the genus *Flavivirus*, family *Flaviviridae*, is the most widespread flavivirus, occurring in Africa, Eurasia, Australia, and North America. Other members of the JEV group flaviviruses are Cacipacore virus (CPCV), Koutango virus (KOUV), JEV, Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), and Yaounde virus (YAOV) (1). Although initially WNV was considered to have minor human health impact, the human and equine outbreaks in Europe (Romania, Russia, France, Italy), Africa (Algeria, Tunisia, Morocco), and Asia (Israel) within the last 10 years, and especially the virus's emergence and spread in North America since 1999, put it into the focus of scientific interest. The distribution and ecology of WNV, as well as clinical features, pathogenesis, and epidemiology of West Nile disease have been reviewed (2–6). Phylogenetic analyses showed 2 distinct lineages of WNV strains (which themselves subdivide into several subclades or clusters), isolated in different geographic regions (7–10).

The presence of WNV in central Europe has been known for some time. Serologic surveys have detected specific antibodies to WNV in several vertebrate hosts in Austria, Czech Republic, Hungary, and Slovakia during the past 40 years, and several virus strains were isolated from mosquitoes, rodents, and migrating birds (3). Human cases of West Nile fever were reported in the Czech Republic in 1997 (11) and in Hungary in 2003 (12). Although these countries are important transit areas or final destinations for migratory birds from the African continent, and hence may play an important role in the circulation and conservation of different WNV strains, genetic information about the strains isolated in central Europe has not been available. We report the complete genome sequence and phylogenetic analyses, as well as antigenic and mouse virulence characteristics, of a unique flavivirus strain (97-103), closely related to WNV, which was isolated by intracranial injection of suckling mice with homogenates of female *Culex pipiens* mosquitoes collected 10 km from Lanzhot, Czech Republic, after a flood in 1997 (11,13,14). The collection site was very close to the Czech-Austrian border, ≈2 km from the small Austrian town of Rabensburg. Consequently, the isolate 97-103 was later tentatively called Rabensburg virus (RabV). Another antigenically identical or very closely related strain (99-222) was isolated from *Cx. pipiens* mosquitoes in the same location 2 years later (14).

Methods

Isolates 97-103 (passage 5 in suckling mouse brain [SMB]) and 99-222 (passage 4 in SMB) were freeze-dried in October 2000 (14). Viral RNA was extracted from 140 μL of virus resuspended in diethylpyrocarbonate

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(DEPC)-treated water, using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For amplification of the complete genome, oligonucleotide primers were designed with the help of the Primer Designer 4 for Windows 95 program (Scientific and Educational Software, version 4.10) and were synthesized by GibcoBRL Life Technologies, Ltd. (Paisley, Scotland, UK). A complete list of the 35 primers used in reverse transcription–polymerase chain reaction (RT-PCR) and sequencing reactions is available upon request. Reverse transcription and amplification were performed with a continuous RT-PCR method with the Qiagen OneStep RT-PCR Kit (Qiagen) following the manufacturer's instructions. Reverse transcription (at 50°C for 30 min) was followed by a denaturation step at 95°C for 15 min, and 40 cycles of amplification (94°C for 40 s, 57°C for 50 s, 72°C for 1 min). Reactions were completed by a final extension for 7 min at 72°C, and the amplicons were kept at 4°C until electrophoresis was carried out. The reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Corp., Wellesley, MA, USA). After RT-PCR, the amplicons were electrophoresed in agarose gel, stained with ethidium bromide, and bands were visualized under UV light. Gels were photographed with a Kodak DS Electrophoresis Documentation and Analysis System (Eastman Kodak Company, New Haven, CT, USA). Product sizes were determined with reference to a 100 – bp DNA Ladder (Promega, Madison, WI, USA). Fluorescence-based direct sequencings were performed in both directions on the PCR products with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer) and an ABI Prism 310 genetic analyzer (Perkin-Elmer) automated sequencing system (15).

The nucleotide sequences were identified by BLAST search against GenBank databases and were compiled and aligned with the help of the Align Plus 4 for Windows 95 (Scientific and Educational Software, version 4.00) and ClustalX Multiple Sequence Alignment (version 1.81) programs. Phylogenetic analysis was performed with the Phylogeny Inference Program Package (PHYLIP) version 3.57c. Distance matrices were generated by the Fitch program, with a translation/transversion ratio of 2.0. Phylogenetic trees were delineated by using the TreeView (Win32) program version 1.6.6.

Results

Both virus strains were identified as WNV by complement fixation and neutralization tests (11,13). Strain 97-103 was compared antigenically in detail with the Egyptian Eg-101 topotype strain of WNV (16), a representative of WNV lineage 1 (clade 1a). In plaque-reduction cross-neutralization tests (PRNT) with homologous and heterologous antisera (produced by injection of ICR mice with 3 intraperitoneal doses at weekly intervals), the serum raised against Eg-101 neutralized both the homologous virus and 97-103 at a titer of 512, while the strain 97-103 specific serum was effective against strain Eg-101 only at a titer of 64, although it neutralized the homologous virus at 512. The average 4-fold difference in cross-PRNT titers indicates certain antigenic heterogeneity of the 2 strains, and the 97-103 isolate was therefore regarded as a subtype of WNV (14).

Virulence of RabV strains 97-103 and 99-222 was determined by intracranial and intraperitoneal injection of specific-pathogen-free (SPF) outbred ICR mice. Central nervous system symptoms (e.g., pareses of hind limbs) developed in suckling mice, which died 7–15 days after intracranial injection (Table 1). Adult mice did not show any clinical symptoms and survived the experimental infection. On the other hand, the WNV topotype strain Eg-101 caused fatal illness in intracranially injected mice, killing them within 4 to 6 days after infection, regardless of their age (11,13). After intraperitoneal injection, strain Eg-101 killed all suckling mice but a <10% of adult mice; RabV strains 97-103 and 99-222 killed approximately one third of suckling mice, and the average survival time was 11 days (range 10–14 days). Thus, both Rabensburg virus strains exhibit clearly lower virulence for mice than the Egyptian WNV topotype strain. In addition, average survival time of suckling ICR mice injected intracranially with RabV was significantly longer than with strain Eg-101.

The genome of strain 97-103 Rabensburg virus (RabV) was investigated by RT-PCR and subsequent direct sequencing of the amplicons. Initially, oligonucleotide primers designed on the consensus sequences of linage 1 and 2 WNV strains were applied to the viral nucleic acid of RabV. On the basis of the sequence information obtained from these PCR products, specific primer pairs were designed to produce overlapping amplicons covering

Table 1. Survival time (days) of suckling mice injected intracranially with Rabensburg virus isolates 97-103 and 99-222

Suckling mouse brain (SMB) passage no.	Strain 97-103		Strain 99-222	
	Average survival time	Range	Average survival time	Range
SMB_0*	12.1	12–13	12.2	9–15
SMB_1	8.5	7–10	11.8	11–13
SMB_2	8.5	7–11	10.0	9–11
SMB_3	8.1	7–9	8.7	7–10

*Represents the original mosquito suspension during virus isolation attempts.

the entire genome. The RT-PCR products were sequenced, and the sequences were compiled, resulting in a 10,972 – nucleotide (nt–) sequence that represented the complete genome of the virus. The sequence was identified by BLAST search against GenBank databases. The highest identity rates of RabV to other flaviviruses (78%–90%) were found with certain regions of WNV strains of lineage 1 and 2.

From the second isolate (99-222), 5 genomic regions have been amplified and sequenced so far, showing a total of 3656 nt. They represent partial coding sections from the core (C), anchored C, premembrane (PreM), and membrane (M) protein regions (between nucleotide positions 117 and 752); NS3 protein region (between nucleotide positions 5294 and 5536, and between nucleotide positions 5565 and 6343); NS4b and NS5 regions (between nucleotide positions 7321 and 8112); and NS5 protein region (between nucleotide positions 9095 and 10305). Partial sequence analysis of isolate 99-222 showed >99% identity to 97-103. Aligned to strain 97-103, only a few nucleotide substitutions were observed, in the following positions: C_{609} to T; C_{720} to A; G_{5727} to A (resulting in an amino acid change Met to Ile); T_{5910} to C (resulting in an amino acid change Ile to Thr); T_{5961} to C; C_{9630} to A; and G_{9843} to T.

Similar to other flaviviruses (17), the nucleotide sequence of RabV contains l open reading frame (ORF) encoding the viral proteins as a large polyprotein precursor. The ORF starts at nucleotide position 97, and codes for a 3,433-amino acid (aa) polypeptide. The putative amino acid sequence of the polyprotein precursor gene of RabV 97-103 has been translated; based on the amino acid alignment with WNV, the putative mature proteins, conserved structural elements, and putative enzyme motifs were localized. The anchored C protein is located between nt 97 and 465; within this region, the C protein is located between nt 97 and 411. The PreM protein is encoded from nt 466 to nt 966, with the M protein between nt 742 and 966. The envelope (E) protein is encoded between nucleotide positions 967 and 2469, followed by the nonstructural proteins NS1 (nt 2470–3525), NS2a (nt 3526–4218), NS2b (nt 4219–4611), NS3 (nt 4612–6468), NS4a (nt 6469–6846), 2K (nt 6847–6915), NS4b (nt 6916–7680), and NS5 (nt 7681–10395), respectively. Amino acid identities with WNV were found at the known conserved positions (i.e., Cys residues involved in

Table 2. Sequences of West Nile virus (WNV) strains and other members of the Japanese encephalitis virus group used for phylogenetic analyses

Virus name	Code	Accession no.*	Isolation			WNV lineage, clade
			Year	Host	Geographic origin	
WNV HNY1999	NY99a	AF202541	1999	Human	New York	1a
WNV NY99flamingo38299	NY99b	AF196835	1999	Flamingo	New York	1a
WNV IS98STD	Is98	AF481864	1998	Stork	Israel	1a
WNV Italy1998Equine	It98	AF404757	1998	Horse	Italy	1a
WNV RO9750	Ro96	AF260969	1996	*Culex pipiens*	Romania	1a
WNV VLG4	Rus99a	AF317203	1999	Human	Volgograd	1a
WNV LEIV-Vlg99-27889	Rus99b	AY277252	1999	Human	Volgograd	1a
WNV PaH001	Tu97	AY268133	1997	Human	Tunisia	1a
WNV PaAn001	Fr00	AY268132	2000	Horse	France	1a
WNV Eg101	Eg51	AF260968	1951	Human	Egypt	1a
WNV Chin-01	Chin01	AY490240	Unknown	Unknown†	China	1a
WNV Kunjin MRM61C	Kunjin	D00246	1960	*Cx. annulirostris*	Australia	1b
WNV Sarafend	Sarafend	AY688948		Laboratory strain		2
WNV B956 (WNFCG)	Ug37	M12294	1937	Human	Uganda	2
WNV LEIV-Krnd88-190	Rus98	AY277251	1998	*Dermacentor marginatus*	Caucasus	4†
Rabensburg virus (97-103)	RabV	AY765264	1997	*Cx. pipiens*	Czech Republic	3†
Japanese encephalitis virus	JEV	NC_001437	–	–	–	–
Murray Valley encephalitis virus	MVEV	NC_000943	–	–	–	–
Usutu virus	USUV	AY453411	–	–	–	–
Saint Louis encephalitis virus	SLEV	*AF013416*	–	–	–	–
Alfuy virus	ALFV	*AF013360*	–	–	–	–
Cacipacore virus	CPCV	*AF013367*	–	–	–	–
Koutango virus	KOUV	*AF013384*	–	–	–	–
Yaounde virus	YAOV	*AF013413*	–	–	–	–

*Partial nucleotide sequences (NS5 protein region) are indicated in italics.
†Unknown, tentative speciation.

intramolecular bonds in the E and NS1 protein, putative integrin binding motif of the E protein, catalytic triad and substrate binding pocket of the trypsin-like serine protease, RNA helicase motif of the NS3 protein, and RNA-dependent RNA polymerase motif of the NS5 protein; 15).

To investigate the phylogenetic relationship of RabV to other WNV isolates, multiple nucleotide and putative amino acid sequence alignments were made involving 16 WNV strains (listed in Table 2). Although several complete WNV nucleotide sequences from previously published studies (10,18) have been deposited in the GenBank databases, only selected representatives of lineages and clades have been included in our alignments, in order to obtain more precise and demonstrative trees.

RabV exhibited 73%–77% nucleotide identity rates to the different WNV strains (Table 3). The relationships between the strains are demonstrated in Figure 1. The 2 lineages of WNV are obviously separated in the tree. Clade 1a viruses form a tight cluster with close genetic relationship among the members. Kunjin virus, the representative of clade 1b, appears as a separate branch of lineage 1. Unfortunately, no complete genome sequence information is available on clade 1c (Indian strains); thus, they are not represented in the tree. The prototype Uganda strain B956 (WNFCG) of lineage 2 is grouped together with the Sarafend strain, a laboratory strain with uncertain origin and passage history. Two viruses proved to be clearly distinct with significant genetic distances to all other WNV strains and also from each other: RabV and strain LEIV-Krnd88-190 (in the phylogenetic trees designated Rus98). The latter virus was isolated from *Dermacentor marginatus* ticks in the northwest Caucasus Mountain valley in 1998 and was regarded as a new variant of WNV (19–21). Because these 2 viruses differ considerably from all WNV strains, the issue is raised about whether classifying these 2 viruses as separate members of the JEV group might be more appropriate.

To elucidate this question, a comprehensive phylogenetic analysis was performed on all representatives of the JEV group. Because only partial common sequence information of the NS5 protein gene region is currently available from SLEV, ALV, CPCV, KOUV, and YAOUV (22), the phylogenetic analysis had to be restricted to this region (Figure 2). Within the investigated genome stretch, RabV showed 77%–78% identity to lineage 1 and 2 WNV strains, 77% identity to strain LEIV-Krnd88-190, and 71%–76% identity to other representatives of the JEV group. In the phylogenetic tree (Figure 2), the separation of the 2 unique strains (RabV and LEIV-Krnd88-190 = Rus98) from WNV is clearly visible. Although RabV

Table 3. Nucleotide and amino acid identity rates between RabV* and other flaviviruses

		Identity to RabV (%)			
		Nucleotide		Amino acid	
Code	WNV lineage and clade	Complete	Partial†	Complete	Partial‡
NY99a	1a	77	78	90	95
NY99b	1a	77	78	90	95
Is98	1a	77	78	90	95
It98	1a	77	78	90	95
Ro96	1a	77	78	90	95
Rus99a	1a	77	78	90	95
Rus99b	1a	77	78	90	95
Tu97	1a	76	78	90	95
Fr00	1a	77	78	90	95
Eg51	1a	77	78	90	95
Chin01	1a	77	78	90	95
Kunjin	1b	75	77	89	94
Sarafend	2	77	78	90	96
Ug37	2	77	78	90	96
Rus98	4 (speculation)	73	77	87	95
JEV	–	68	74	75	86
MVEV	–	69	74	76	86
USUV	–	68	72	75	83
SLEV	–	–	71	–	78
ALFV	–	–	74	–	88
CPCV	–	–	71	–	79
KOUV	–	–	76	–	90
YAOV	–	–	75	–	87

*RabV, Rabensburg virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; USUV, Usutu virus; SLEV, St. Louis encephalitis virus; ALFV, Alfuy virus; CPCV, Cacipacore virus; KOUV, Koutango virus; YAOV, Yaounde virus.
†Partial alignment between nucleotide positions 9067 and 10101.
‡Partial alignment between amino acid positions 2991 and 3335.

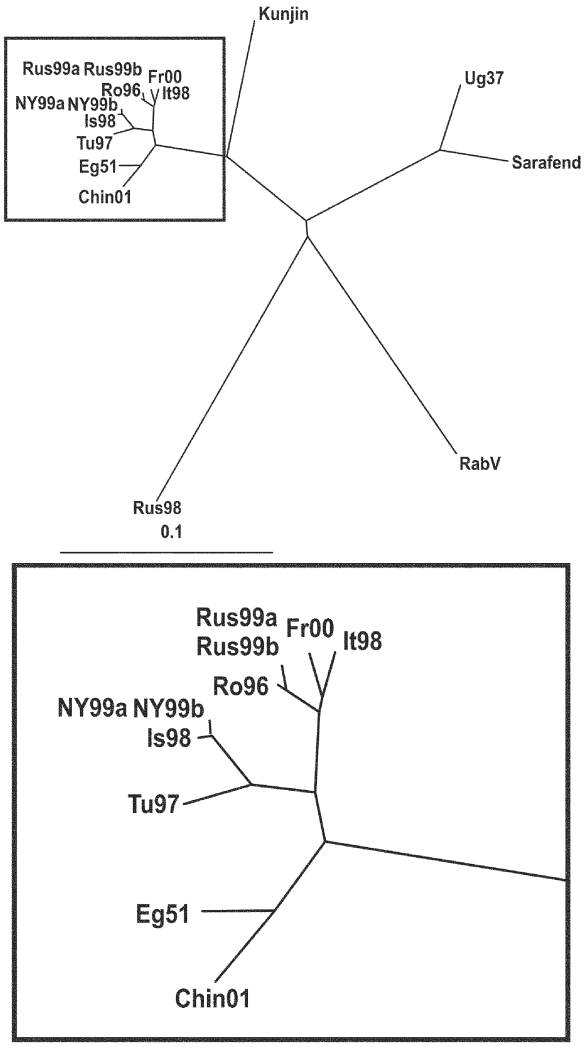

Figure 1. Phylogenetic tree illustrating the genetic relationship between selected West Nile virus strains based on their complete genome sequences. Bar on the left demonstrates the genetic distance. (Abbreviations and accession numbers are listed in Table 2.)

exhibits the closest relationship to the WNV representatives, similar identity rates (76%) exist between MVEV and USUV, as well as between JEV and ALFV, and these viruses have been taxonomically classified as separate viruses. The Rus98 virus clusters together with KOUV, a virus isolated originally from a Kemp's gerbil (*Tatera kempi*) in Senegal 1968 and subsequently recovered from other rodent species and several genera of ticks (*Rhipicephalus*, *Hyalomma*, *Alectorobius*) in central Africa (23). The Rus98 strain was also isolated from ticks.

The putative amino acid sequence of RabV was also compared with the corresponding sequences of representatives of WNV lineages and clades, as well as with other JEV group viruses on the available polypeptide sequence regions. RabV shared 89%–90% identity on the complete polypeptide precursor region with the WNV strains, 87% identity with the Rus89 strain, and 75%–76% identity with JEV, USUV, and MVEV. The alignments of the partial amino acid sequences of the NS5 region (between aa 2991 and 3335) showed 94%–96% identity rates with the WNV strains, 95% with strain Rus98, and 78%–90% with the other members of the JEV group (Table 3). Phylogenetic trees, based on the amino acid alignments, displayed nearly identical topology to nucleotide sequence–based trees (data not shown). The complete genome sequence of RabV (flavivirus strain 97-103) has been deposited in GenBank under accession no. AY765264.

Discussion

WNV strains of different lineages exhibit considerable genomic diversity (76%–77% nucleotide identity only). At the same time, WNV is not sharply delimited genomically from the other members of the JEV group. The available partial sequences of the NS5 gene region from other viruses of the group show 71%–76% nucleotide and 78%–90% amino acid identities to WNV strains. The closest relatives of WNV are KOUV and YAOV (10,22–24).

Lineage 1 of WNV comprises strains from several continents and is subdivided into at least 3 clades. In clade 1a, several subclades or clusters are formed by closely related strains, such as strains isolated 40–50 years ago in Europe and Africa; strains isolated 20–30 years ago in Africa; strains isolated within the last 10 years in Europe and Africa; and strains isolated within the last 5 years in the United States and Israel. Clade 1b consists of the Australian isolates (Kunjin), while clade 1c contains strains from India. Lineage 2 is composed of WNV strains that have been isolated, so far exclusively, in the sub-Saharan region of Africa and in Madagascar (18). The genetic distance between the 2 lineages is relatively great in contrast to that within some representatives of lineage 1 that were isolated in distant geographic locations and within considerable time intervals. While the viruses in clade 1a share 95.2%–99.9% nucleotide and 99.3%–100% amino acid identity to each other, and also 86.6%–87.8% nucleotide and 97.4%–97.7% amino acid identity to the clade 1b viruses, the overall identity rates between lineage 1 and 2 are only 75.7%–76.8% on nucleotide level and 93.2%–94.0% on amino acid level (18), identity rates that resemble those between RabV and either lineage 1 or lineage 2 WNV strains. Besides genomic differences, antigenic variability can be observed in cross-neutralization analyses and monoclonal antibody binding assays (8,18).

The results of the phylogenetic analyses indicate that viruses closely related to WNV are present in central Europe and southern Russia. Although these viruses have initially been identified as WNV, they can be regarded, on

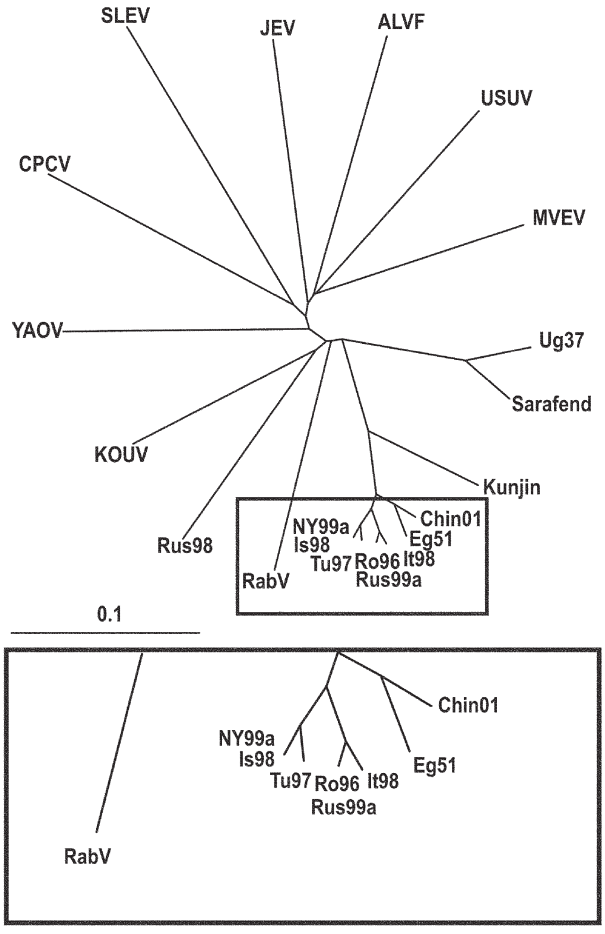

Figure 2. Phylogenetic tree illustrating the genetic relationship between representatives of the Japanese encephalitis virus complex and selected West Nile virus strains based on partial genome sequences of the NS5 protein gene. Bar on the left demonstrates the genetic distance. (Abbreviations and accession numbers are listed in Table 2.)

the basis of their genetic distances, either as separate lineages of WNV (RabV: lineage 3; LEIV-Krnd88-190 = Rus98: lineage 4) or as new viruses within the JEV group. The antigenic and biologic differences between RabV and the WNV reference strain Eg-101 also support this opinion. Isolation of RabV in 1997 was obviously not an isolated event; rather, flaviviruses of the RabV type seem to be present or persist in this area, as demonstrated by the isolation of an almost identical virus strain (99-222) 2 years later (14). The ecology of RabV needs further investigation. Other unanswered questions concern the pathogenicity and host spectrum of the virus, especially regarding possible human infections.

To summarize, a novel flavivirus strain of unknown human pathogenicity, repeatedly isolated from *Cx. pipiens* mosquitoes in central Europe, has been molecularly characterized, including determination of its complete nucleotide and deduced amino acid sequences. Based on the analysis of the virus and comparison with related viruses including phylogenetic relationships, we suggest that RabV be classified either as a new (third) lineage of WNV or as a novel flavivirus within the JEV group.

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PRÁCE 18

Meister T., **Lussy H.**, Bakonyi T., **Šikutová S.**, **Rudolf I.**, **Vogl W.**, **Winkler H.**, **Frey H.**, **Hubálek Z.**, **Nowotny N.**, **Weissenböck H**. 2008. Serological evidence of continuing high Usutu virus (*Flaviviridae*) activity and establishment of herd immunity in wild birds in Austria. *Vet. Microbiol*. 127: 237–248.

Stručná charakteristika: Usutu virus (USUV) (čel. *Flaviviridae*) je arbovirus, který se endemicky vyskytuje v Africe, kde cirkuluje mezi komáry a ptáky. Pro některé ptačí druhy je vysoce patogenní. V roce 2001 došlo k emergenci USUV mimo africký kontinent (podle jedné retrospektivní studie došlo k introdukci do Evropy již dříve – viz. Weissenboeck a kol., 2013), kdy byla zaznamenána rozsáhlá epizootie převážně u kosí populace ve Vídni a jejím okolí. Tato sérologická studie si kladla za cíl vyšetřit některé ptačí druhy v místě introdukce viru a zhodnotit celkovou aktivitu USUV (přítomnost protilátek) včetně monitoringu/zjištění tzv. 'herd immunity'.

Hlavní přínos práce: bylo vyšetřeno značné množství sér volně žijících ptáků (442 jedinců náležejících do 55 druhů) a také 86 dravců a sov z rehabilitačního centra na přítomnost protilátek proti USUV pomocí hemaglutinačně inhibičního a neutralizačního testu. Práce potvrdila vysokou 'kontinuální' aktivitu USUV v daném regionu (byly sledovány roky 2003-2006).

Příspěvek autora k dané práci: autor se podílel na hodnocení neutralizačního testu a přípravě rukopisu.

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Serological evidence of continuing high *Usutu virus* (*Flaviviridae*) activity and establishment of herd immunity in wild birds in Austria

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Abstract

Usutu virus (USUV), family *Flaviviridae*, has been responsible for avian mortality in Austria from 2001 to 2006. The proportion of USUV-positive individuals among the investigated dead birds decreased dramatically after 2004. To test the hypothesis that establishment of herd immunity might be responsible, serological examinations of susceptible wild birds were performed.

Blood samples of 442 wild birds of 55 species were collected in 4 consecutive years (2003–2006). In addition, 86 individuals from a birds of prey rehabilitation centre were bled before, at the peak, and after the 2005 USUV transmission season in order to identify titre dynamics and seroconversions. The haemagglutination inhibition test was used for screening and the plaque reduction neutralization test for confirmation. While in the years 2003 and 2004 the proportion of seropositive wild birds was <10%, the percentage of seroreactors raised to >50% in 2005 and 2006. At the birds of prey centre, almost three quarters of the owls and raptors exhibited antibodies before the 2005 transmission season; this percentage dropped to less than half at the peak of USUV transmission and raised again to almost two thirds after the transmission season.

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These data show a from year to year continuously increasing proportion of seropositive wild birds. The owl and raptor data indicate significant viral exposure in the previous season(s), but also a number of new infections during the current season, despite the presence of antibodies in some of these birds. Herd immunity is a possible explanation for the significant decrease in USUV-associated bird mortalities in Austria during the recent years.

Keywords: *Usutu virus*; USUV; Serology; Wild birds; Herd immunity

1. Introduction

Usutu virus (USUV), a member of the Japanese encephalitis virus (JEV) antigenic group within the mosquito-borne cluster of the genus *Flavivirus* (Kuno et al., 1998) was isolated for the first time from mosquitoes (*Culex univittatus*) in South Africa in 1959 and named after a river in Swaziland. Although the virus had been detected several times in different mosquito and bird species in Africa, it had never been associated with clinical disease in birds or mammals and was therefore widely scientifically ignored. In summer 2001, however, USUV emerged unexpectedly in central Europe and was responsible for an episode of mortality among Eurasian blackbirds (*Turdus merula*) and great grey owls (*Strix nebulosa*) in and around Vienna, Austria (Weissenböck et al., 2002). In the following years the same virus strain continued to kill birds in eastern Austria (Weissenböck et al., 2003b; Chvala et al., 2007). This observation showed that USUV had managed to overwinter and had been able to establish an efficient local bird–mosquito transmission cycle (Weissenböck et al., 2003a). Meanwhile, USUV-associated bird mortality has been registered in other central European countries like Hungary (Bakonyi et al., unpublished data), Switzerland (ProMED-mail) and Italy (Dorrestein et al., 2007). Surveillance data of USUV-associated bird deaths in Austria indicated that seasons of massive USUV-associated bird losses (2001–2003) were followed by seasons with significant decline of USUV-linked avian mortality (2004–2006) (Chvala et al., 2007). In addition to climatic reasons (the summers of 2004 and 2005 had unusually low average temperatures in Austria, http://www.zamg.ac.at) or decreased virulence of the circulating virus another possible explanation for this phenomenon would be a progressive seroconversion in the Austrian wild bird population.

As it has to be expected that USUV will continue to expand its area of activity during the next years, data on seroprevalence and potential herd immunity in the European area affected first, i.e. eastern Austria, might be useful for other scientists and wildlife conservationists having to deal with this phenomenon in the future.

The aims of the present study were first to evaluate the proportion of USUV antibody positives among wild birds in Austria and to record changes during the course of time. Second, we intended a longitudinal serological study with three blood collection time-points from the same individuals during one transmission season in order to determine the dynamics of change in antibody titre to USUV in naturally infected birds. For this part of the study an owl and raptor rehabilitation centre situated within the USUV-endemic area in eastern Austria was chosen because (i) some owl species (great grey owl, *Strix nebulosa*, Tengmalm's owl, *Aegolius funereus*) easily acquired USUV infection and also succumbed to it, (ii) birds of prey and owls were found to be frequently affected by the related *West Nile virus* (WNV) in North America (Fitzgerald et al., 2003; Gancz et al., 2004; Wünschmann et al., 2004) and (iii) because the centre offered a large collection of wild birds in an open mosquito-accessible environment with the opportunity of repeated blood collections of the same birds, something not easily done with wild birds.

2. Materials and methods

2.1. Sera for seroprevalence study

Bird sera were collected in 4 consecutive years, between August 2003 and May 2006. As the transmission season of USUV is most likely restricted

to the months July to September, the data of the 2006 sera reflect viral exposure which had happened up to the 2005 transmission season. In total, sera of 442 birds were included. A total of 113 sera were collected in 2003 (between August and December), 109 sera in 2004 (January to October), 197 sera in 2005 (March to October), and finally, 23 sera were collected in the first 5 months of 2006. As it significantly influences the interpretation of the results, a possible exposure in the previous year(s) was especially considered for the 2005 and 2006 sera. The sources of sera were (i) wild birds captured in mist nets or other trapping devices especially for the purpose of USUV serosurveillance (2003: 14; 2004: 27; 2005: 91; 2006: 23), (ii) sick or injured birds brought to the bird clinic of the University of Veterinary Medicine, Vienna, for treatment (2003: 28; 2004: 2; 2005: 45), (iii) birds from the above mentioned owl and raptor rehabilitation centre (2003: 28; 2005: 38), and (iv) dead birds submitted for necropsy (2003: 43; 2004: 80; 2005: 23). The sera originated from 55 different species of birds**.** The huge majority of the birds were from USUV-endemic areas in Vienna, Lower Austria and Burgenland. Only seven birds were from areas where USUV activity has not been found so far.

2.2. Longitudinal serosurvey in captive birds of prey

All birds originated from a birds of prey rehabilitation centre which is located in the village Haringsee (48°11′N, 16°46′E) in the geographic area Marchfeld in Lower Austria. The entire area is 11,000 m^2 in size. There are 70 separate aviaries covering a total of 3000 m^2. The birds were separated according to species, and aviaries with birds of the same species were located in close proximity to each other. The station mainly provides medical care and shelter for injured bird foundlings and confiscated animals, and information for the interested public. USUV activity has been recognized in the area since 2003 with the virus found in dead blackbirds and in mosquitoes (Chvala et al., 2007).

Blood samples were collected from 86 birds belonging to 9 species: 6 species of the family *Strigidae*: 8 eagle owls (*Bubo bubo*), 18 barn owls (*Tyto alba*), 14 tawny owls (*Strix aluco*), 4 little owls (*Athene noctua*), 5 long-eared owls (*Asio otus*), 1 Ural owl (*Strix uralensis*), 2 accipitrid species: 20 common buzzards (*Buteo buteo*) and 4 marsh harriers (*Circus aeruginosus*), and 1 falcon species, namely 12 common kestrels (*Falco tinnunculus*). From each bird three blood samples were taken at approximately 2-month intervals during 2005: the first blood samples prior to any anticipated USUV activity (May 25), the second sample on August 29 at the time when in the previous years USUV activity had reached its peak, and the final sample was taken October 17, 2005, when, according to the experiences from the previous years, USUV activity should have ceased and antibodies due to recent exposure should have developed. All birds were after hatch-year birds (older than 1 year), except for one Ural owl, which was a hatch-year fledgling. None of the birds showed clinical signs during the surveillance period. For USUV antibody assays 0.2–0.5 ml of blood was drawn from the cutaneous ulnar vein. The blood was transferred into heparin–lithium tubes (Sarstedt, Nürnbrecht, Germany) and centrifuged at 2000 × *g* for 15 min. The plasma was separated from the clot and stored at −20 °C until use. In order to rule out test variabilities, all three blood collections of the birds of prey rehabilitation centre were tested in one investigation and carried out and read by the same investigator.

2.3. Serological tests

The majority of the bird sera obtained for the seroprevalence study were examined by the haemagglutination inhibition test (HIT). Whenever possible, HIT positives were confirmed by the plaque reduction neutralization test (PRNT). However, due to the small quantity of some sera, either this confirmation could not be performed or it was decided to use the PRNT only.

All serum samples of the longitudinal study were analysed by HIT for initial screening. Positive samples (titre $\geq$1:20) were also tested by PRNT to evaluate the specificity of the HIT. To rule out a possible cross-reaction of the tests with *tick-borne encephalitis virus* (TBEV) and WNV a number of randomly selected USUV-positive sera (TBEV: 55; WNV: 49) were also tested with serological test systems established for detection of antibodies to these viruses.

2.4. HIT for USUV and TBEV antibodies

The standard HIT was performed as previously described by Clarke and Casals (1958) and as adapted for USUV by Chvala et al. (2005). In brief, non-specific inhibitors and natural haemagglutinins were removed by kaolin treatment and absorption with goose erythrocytes, respectively. Serial dilutions of kaolin-treated bird sera were mixed with eight haemagglutinating (HA) units of USUV strain Vienna 2001-blackbird or TBEV strain KEM_1 antigen (Molnar, 1982), respectively. Tests were performed in U-shaped microtitre plates. The HIT titre was determined as the highest serum dilution that caused complete inhibition of erythrocyte agglutination. Sera with a titre of 1:20 and higher were considered positive.

2.5. PRNT for USUV and WNV antibodies

The PRNT method for USUV and WNV antibodies was performed as described by de Madrid and Porterfield (1974), adopted to a microtechnique (Hubálek et al., 1979).

The sera were inactivated at 56 °C for 30 min prior to testing. The PRNTs were run in microtitre plates with flat-bottomed wells. For USUV the above mentioned virus strain Vienna-2001 blackbird (Bakonyi et al., 2004) and porcine kidney (PK) cells, and for WNV the WNV topotype strain Eg-101 and the pig kidney embryo cell line SPEV was used. Twofold serum dilutions were made in Minimal Essential Medium (MEM), or in case of WNV in L-15 medium; 30 μl of diluted sera were mixed with 30 μl of virus suspension containing 100 plaque-forming units of the virus and incubated for 60 min at 37 °C. Then 60 μl of cell suspension in MEM with 3% foetal calf serum (in case of WNV L-15 medium with 2% foetal calf serum) was added to each well and incubated at 37 °C for 4 h. Thereafter 120 μl of a carboxy-methyl cellulose overlay was added to each well and incubated at 37 °C for 3 days (5 days in case of WNV). The fluid was removed and 150 μl of the colouring naphtol blue black solution was added for 40 min at room temperature. The PRNT titre was determined as the highest serum dilution with a 90% reduction of the number of plaques. Sera with a titre of at least 1:20 were considered positive. The specificity of this assay for antibodies to the viruses tested (i.e. USUV and WNV) had been validated by using WNV- and USUV-positive test sera. Cross-reactivity was minimal and only occurred in sera with high titres to one of the viruses to a titre of at least four dilution steps less than the homolog virus.

2.6. RT-PCR for detection of viraemia

At the assumed peak of USUV activity (August), we also took blood samples from 32 larger birds (8 eagle owls, 20 buzzards, and 4 marsh harriers) for determination of viraemia. From these birds, blood was drawn from the ulnar vein into EDTA-treated tubes (Sarstedt, Nürnbrecht, Germany), centrifuged at 6700 × *g* for 5 min, and the plasma was saved for serological studies. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coat using erythrocyte lysis buffer (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was extracted from the PBMCs using the QiaAmp Viral RNA Mini Kit, and RT-PCRs were performed in a continuous one-step RT-PCR system employing USUV-specific primer pairs (Bakonyi et al., 2004; Weissenböck et al., 2004).

3. Results

3.1. Antibodies to USUV are found in an increasing proportion of wild birds between 2003 and 2006

Of the 222 birds tested in 2003 and 2004, 19 (8.5%) were positive for USUV by HIT. The titres ranged from 1:20 to 1:1280, with a geometrical mean titre of 51.8. All positives except one were confirmed by PRNT. Four of the positive birds were necropsy cases with an acute USUV infection. Among the 19 examined owls 6 (31.6%) were positive. The USUV positive sera were also tested by HIT for antibodies to TBEV. One serum (with an USUV titre of 1:1280) showed a positive reaction (1:80). All other sera were TBEV antibody negative.

In 2005 and early 2006 a total of 220 sera was tested by HIT (150) and/or PRNT (157). In 87 cases a comparative evaluation of both tests could be performed. In these years 119 (54%) of the samples

Table 1
Compilation of all wild birds, sorted according to numbers, species and years, which were subjected to serological investigation

No	Common name	Scientific name		Total	2003 postive/ total	2004 positive/ total	2005 positive/ total	2006 positive/ total	All years positive/ total
1	Eurasian blackbird	*Turdus merula*		165	3/33	8/83	20/35	6/14	37/165
2	Blackcap	*Sylvia atricapilla*	S	23			12/23		12/23
3	Ural owl	*Strix uralensis*		22			16/22		16/22
4	Eurasian collared dove	*Streptopelia decaocto*		20	0/11		6/9		6/20
5	Great tit	*Parus major*		19	0/1	0/9	3/9		3/19
6	Long-eared owl	*Asio otus*		17	3/11		5/6		8/17
7	Great spotted woodpecker	*Dendrocopos major*		16			2/7	3/9	5/16
8	Kestrel	*Falco tinnunculus*		12	0/7		4/5		4/12
9	European robin	*Erithacus rubecula*	S	11			5/11		5/11
10	Tawny owl	*Strix aluco*		10	3/8	0/1	1/1		4/10
11	Jackdaw	*Corvus monedula*		10	0/3		6/7		6/10
12	Song thrush	*Turdus philomelos*	S	9	0/4	0/1	4/4		4/9
13	Tree sparrow	*Passer montanus*		9	0/8		0/1		0/9
14	Jaybird	*Garrulus glandarius*		8	1/2	1/4	2/2		4/8
15	Bearded vulture	*Gypaetus barbatus*		7	0/2		2/5		2/7
16	Blue tit	*Parus caeruleus*		7	0/1	0/6			0/7
17	Reed warbler	*Acrocephalus scirpaceus*	L	7			3/7		3/7
18	Common buzzard	*Buteo buteo*		6	0/2	0/2	0/2		0/6
19	Hooded crow	*Corvus corone cornix*		6	0/2		3/4		3/6
20	Rook	*Corvus frugilegus*	W	5	0/3	0/2			0/5
21	Nuthatch	*Sitta europaea*		4			1/4		1/4
22	Eagle owl	*Bubo bubo*		3			1/3		1/3
23	Marsh harrier	*Circus aeruginosus*	L	3	0/2		1/1		1/3
24	Yellowhammer	*Emberiza citrinella*		3	0/3				0/3
25	Barn-swallow	*Hirundo rustica*	L	2			2/2		2/2
26	European goldfinch	*Carduelis carduelis*		2	0/2				0/2
27	Kingfisher	*Alcedo atthis*		2	0/2				0/2
28	Lesser whitethroat	*Sylvia curruca*	L	2			1/2		1/2
29	Middle-spotted woodpecker	*Dendrocopos medius*		2			0/2		0/2
30	Mute swan	*Cygnus olor*		2			0/2		0/2
31	Pheasant	*Phasianus colchicus*		2	0/1		1/1		1/2
32	Reed bunting	*Emberiza schoeniclus*	S	2			1/2		1/2
33	Whitethroat	*Sylvia communis*	L	2			1/2		1/2
34	Barn owl	*Tyto alba*		1			1/1		1/1
35	Black redstart	*Phoenicurus ochruros*	S	1			1/1		1/1
36	Brambling	*Fringilla montifringilla*	W	1			0/1		0/1
37	Capercaillie	*Tetrao urogallus*		1			0/1		0/1
38	Chaffinch	*Fringilla coelebs*		1		0/1			0/1
39	Chiffchaff	*Phylloscopus collybita*	S	1			0/1		0/1
40	Crossbill	*Loxia curvirostra*		1			0/1		0/1
41	Garden warbler	*Sylvia borin*	L	1			1/1		1/1
42	Greenfinch	*Carduelis chloris*		1	0/1				0/1
43	House martin	*Delichon urbica*	L	1			1/1		1/1
44	Mallard duck	*Anas platyrhynchos*		1	0/1				0/1
45	Nightingale	*Luscinia megarhynchos*	L	1			0/1		0/1
46	Indian peafowl	*Pavo cristatus*		1			1/1		1/1
47	Penduline tit	*Remiz pendulinus*	S	1			0/1		0/1
48	Pied flycatcher	*Ficedula hypoleuca*	L	1			1/1		1/1
49	Quail	*Coturnix coturnix*	L	1			0/1		0/1
50	Red-backed shrike	*Lanius collurio*	L	1			0/1		0/1
51	Seagull	*Larus sp.*		1	0/1				0/1

Table 1 (*Continued*)

No	Common name	Scientific name		Total	2003 postive/ total	2004 positive/ total	2005 positive/ total	2006 positive/ total	All years positive/ total
52	Sparrow hawk	*Accipiter nisus*		1	0/1				0/1
53	Starling	*Sturnus vulgaris*	S	1			1/1		1/1
54	Waxwing	*Bombycilla garrulous*	W	1			0/1		0/1
55	Woodcock	*Scolopax rusticola*	S	1	0/1				0/1
				442	10/113	9/109	110/197	9/23	138/442

Positive means titres ≥1:20 to USUV, either with HIT or PRNT. S: short distance migrant (winter habitat: mediterranian); L: long distance migrant (winter habitat: sub-saharan Africa); W: winter guest.

were found positive: 11 exclusively by HIT (no PRNT performed), 29 exclusively by PRNT (no HIT performed), 68 with correspondingly positive HIT and PRNT results, and 11 cases with positive HIT and negative (7) or not analysable (4) PRNT. These results are compiled in Table 1 and Fig. 1. Seventy-one (43 of which were positive) of the 2005 samples and all 23 (9 of which were positive) 2006 samples were taken before July, i.e. before the actual year's transmission season (Table 2). Thus these samples indicate antibody titres acquired the years before. Of the sick or dead birds examined 29 had an acute USUV infection with characteristic lesions and presence of virus in a number of tissues. Out of these birds only four were serologically positive.

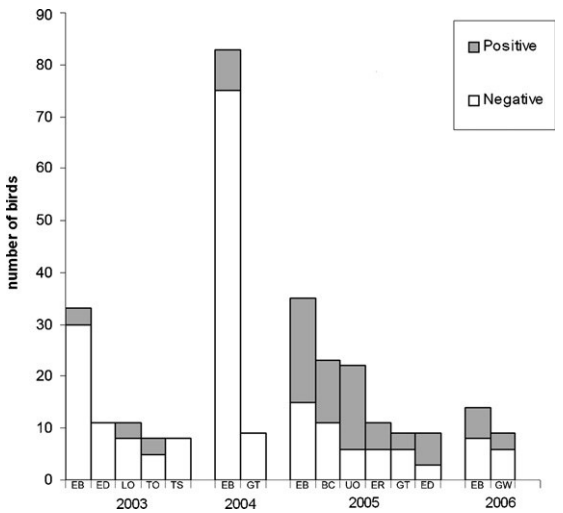

Fig. 1. Histogram showing the ratio of serologically USUV-positive and USUV-negative (based on both HIT and PRNT data) birds among the animals examined from 2003 to 2006. Only bird species of which more than seven individuals were examined are included. Two hundred and ninety five examined birds (66.7% of the total) are presented in this figure. EB: Eurasian blackbird, TO: tawny owl, LO: long-eared owl, ED: Eurasian collared dove, TS: tree sparrow, GT: great tit, ER: European robin, GW: great spotted woodpecker, BC: blackcap, UO: Ural owl.

An interesting aspect of this study were the serological data of the 78 examined juvenile birds (Table 2). Forty-two (54.5%) of them were serologically USUV antibody positive. Among them were five Ural owls whose antibody titres were 1:20 (1), 1:40 (2), and 1:80 (2). The adult females that produced these six nestlings had titres of 1:320 and 1:2560, respectively. The mother of the other juveniles was unknown.

3.2. Captive birds of prey show a high proportion of USUV antibody positives and considerable HIT titre dynamics during one transmission season

In May 2005, 63 (73.3%) out of 86 birds exhibited HIT antibodies to USUV (titres ≥1:20). The titres ranged from 1:20 to 1:640, with the majority (69.8%) having a titre of 1:80 or lower. In August 2005, the number of seropositives declined to 39 (45.3%), the majority of which (56.4%) had low titres of 1:20 or 1:40. In October 2005, 56 (65.1%) were serologically positive, with a higher proportion of medium and high titres (almost 60.7% with titres ≥1:80) compared to the previous two timepoints (Figs. 2 and 3).

A total of 143 sera, which showed a HIT titre of least 1:20 were tested by PRNT for confirmation. 85.3% of the PRNT titres were in accordance with the HIT results. Sixty-two of the sera sampled in May were tested by PRNT. Of these, 25 showed a lower titre compared to HIT, four HIT positives were

Table 2
Serologically investigated wild birds, grouped according to sampling timepoint (before/after start of USUV transmission season), age (born in the year of sampling or earlier) and presence of migratory birds among the sampled individuals

	Year 2003				Year 2004				Year 2005				Year 2006			
	Before July[a]		After July[a]		Before July[a]		After July[a]		Before July[a]		After July[a]		Before July[a]		After July[a]	
	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]	Neg[b]	Pos[b]
Juveniles	0	0	1	0	0	0	0	0	5[c]	11[c]	30[c]	31[c]	0	0	0	0
Migrants	0	0	1	0	0	0	0	0	0	0	8[d]	8[d]	0	0	0	0
Total	0	0	103	10	18	2	82	7	28	43	59	67	14	9	0	0

[a] Sampling timepoint.
[b] Serological result.
[c] Juvenile seropositive birds belonged to the following species: 10 Eurasian blackbirds (*Turdus merula*), 5 blackcaps (*Sylvia atricapilla*), 5 Ural owls (*Strix uralensis*), 3 Eurasian collared doves (*Streptopelia decaocto*), 2 barn swallows (*Hirundo rustica*), 2 bearded vultures (*Gypaetus barbatus*), 2 European robins (*Erithacus rubecula*), 2 reed warblers (*Acrocephalus scirpaceus*), 1 black redstart (*Phoenicurus ochruros*), 1 garden warbler (*Sylvia borin*), 1 great tit (*Parus major*), 1 house martin (*Delichon urbica*), 1 jaybird (*Garrulus glandarius*), 1 kestrel (*Falco timunculus*), 1 lesser whitethroat (*Sylvia curruca*), 1 nuthatch (*Sitta europaea*), 1 pied flycatcher (*Ficedula hypoleuca*), 1 song thrush (*Turdus philomelos*), 1 whitethroat (*Sylvia communis*).
[d] All juveniles.

negative by PRNT, and one PRNT titre could not be analyzed due to cytotoxicity of the serum. Of the sera taken in August 34 were tested by PRNT. In 10 of the samples the PRNT titre was lower than the HIT titre. Two sera were negative by PRNT and 10 were cytotoxic. Of the October samples, 47 were tested by PRNT. Twenty-five sera had a lower PRNT titre compared to HIT. Five sera were negative and six were cytotoxic.

3.3. Low titre haemagglutinating antibodies to TBEV and neutralizing antibodies to WNV are present in a few birds

A portion of USUV antibody positive sera from the third bleeding time were also tested by HIT for TBEV antibodies. Only 7 of 55 exhibited a low-range titre of 1:20 and 1:40, respectively.

Forty-nine USUV antibody positive birds were tested by PRNT for WNV antibodies. Of 19 birds from the first bleeding time, 15 were negative, 1 kestrel showed a titre of 1:40, and 2 marsh harriers and 1 barn owl had titres of 1:20. Of the 11 tested birds of the second bleeding time, 7 were negative, 7 birds exhibited titres of 1:20 (common buzzard, Ural owl) and 2 kestrels had titres of 1:80 and 1:160, respectively. In October, the third bleeding, 19 birds were tested, 12 of which were negative; 5 had titres of 1:20, and 2, both kestrels, showed titres of 1:80 and 1:160, respectively.

3.4. No evidence of viraemia in the sampled birds at the peak of the transmission period

USUV nucleic acid sequences were not detected in any of the examined PBMC samples by RT-PCR.

4. Discussion

Since its first documented emergence in central Europe in 2001, USUV has been associated with rising avian mortality in the affected areas which was followed by a rapid decline of USUV-associated deaths by 2004 until present. A major aim of the study was to discern, whether an increasing number of seroreactors in the wild bird population might have contributed to this phenomenon. The data point towards a low USUV antibody prevalence in samples from 2003 to 2004, and

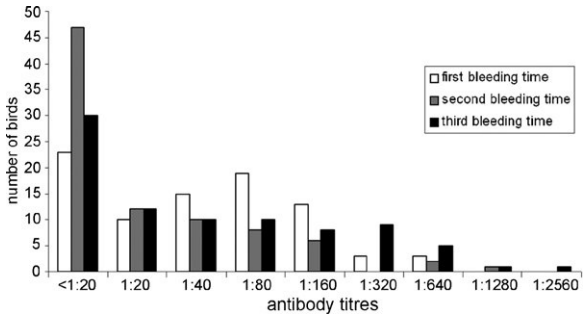

Fig. 2. Histogram depicting the percentage of captive birds of prey with certain HIT antibody titres at the different timepoints of sampling.

a clearly increased antibody prevalence in samples taken in 2005 and 2006. This change is not likely to be due to biased sample selection. Especially two subpopulations of examined birds – blackbirds and owls – originated from comparable habitats during the entire investigation period.

In the cases in which comparative investigations of sera were carried out by HIT and PRNT the majority of the HIT titres were confirmed by PRNT. Generally the PRNT titres were lower. Although the HIT is not considered to be highly specific, it proved useful as initial screening test in the present study. Possible cross-reactions or false positive reactions did not occur on a grand scale. The only other flavivirus known to be enzootic in Austria is TBEV. The most likely explanation for the few seroreactors to TBEV in the used HIT is cross-reactivity with USUV, as the TBEV titres were generally 8–16 times lower than those to USUV. HIT cross-reactivity between these two distantly related flaviviruses has also been previously noticed (Casals and Brown, 1954; de Madrid and Porterfield, 1974; Stiasny et al., 2006). Also cross-reactivity of USUV with WNV including associated lineages (e.g. *Rabensburg virus* (RabV) (Bakonyi et al., 2005)) is very likely. Using the less specific HIT, distinction of USUV- and WNV-titres might have been difficult or impossible. Therefore, the more specific PRNT was used in the search for WNV antibodies. The WNV serological data of a randomly chosen subset of samples showed several reactors, the majority of which had a low titre. These low titres are explainable by cross-reactivity to USUV, as all these cases had high USUV titres. The few birds with a moderate or high titre to WNV (e.g. common kestrel) could represent WNV- (or RabV-) infected animals, because the locality, where RabV was isolated, is situated very closely to the USUV study site (Hubálek et al., 1998). As the vast majority of these birds had

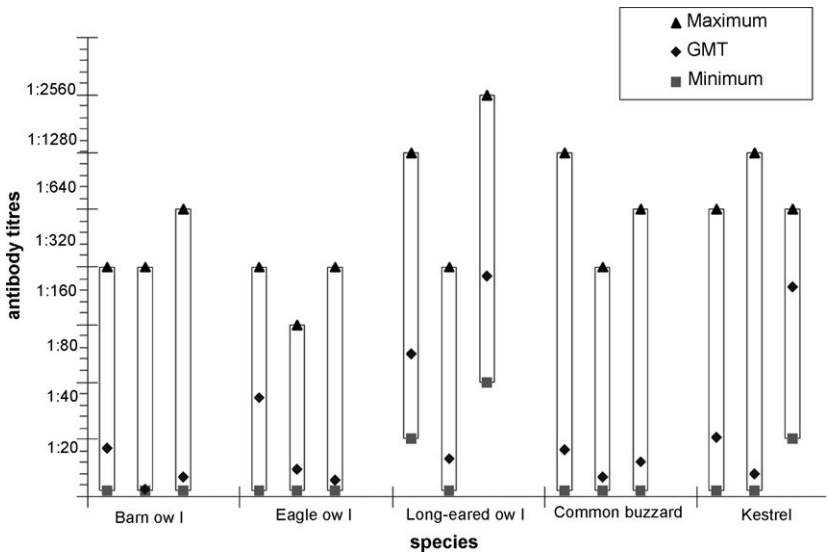

Fig. 3. Histogram depicting the HI titre dynamics in the birds of prey during timecourse. For each species which comprised more than seven individuals the geometric mean titre (GMT), and the minimum and maximum titres are shown in columns. The three columns for each bird species demonstrate the values at the three sampling timepoints.

high haemagglutinating and moderate neutralizing antibody titres to USUV, cross-reactivity of WNV antibodies in the USUV assays seem rather unlikely. Thus, these kestrels might represent double infections with USUV and a representative of one of the WNV lineages. The presence of a few seroreactors to WNV is not surprising and is in line with previous seroepidemiological studies from comparable geographical regions (Hubálek and Halouzka, 1999; Hubálek et al., 2005).

The serological data indicate that species do not differ in the likelihood to acquire USUV infection. However, there seem to be great differences with respect to the expression of clinical symptoms: while certain species, like blackbirds, great grey owls and obviously house sparrows – as recently shown in Switzerland (Steinmetz et al., 2007) – succumb in high numbers to the infection, other species never exhibited significant USUV-associated mortality.

A small number of seropositive birds, especially among those captured in 2005, were long distance migrants, i.e. birds with wintering habitats in sub-Saharan Africa. Adult birds of this group could well have acquired USUV antibodies in Africa. However, the vast majority of these birds were identified as juveniles, i.e. they had hatched in Austria several weeks or months prior to sampling, and provided that maternally transferred antibodies do not last until several months of age, they most likely have been exposed to the virus in Austria. Data concerning persistence of maternally transferred antibodies in wild birds are scarce (Müller et al., 2004; Hahn et al., 2006); thus it cannot be definitely excluded that some of these antibodies have their origin in Africa. The few seropositive juvenile birds for which the mother was known were five Ural owl nestlings with an age of 62 days at sampling. The USUV antibody levels of these birds were markedly lower than those of their mothers. As sampling in these nestlings took place before the transmission season the results suggest that they might have acquired antibodies through passive transmission and that detectable amounts of passively transferred USUV antibodies are detectable up to 2 months. In contrast, Gibbs et al. (2005) found maternal WNV antibodies in rock pigeons only up to 30 days after hatching. Alternatively, it cannot be ruled out that the juvenile Ural owls were exposed to one of the alternative transmission routes (see below), which are not necessarily linked with mosquito activity.

While in 2003 the proportion of USUV-positives among dead birds collected during a surveillance program was more than 50%, this percentage dropped to 5% and less in 2004 and 2005 (Chvala et al., 2007). One possible explanation for such a phenomenon could be establishment of herd immunity resulting in an increasing number of birds born with passive immunity under the protection of which active immunity can develop in the case of exposure. Although the serological data of the 2004 birds did not yet suggest such a phenomenon, the closer inspection of the 2005 data shows that more than a third of the samples were taken before the transmission season and thus indicate titres acquired in the previous year(s) or through maternal antibodies in hatchlings. In fact, 60% of this subset were positive which indicates that already in (late) 2004 many more birds were exposed to the virus and subsequently seroconverted than the samples taken in 2004 suggest. From this point of view it becomes evident that in parallel with the significant decline of USUV-associated avian mortality the number of seropositive birds in the endemic areas increases steadily. Therefore, it is a likely possibility that a rather rapid establishment of herd immunity has been responsible for apparent disappearance of USUV-associated bird deaths, despite continuing viral circulation. The high percentage of seropositives to a circulating arbovirus with a bird–mosquito transmission cycle is unparalleled in other endemic transmission cycles so far. Seroprevalence rates of WNV, *Saint Louis Encephalitis virus*, and *Sindbis virus* usually only reach 1.5–9.7% (McLean et al., 1988; Antipa et al., 1984; Juricova et al., 1987; Juricova et al., 1989; Beveroth et al., 2006). The only other paper which claims a similar high transmission rate, however using the more sensitive 50% PRNT (compared to the 90% PRNT used in the present study), does not only suggest local transmission but also continuous introduction of virus by migratory birds to the British Isles (Buckley et al., 2003). In the case of USUV, however, one genetically stable virus strain established a local transmission cycle in local birds and mosquitoes in Austria with a tendency of slow but steady spread to adjacent areas (Chvala et al., 2007).

In addition to the indisputable increase of seroreactors within the wild bird population also

other factors could have contributed to the rapid decline of USUV-associated avian deaths registered during a 3-year period of dead bird surveillance (Chvala et al., 2007). On the one hand climate factors could have been influential, on the other hand decreased virulence of the circulating USUV strain could also have played a role. Data from other flaviviruses (e.g. WNV) showed that virulence for certain bird species is strain-dependent (Brault et al., 2004) and it has been suggested that especially mutations in certain E-protein gene regions resulting in loss of glycosylation were responsible for reduced virulence or neuroinvasiveness (Beasley et al., 2005). For USUV, currently no complete sequences or experimental data of virus strains isolated in different years are available. However, sequencing of 88% of the E coding region of 12 USUV isolates from 3 consecutive years (2003–2005) revealed only single random mutations, all of which except one did not result in amino acid changes (Chvala et al., 2007).

The fact that already in 2003 the proportion of seropositives among the surveyed owl species tawny owl and long-eared owl was significantly above the average prompted us to undertake a more thorough investigation among the birds in this rehabilitation centre. The overall seroprevalence among these birds almost doubled after 2 years. We expected new insights into the infection dynamics of USUV infections from the comparative examination of three blood samples per bird taken at three different timepoints during one transmission season. Already in May, well before the start of the transmission season, a high percentage of the blood samples exhibited antibodies to USUV. This observation correlates well with the generally high seroprevalence in the wild bird population, indicating again viral exposure in the previous season(s). Transmission of mosquito-borne flaviviruses occurs predominantly from viraemic birds to mosquitoes which after completion of the extrinsic incubation period are capable of transmitting the virus to a new avian host. Under natural conditions this is certainly the most efficient and most common transmission route. In more artificial settings, such as the case for caged wild birds, also other modes of flaviviral transmission have been observed. WNV, for example, can also be transmitted by direct contact (Komar et al., 2003), by eating infected reservoir hosts (Austgen et al., 2004; Nemeth et al., 2006) and especially in owls, it has been speculated that louse flies might serve as additional vectors (Gancz et al., 2004). Many of the owls of the present study were infested with louse flies, too, and they probably might have contributed to the viral distribution among the birds within certain aviaries. However, there is no formal proof as yet that louse flies are competent vectors for flaviviruses. These transmission modes are not restricted to seasons of mosquito activity and could theoretically have occurred within this bird collection at any time of the year.

During the following 6-month observation period some interesting changes in titre development were noticed. From the first to the second bleeding the geometric mean titre of most bird species markedly dropped as did the total number of seropositives. This can be explained by a natural decline of antibody titres during a period without viral activity. In several birds the titre decline within this rather short time interval was intriguingly pronounced. This observation suggests that even after natural infection flaviviral titres in birds are generally not very robust and long lasting, but subject to considerable variations within short times and it can certainly not be assumed that such antibodies persist life-long. After the transmission season, which – based on dead bird surveillance data – ends in mid-September, seroconversions were noted in several birds. Some had not had any detectable antibodies before and some had had low titres. In several birds the serotitres continued to drop until the last bleeding which might either indicate lack of exposure or protective titres preventing infection and viral replication. However, despite the fact that seroconversions obviously occurred, by RT-PCR of PBMCs of selected birds taken during the transmission season no evidence of viraemia was found. Taking into account that viraemia in flavivirus infections of birds is usually short-lived, i.e. not longer than a few days (Nemeth et al., 2006) it simply seems to have been bad luck that no viraemic bird had been detected by examining a single blood sample during the entire transmission season. Taking all data together, the number of seropositives had risen between the second and third bleeding and the proportion of medium and high titres was highest at the last bleeding. These data clearly indicate that despite a high pre-existing herd immunity viral activity still leads to new infections and seroconversions. This fact that flaviviral circulation despite the presence of significant immunity is easily possible is a significant observation which is especially

important for the understanding of concepts of flavivirus epidemiology. This study also clearly shows the lack of pathogenicity of USUV for the particular species of owls and birds of prey kept in captivity. Since the first detection of viral activity in the area in 2003, no diseases or deaths of birds which could be attributed to USUV infection were noticed in this particular region. This observation is in sharp contrast to the documented vulnerability of one owl species (great grey owl) (Weissenböck et al., 2002) with its natural habitat in periarctic zones. This species has also proved to be highly vulnerable to infection with the related WNV (Gancz et al., 2004).

In conclusion, the findings presented in this paper suggest that USUV circulates very efficiently between local birds and mosquitoes in eastern Austria. After a few years of presence with an initial severe bird mortality the virus produced a high seroprevalence in the susceptible hosts which seems to be sufficient for establishment of an (at least currently) stable herd immunity.

Acknowledgements

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PRÁCE 19

Hubálek Z., Wegner E., Halouzka J., Tryjanowski P., Jerzak L., Šikutová S., **Rudolf I.**, Kruszewicz A.G., Jaworski Z., Wlodarczyk R. 2008. Serologic survey of potential vertebrate hosts for West Nile virus in Poland. *Viral Immunol*. 21: 247–253.

Stručná charakteristika: neutralizačním testem bylo vyšetřeno 78 koní, 20 kuřat a 97 volně žijících ptáků na přítomnost protilátek k WNV.

Hlavní přínos práce: jde o vůbec první významnou práci zkoumající aktivitu WNV v Polsku. Celková séropozitivita k WNV byla 5,2%. WNV protilátky byly zjištěny u čápů bílých (*Ciconia ciconia*), labutě velké (*Cygnus olor*) a vrány šedé (*Corvus corone cornix*). Dokonce byly poprvé v Polsku detegovány protilátky k USUV u racka chechtavého (*Larus ridibundus*).

Příspěvek autora k dané práci: autor se podílel na hodnocení neutralizačního testu a přípravě rukopisu.

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Serologic Survey of Potential Vertebrate Hosts for West Nile Virus in Poland

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ABSTRACT

A survey for antibodies to West Nile virus (WNV; genus *Flavivirus*) was carried out by plaque-reduction neutralization microtesting in 78 horses, 20 domestic chickens, and 97 wild birds belonging to 10 species from different areas in Poland. Specific antibodies were detected in five juvenile (hatching-year) birds collected in 2006: three white storks (*Ciconia ciconia*) in a wildlife rehabilitation center (5.4% of all examined storks; the antibody titers in each bird were 1:320, 1:160, and 1:20), one free-living mute swan (*Cygnus olor*; the titer was 1:20), and one hooded crow (*Corvus corone cornix*; the titer 1:20) in a wildlife rehabilitation center; thus the overall seropositivity to WNV was 5.2% among all the birds sampled. These data do not rule out the presence of WNV activity in Poland with 100% certainty, but they indicate a significant trace that demands verification. In addition, one black-headed gull (*Larus ridibundus*) had neutralizing antibodies for the Usutu *Flavivirus*, the first case recorded in Poland.

INTRODUCTION

West Nile virus (WNV; family Flaviviridae) circulates in natural foci between birds and bird-feeding mosquitoes, principally *Culex pipiens*, *C. modestus*, and *Coquillettidia richiardii* in Europe (13). WNV has not yet been isolated in Poland, unlike neighboring Czechland (14,15) and Slovakia (7,18). Antibodies inhibiting hemagglutination of WNV were detected in sparrows (*Passer domesticus* and *P. montanus*) near Warsaw (16), though this result was not verified by virus neutralization testing.

WNV is occasionally introduced by birds migrating to temperate countries of the Northern Hemisphere from tropical and subtropical countries (7,10,25,30). Moreover, there are predictions according to climate change models that transmission of WNV may soon become more intense and rapid (6).

The aim of this study was to assess the level of WNV activity in Poland, by performing a serologic survey of vertebrates (horses and birds) that are prone to viral exposure in the field. Horses are especially susceptible to WNV and form antibodies easily; white storks are also susceptible, as are corvids (23,28).

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MATERIALS AND METHODS

Blood sampling

Blood samples from 50 horses of the Polish konik variety originating from Popielno (Mazurian Lakeland) (site A on the map shown in Fig. 1), and 28 horses of the same breed from Roztocze National Park (site B on Fig. 1) were collected in October 2006 (Table 1). These horses lived free in their natural habitats. The Polish konik is a local horse breed originating directly from the wild Tarpan horse (*Equus caballus gmelini* form *silvatica*). These horses are highly resistant to tough environmental conditions, and have lower feeding needs and a higher level of natural immunity compared to other breeds of horses (17). Blood samples from free-living birds were collected in 2006 by puncturing the jugular or brachial vein, and the birds were released after sample collection. Swans were captured for banding at two breeding territories near Sieradz: on the ponds in the village of Jeziory and an oxbow lake near the village of Bilew (site 3 on Fig. 1). White storks were sampled either as nestlings from the Wielkopolska region (site 5 on Fig. 1; 48 individuals) during two breeding seasons (2002 and 2003), or as birds living in a few wildlife rehabilitation centers (8 hatching-year birds in 2006; Table 1). Some other birds (crows, rooks, jackdaws, and pigeons) also visited the Wildbird Rehabilitation Centre in Warsaw (site 4 on Fig. 1) between July 25 and September 13, 2006, where they were brought after being found injured elsewhere in the country, most being from the Mazovian Lowland (near Warsaw). Other wild birds were caught in Japanese nets in the region of Podlasie (site 2 on Fig. 1) in the Siemianówka water reservoir (near Białowieża National Park and the Belarussian border) at the end of July 2006. Domestic chickens were sampled on an ecological farm in the region of the Mazurian Lakeland at Pokrzydowo (site 1 on Fig. 1) in October 2006. All Polish laws and regulations regarding protection, conservation, and animal welfare were adhered to during all sampling procedures, which were endorsed by Polish local ethics committees and the Ministry of the Environment. Blood samples were centrifuged in the laboratory within 3–6 h after collection, and the separated sera were then stored at −20°C until use.

Plaque reduction neutralization microtest (PRNμT)

This method was originally proposed by Madrid and Porterfield (21,22), and was later adapted into a microtechnique done in flat-bottomed, 96-well disposable sterile microplates (Sarstedt, Newton, NC) used for tissue culture (12). Vero E6 cells were cultivated in Lei-

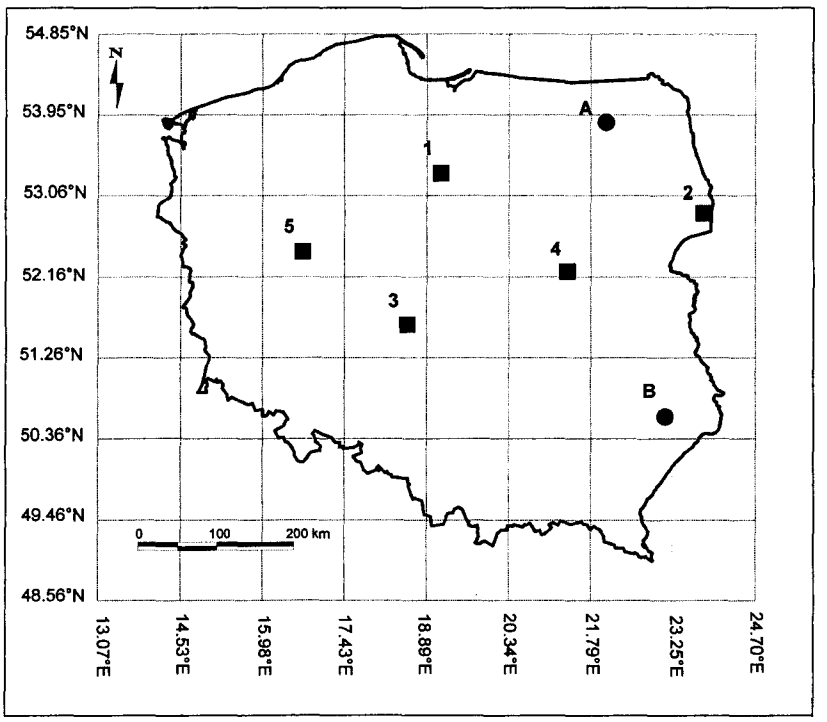

FIG. 1. Map of Poland with the study sites. (**A**) Popielno (Mazurian Lakeland). (**B**) Roztocze National Park. (**1**) Pokrzydowo (Mazurian Lakeland). (**2**) Siemianówka water reservoir close to Białowieża National Park (region of Podlasie). (**3**) Sieradz: ponds in Jeziory village and an oxbow lake near Bilew village (region of Łódź). (**4**) Wildbird Rehabilitation Centre in Warsaw. (**5**) Wielkopolska region.

TABLE 1. WEST NILE VIRUS NEUTRALIZING ANTIBODIES (PRNμT$_{90}$): NUMBERS OF POSITIVE AND UNDETERMINED REACTIONS

Animal species	*No. examined*	*No. positive*	*No. undetermined*
Horse (*Equus caballus*)	78	0	0
Domestic chicken (*Gallus domesticus*)	20	0	0
Feral pigeon (*Columba livia* f. *domestica*)	8	0	1
Wood pigeon (*Columba palumbus*)	2	0	0
White stork (*Ciconia ciconia*) juveniles	8	3	2
White stork nestlings	48	0	0
Mute swan (*Cygnus olor*)	9	1	1
Black-headed gull (*Larus ridibundus*)	6	0	5
Common tern (*Sterna hirundo*)	3	0	2
Rook (*Corvus frugilegus*)	4	0	1
Hooded crow (*Corvus corone cornix*)	2	1	1
Jackdaw (*Corvus monedula*)	6	0	1
Jay (*Garrulus glandarius*)	1	0	0
Total	195	5	14

bovitz L-15 medium (Sigma, USA) supplemented with 5% of fetal calf serum (FCS; UK Gibco Bio-Cult, Scotland) and antibiotics. Tested sera were inactivated at 56°C for 30 min and diluted 1:10 for screening in L-15 with 3% inactivated FCS, then 30 μL of the diluted sera was mixed with 30 μL of test dose of the virus (containing 20–30 PFU of the Eg-101 SM_{17} strain of WNV) in L-15 supplemented with 3% inactivated FCS. The plates were then covered and incubated at 37°C for 60 min; then 60 μL of the Vero cell suspension was added to each test well (20,000–30,000 cells/well), and after incubating covered at 37°C for 4 h, 120 μL of carboxymethylcellulose (CMC) sodium salt were added (1.5% CMC of medium viscosity BDH in PBS mixed with the same volume of L-15 mixed with 3% inactivated FCS). Controls included a test dose of the virus and its serial twofold dilutions and immune WNV reference serum; control negative serum; and cells without virus. Incubation of the plastic-sealed cultures at 37°C lasted 5 d, and the microplate cultures were then stained with a 0.1% acidic solution of naphthol blue black (FLUKA, Switzerland) at room temperature for 40 min.

Sera that were reactive with WNV, revealing 90% or greater reduction of plaques at the 1:10 dilution (corresponding to the 1:20 final dilution of the serum, after mixing with the virus test dose), were titrated by twofold dilutions, and dilutions corresponding to 90% reduction of PFU were regarded as the serum titers (PRNμT$_{90}$). Reciprocal titers $\geq$20 were considered positive. Positive sera were also tested in parallel (PRNμT$_{90}$) against *Flavivirus* Usutu (USUV; strain 939/01 $Vero_3$) to exclude cross-reaction with this related mosquito-borne virus found in Central Europe (35).

RESULTS

None of the 78 horses examined had antibodies to WNV (one animal showed a titer of 1:10, which was considered negative). Similarly, none of the 20 chickens and 48 nestling white storks tested positive for WNV (Table 1). Although sera from 19 of the 97 free-living birds examined (19.6%) reacted on PRNμT$_{90}$ with WNV titers of at least 1:20, most of them also had USUV titers at similar or higher levels (Table 2), and these results were interpreted as cross-reactions non-specific for WNV. In all only five birds (5.2%) reacted with WNV specifically, namely juvenile white storks (nos. 126, 127, and 128), which were kept in the wildlife rehabilitation center (WRC) after being injured in the field. Their antibody titers against WNV were 1:320, 1:160, and 1:20, respectively, while titers to USUV were low or absent (in total, 5.4% of the 56 storks tested had antibodies to WNV); USUV titers were also present in one juvenile mute swan (no. 6551; titer 1:20) and one juvenile hooded crow (no. 27; titer 1:20) from a WRC. One bird, a young black-headed gull (no. 16), reacted with USUV at a titer of 1:80, which was higher than that against WNV (1:20–1:40).

DISCUSSION

There is speculation about the possible presence of WNV in Poland (31). Some bird species that are known to carry WNV are common in Poland (e.g., the white stork [*Ciconia ciconia*], garganey [*Anas querquedula*], common coot [*Fulica atra*], lapwing [*Vanellus vanellus*], black-headed gull [*Larus ridibundus*], turtle dove [*Strep-*

TABLE 2. DETAILED COMPARISON OF RECIPROCAL PRNμT$_{90}$ TITERS AGAINST WEST NILE AND USUTU VIRUSES

No.	*Species*	*Age*	*Locality*	*Date*	*WNV titer*	*USUV titer*
124	White stork	Juvenile	WRC[a]	Aug. 23, 2006	20–40	20–40
163	White stork	Juvenile	WRC	Aug. 23, 2006	20	20
126	White stork	Juvenile	WRC	Sept. 13, 2006	320	20
127	White stork	Juvenile	WRC	Sept. 13, 2006	160	20
128	White stork	Juvenile	WRC	Sept. 13, 2006	20	<20
6551	Mute swan	Juvenile	Bilew	Oct. 11, 2006	20	<20
6552	Mute swan	Juvenile	Bilew	Oct. 11, 2006	20	20
8	Common tern	Juvenile	Siemianówka	July 27, 2006	20	20
9	Common tern	Juvenile	Siemianówka	July 27, 2006	20–40	40
11	Black-headed gull	Adult	Siemianówka	July 27, 2006	40	40
13	Black-headed gull	Juvenile	Siemianówka	July 27, 2006	20–40	20
14	Black-headed gull	Juvenile	Siemianówka	July 27, 2006	20	20
15	Black-headed gull	Juvenile	Siemianówka	July 27, 2006	20	40
16	Black-headed gull	Juvenile	Siemianówka	July 27, 2006	20–40	80
27	Hooded crow	Juvenile	WRC	Aug. 9, 2006	20	<20
28	Hooded crow	Juvenile	WRC	Aug. 23, 2006	20–40	≤20
29	Jackdaw	Juvenile	WRC	July 27, 2006	20	≤20
30	Rook	Juvenile	WRC	Sept. 13, 2006	20–40	20

Abbreviation: WRC, wildlife rehabilitation center.

topelia turtur], hooded crow [*Corvus corone*], rook [*C. frugilegus*], and European starling [*Sturnus vulgaris*]). Most of them are migratory and can potentially spread the pathogen along their migration routes. Moreover, six mosquito species that were previously reported positive for WNV in other European countries (13), are common in Poland: *Culex pipiens*, *C. modestus,* and *Coquillettidia richiardii*—species believed to be common vectors in Europe—as well as *Ochlerotatus dorsalis, O. caspius,* and the flood-water mosquito, *Aedes vexans* (32). However, an outbreak of human cases of West Nile fever (WNF) has never been reported in Poland, although it is possible that human WNF cases may go unnoticed in this country. For instance, epidemic reports showed a three- to fivefold increase of the incidence of aseptic meningitis in the areas flooded in 1997 and in the district of Gdańsk, where a flood occurred in 2001 (31), and where enormous numbers of mosquitos subsequently appeared. At the same time, there were no mass deaths of birds observed, which some researchers regard as a sign of the presence of WNV (however, this is true only for North America, and not for Europe).

Only one paper indicated the possible presence of WNV in Poland by detection of antibodies to WNV in 3%–12% of house sparrows (*Passer domesticus*) and tree sparrows (*P. montanus*) captured near Warsaw (16), using a hemagglutination-inhibition test (HIT). Unfortunately, this finding could not be verified by a more specific neutralization test. In HIT (and ELISA as well), cross-reactions among flaviviruses are very common and well known (e.g., even cross-reactions between WNV and tick-borne encephalitis virus). The plaque-reduction neutralization test is regarded as the gold standard in flavivirus serology, and is generally more specific than other serological techniques (33,34). However, significant serological *Flavivirus* cross-reactivity can sometimes occur even in the neutralization test (4,5,22,24,28). Often several antigenically related flaviviruses of the same antigenic group co-occur in one area (e.g., in central Europe Usutu virus together with WNV, both members of the Japanese encephalitis group) (20,35). It is sometimes difficult to decide which particular antigen was responsible for antibody production, and thus controversial results may be published. It is always necessary to interpret results of flavivirus serology with great care, especially during serosurveys in birds and wild mammals, where nonspecific inhibitors of hemagglutination and neutralization can occasionally occur (11,28).

In this study, we used neutralization (PRNμT) with the standard, topotype Egyptian strain Eg-101 of WNV (its suckling mouse brain homogenate), and carefully stored and thermally inactivated avian serum (not plasma) samples devoid of heparin, citrate, or any stabilizing substances like merthiolate. We estimated the results conservatively, and used 90% reduction in the number of plaques (not 50% reduction, which is sometimes used), and 1:20 dilution (instead of the usual 1:10) as a titer cut-off point. In addition, the fetal calf serum used in the

PRNμT was tested for antibodies against WNV in a separate assay.

Our data indicate only limited WNV activity in Poland in 2006. The three juvenile white storks with specific antibodies to WNV, sampled on September 13, 2006, were either born in Poland or arrived from nearby countries endemic for WNV in northeastern or eastern Europe (e.g., Latvia, Belarus, or the Ukraine) (13). White storks are susceptible to WNV infection, and WNV was isolated in Israel from migrating white storks, supposedly arriving from eastern or central Europe (23). It is noteworthy to mention that white storks from Poland and eastern parts of Germany migrate via the Middle East, along the flyway leading southeast through the Marmara Sea and Turkey, then turning to the south near the Gulf of Iskenderun and through the Sinai, reaching the Suez Bay and approaching the Nile; they then disperse across eastern and southern Africa (29).

Maternal antibodies against WNV, transferred passively via egg yolks from seropositive females, can persist in nestling birds for 2–4 weeks (1,8,9). Identical results were described for the related mosquito-borne flavivirus causing Japanese encephalitis (3,27). Because the hatching season of the seropositive bird species is from April to June in Central Europe, the antibodies found in young birds in August and September (i.e., $\geq$2 months after hatching; egg laying occurs at the end of April, and the majority of eggs are hatched in May) should not be regarded as maternal ones, but as antibodies formed after natural WNV infection.

The relatively high proportion of positive serological results among patients in wild bird rehabilitation centers (three storks and one hooded crow out of 21 avian patients) is not surprising, since birds with CNS disorders are most susceptible to accidents. On the other hand, the seropositive young mute swan (although the titer was low) was apparently healthy and free-living, and a number of healthy individuals captured on the water reservoirs (Siemianówka and Bilew) were classified as serologically undetermined, and most of them were juveniles. These results are essential to understanding how WNV spreads, especially in light of the fact that summer 2006 was extremely dry and therefore unfavorable for growth of mosquito vectors.

The results of our study correlate well with findings from some other European studies done between 2000 and 2005. No or very low WNV activity was observed in free-living birds near Lyon in France (19), as all 364 birds examined were seronegative, and in Germany (20), where seroprevalence was 1.6% in 3399 birds. However, the German study showed a relatively high frequency of antibodies to WNV in predatory birds, especially in the migratory osprey (*Pandion haliaetus*), for which 20% of 140 birds examined had neutralizing antibodies. In addition, 2.2% of mute swans, 2.3% of white storks, and six other avian species were seropositive as well. The only study deviating from this trend is one done in Great Britain in 2001–2002, where as many as 14.7% of 353 wild birds reacted with WNV in $PRNT_{90}$ (2), but the authors considered titers as low as 1:10 to be positive, and they did not use the topotype WNV strain, but instead used three other WNV strains that yielded varying results.

Negative results obtained in white stork nestlings cannot exclude the possible presence of WNV in the study area. Malkinson *et al.* (23) did not find antibodies in white stork nestlings in the Golan Heights in 2000, although WNF cases were frequent among geese and humans and the virus circulated in the area. German observations also show that the nestlings (2–9 wk old) of white storks were mostly serologically negative, although very low antibody titers (1:10–1:15, and only one bird had a titer of 1:30) against WNV were detected in 10 of them, most probably due to maternal immunity passed vertically to the young. At the same time several post-hatching year storks and ospreys (*Pandion haliaetus*) showed much higher titers of antibodies against WNV (20). On this basis it can be stated that nestlings of the white stork are not suitable for a serologic survey for WNV.

The negative results found in horses also deserve comment. The horse—Polish konik—is a breed closely related to a species of wild horse (tarpan), and is a descendant of a primitive, wild species. Recent studies of the Polish konik show that it has a high level of natural immunity (26). In a similar central European study serum samples from 350 horses from eastern Austria were examined for WNV, and all were also negative, while 4 of 35 horses transported from Hungary to Germany were seropositive, although these animals had no obvious clinical signs on examination (34).

CONCLUSION

Our study has not confirmed with 100% certainty the circulation of WNV in Poland, but the results show tangible traces of seropositivity that demand verification. This study also revealed for the first time in Poland antibodies to Usutu virus, a mosquito-borne *Flavivirus* recently recognized as occurring in Central Europe (35). The bird that tested positive, a black-headed gull, was a hatching-year specimen, and it could have been born in Poland or in any other central European country, including Austria.

Further study is needed to more accurately define how West Nile virus spreads in Europe, as well as how to control its spread.

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PRÁCE 20

Hubálek Z., Halouzka J., Juřicová Z., Šikutová S., **Rudolf I.**, Honza M., Janková J., Chytil J., Marec F., Sitko J. 2008. Serologic survey of birds for West Nile flavivirus in southern Moravia (Czech Republic). *Vector-borne and Zoonotic Dis*. 8: 659–666.

Stručná charakteristika: WNV byl na jižní Moravě poprvé izolován po povodních v roce 1997 (kmen Rabensburg), současně byly zjištěny protilátky k viru u místních obyvatel a také několik případů onemocnění u lidí. Sérologická surveillance obratlovčích hostitelů (ptáků) v ohnisku nákazy je klíčová pro posouzení aktivity WNV ohniska a také rizika nákazy u lidí.

Hlavní přínos práce: první extenzivní WNV sérosurveillance volně žijících ptáků na jižní Moravě, která je charakteristická endemickým výskytem WNV. Byl potvrzen výskyt protilátek k WNV u 5,9% volně žijících ptáků (vyšetřeno celkem 391), a absence protilátek u ptáků chovaných v zajetí (vyšetřeno 54). Byly poprvé na našem území zjištěny protilátky k USUV u lysky černé (*Fulica atra*).

Příspěvek autora k dané práci: autor se podílel na hodnocení neutralizačního testu a přípravě rukopisu.

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Serologic Survey of Birds for West Nile Flavivirus in Southern Moravia (Czech Republic)

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ABSTRACT

A serosurvey for West Nile virus (WNV) was carried out in 54 domestic birds (geese and ducks bred on fishponds) and 391 wild birds representing 28 migratory and resident species, using a plaque-reduction neutralization microtest with Vero cells and Egyptian topotype Eg-101 strain as test virus. The birds were sampled in the South-Moravian fishpond ecosystem between 2004 and 2006. Antibodies to WNV were not detected in domestic waterfowl, but 23 (5.9%) free-living birds of 10 species showed a positive response. These were the common coot (*Fulica atra*, 5 positive/18 examined), common kingfisher (*Alcedo atthis*, 1/1), reed warbler (*Acrocephalus scirpaceus*, 2/80), sedge warbler (*A. schoenobaenus*, 3/80), marsh warbler (*A. palustris*, 2/28), Savi's warbler (*Locustella luscinioides*, 3/12), reed bunting (*Emberiza schoeniclus*, 1/28), blackcap (*Sylvia atricapilla*, 2/11), penduline tit (*Remiz pendulinus*, 1/14), blue tit (*Parus caeruleus*, 1/1), and starling (*Sturnus vulgaris*, 2/4). The antibody titers were comparatively low (1:20–1:40), and the only high titer (1:160) was found in an adult marsh warbler. When 14 of the sera reacting with WNV were titrated in parallel with Usutu *Flavivirus*, 12 were interpreted as having specific antibodies to WNV, one coot had a higher titer against Usutu virus, and another one could not be attributed to either of the two viruses. In conclusion, 13 (3.3%) of 391 wild birds had specific antibodies to WNV. The results indicate that WNV activity in southern Moravia was limited during 2004–2006. Key Words: Birds—Field studies—Immunology—Mosquito(es)—West Nile

INTRODUCTION

West Nile virus (WNV, family *Flaviviridae*) circulates in natural foci between birds and bird-feeding mosquitoes largely of the genus *Culex* (*Cx. pipiens* and *Cx. modestus* in Europe). This virus could be occasionally transported by migratory birds to temperate countries of the northern hemisphere from (sub)tropical countries, or vice versa (Hannoun et al. 1972; Watson et al. 1972; Malkinson et al. 2002).

The aim of the present study was to evaluate the present activity of WNV in southern Moravia (Czech Republic), using an indirect method of serological survey of birds thought to be potentially exposed to the virus in the field. West Nile virus had already been isolated in this region (Hubálek et al. 1998; Bakonyi et al. 2005), as well as in neighboring Slovakia (Labuda et al. 1974), and five human cases of WNV fever were described after floods in southern Moravia in 1997 (Hubálek et al. 2000).

MATERIALS AND METHODS

Study sites

All sampling localities (Fig. 1) were situated in habitats with abundant mosquito popula-

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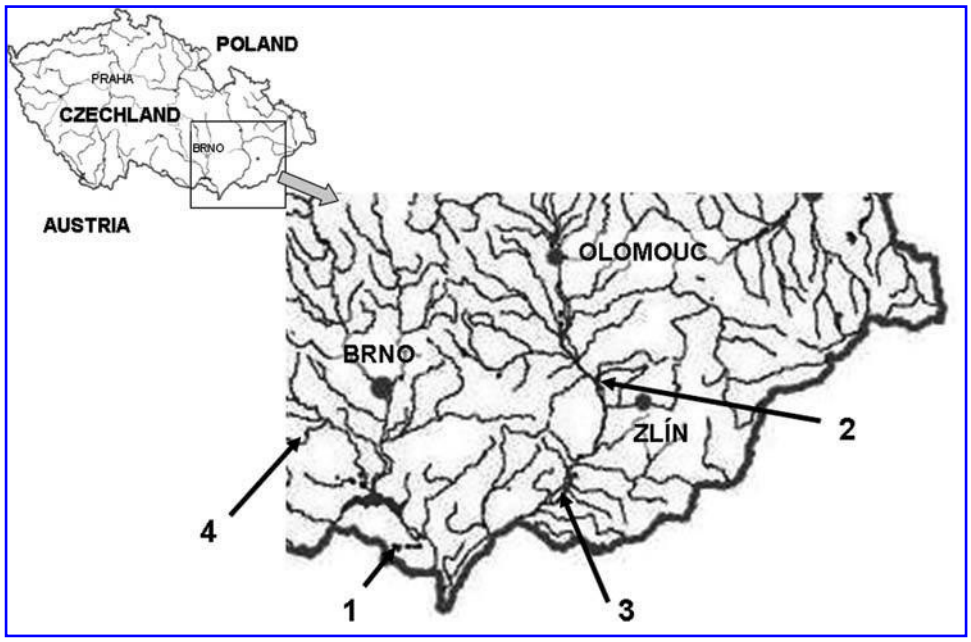

FIG. 1. The study sites in southern Morovia. (**1**) Fishpond Neoyt at Sedlec; (**2**) Fishponds at Za'hlinice; (**3**) fishponds at Hodonín; (**4**) fishpond at Morovsky, Krumlov.

tions, including *Culex pipiens*, *Cx. modestus*, *Aedes*, and *Ochlerotatus* spp. (Kramář 1958; Vaňhara 1985; Olejníček et al. 2003).

- *Site 1.* Fishpond Nesyt (48°46′ N, 16°43′ E) at Sedlec near Mikulov (reedbelt habitat, *Phragmites communis*): 319 wetland birds (largely passerines) sampled 15–23 July 2006.
- *Site 2.* Fishponds at Záhlinice (49°17′ N, 17°28′ E) near Přerov (reedbelt habitat): 33 adult wild waterfowl (3 *Anas platyrhynchos*, 3 *Anas strepera*, 10 *Aythya ferina*, 3 *Netta rufina*, 1 *Tachybaptus ruficollis*, and 13 *Fulica atra*) sampled from 25 April to 10 May 2006.
- *Site 3.* Fishponds at Hodonín: 18 domestic geese bred on the shore of fishpond Písečnický (48°51′ N, 17°04′ E), sampled on 7 November 2005; 24 domestic ducks bred on fishpond Novodvorský (48°52′ N, 17°04′ E), sampled on 7 November 2005; and 34 adult wild waterfowl (18 *Aythya ferina*, 12 *Aythya fuligula*, and 4 *Larus ridibundus*) sampled in reedbelt habitat of Jarohněvický fishpond (48°55′ N, 17°03′ E), May–June 2004.
- *Site 4.* A fishpond near Moravský Krumlov (49°02′ N, 16°18′ E): 12 domestic ducks bred on a waterfowl farm, sampled on 7 November 2005.

Sampling

Blood samples (in passerines and other small birds 50–150 μL, in waterfowl about 1 mL) were collected from the brachial vein (in small passerines by its puncturing with miniature syringe needle and aspiration into hematocrit capillaries: Juřicová et al. 1986) of captured birds. Wild birds were trapped in mist-nets during a regular ringing action of the Czech Society for Ornithology managed in accord with the Czech Animal Protection Act (no. 246/92). The wild birds were aged, sexed, ringed, and released after the blood collection. The waterbirds on site 2 were sampled during spring hunts under relevant permits. The blood samples were centrifuged in the laboratory 3–6 hours after collection, and the separated sera were stored at −20°C until use.

Plaque-reduction neutralization microtest (PRNμT)

The method was originally proposed by Madrid and Porterfield (1969, 1974), and adapted to a microtechnique on 96-well (flat-bottomed) sterile microplates (Sarstedt) for cell culture (Hubálek et al. 1979). Vero E6 cells were serially propagated in Leibovitz L-15 medium (Sigma) supplemented with 10% fetal calf

serum (FCS, Gibco Bio-Cult) and antibiotics. Tested sera were inactivated at 56°C for 30 minutes, and for screening were diluted 1:10 in L-15 medium; 30-μL of the serum (in duplicate) was mixed in a microplate well with a 30-μL test dose of the virus (containing 20–30 plaque forming units (PFU) of Eg-101 strain of WNV, passaged 17 times in suckling mouse brain, and homogenized in phosphate buffered saline (PBS) with 0.4% bovine serum albumin fraction V, Sigma, and centrifuged) in L-15 supplemented with 3% inactivated FCS, and incubated at 37°C for 60 minutes. Vero cell suspension (in L-15 with 3% FCS) was then added to each test well (60 μL with 20,000–30,000 cells per well). After an incubation at 37°C for 4 hours, 120 μL of carboxymethylcellulose sodium salt overlay (1.5% CMC of medium viscosity BDH in PBS mixed with the same volume of L-15 with 3% of inactivated FCS) was added to each well. Controls included the virus test dose and its titration, immune WNV reference serum, control negative serum, and cells without virus. The microplates, sealed in small polyethylene bags, were incubated at 37°C for 5 days, and the cultures were then stained with 0.1% acidic solution of naphthalene black (Fluka).

Sera reactive with virus, revealing 90% or greater reduction in the number of plaques at the 1:10 dilution at screening (corresponding to the 1:20 final dilution of the serum after mixing with the virus test dose), were titrated in duplicate by twofold dilutions, and those dilutions corresponding to 90% reduction of plaque numbers were regarded as the serum titers ($PRN\mu T_{90}$). Reciprocal titers $\geq$20 were considered positive. The positive sera were also tested against *Flavivirus* Usutu (USUV; strain 939/01 $Vero_3$) in order to exclude cross reactions with this related mosquito-borne virus that occurs occasionally in Central Europe.

RESULTS

All tested domestic waterfowl (18 geese, 36 ducks) at sites 3 and 4 were negative at 1:20 dilution. Similarly, all wild anseriform birds were seronegative at sites 2 and 3. Overall, 23 of 391 (5.9%) examined wild birds belonging to 28 species collected in the reedbelt habitat of several fishponds reacted with WNV at titers 1:20 or higher (Table 1): 5/18 (no. positive/no.examined) common coots, 2/80 reed warblers, 3/80 sedge warblers, 2/28 marsh warblers, 3/12 Savi's warblers, 1/28 reed buntings, 1/14 penduline tits, 2/11 blackcaps, 1/1 blue tit, 2/4 starlings, and 1/1 common kingfisher. Fourteen of these seroreacting birds were examined in $PRN\mu T_{90}$ with USUV in parallel, and two of them (common coots) also reacted with the latter virus at titers similar to WNV or higher (Table 2): whereas coot #Z22 was interpreted as having specific antibodies to USUV, coot #Z32 revealed a flavivirus cross reaction not specific for WNV or for USUV, and its antibodies have been regarded as undetermined. In contrast, 12 birds have been regarded as reacting specifically with WNV. The remaining 9 seropositive birds could not be tested with USUV because of the small amount of sera, but the high titer (1:160) in an adult marsh warbler (#N104) was also attributed to specific WNV antibodies. Therefore at least 13 wild birds (3.3%) reacted with WNV specifically, although the titers were, with one exception low, 1:20 to 1:40 (Table 2). Eight of these birds with specific WNV antibodies were adults, and five were young (hatching-year) individuals.

Of 445 tested birds, 192 were young (hatching-year) birds, and 253 were adult (after hatching-year) birds. There was no significant difference in the seroprevalence rate between young and adult birds overall, nor between the young and adult passerines (Table 1; χ^2 test). Also, when only specific WNV-seroreactors were taken into account (Table 2), there was no significant difference in the seroprevalence rate: 2.6% in young birds versus 3.2% in adults.

DISCUSSION

The plaque-reduction neutralization test is regarded as the gold standard in flavivirus serology and is used for verification of other serological tests (enzyme-linked immunosorbent assay [ELISA], hemagglutination-inhibition test [HIT]) because it is generally more specific and discriminatory. However, it is well known that flaviviruses present a high degree

TABLE 1. BIRDS SEROREACTING WITH WEST NILE VIRUS IN PRNMT$_{90}$ (TITER AT LEAST 1:20)

Bird species	*Examined birds* Juvenile	Adult	*WNV seroreactors* Juvenile	Adult
Domestic goose, *Anser anser* f. *domestica*	0	18	—	0
Domestic duck, *Anas platyrhynchos* f. *domestica*	0	36	—	0
Wild mallard, *Anas platyrhynchos*	0	3	—	0
Gadwall, *Anas strepera*	0	3	—	0
Common pochard, *Aythya ferina*	0	28	—	0
Tufted duck, *Aythya fuligula*	0	12	—	0
Red-crested pochard, *Netta rufina*	0	3	—	0
Little grebe, *Tachybaptus ruficollis*	0	1	—	0
Common coot, *Fulica atra*	0	18	—	5
Black-headed gull, *Larus ridibundus*	4	0	0	—
Common sandpiper, *Tringa hypoleucos*	1	0	0	—
Common kingfisher, *Alcedo atthids*	0	1	—	1
Swallow, *Hirundo rustica*	11	9	0	0
Savi's warbler, *Locustella luscinioides*	7	5	1	2
Reed warbler, *Acrocephalus scirpaceus*	41	39	0	2
Great reed warbler, *Acrocephalus arundinaceus*	10	20	0	0
Sedge warbler, *Acrocephalus schoenobaenus*	60	20	1	2
Marsh warbler, *Acrocephalus palustris*	16	12	1	1
Icterine warbler, *Hippolais icterina*	2	0	0	—
Blackcap, *Sylvia atricapilla*	6	5	1	1
Barred warbler, *Sylvia nisoria*	1	0	0	—
Stonechat, *Saxicola torquatus*	1	0	0	—
Blackbird, *Turdus merula*	1	0	0	—
Song thrush, *Turdus philomelos*	0	2	—	0
Bearded titmouse, *Panurus biarmicus*	0	1	—	0
Penduline tit, *Remiz pendulinus*	14	0	1	—
Blue tit, *parus caeruleus*	1	0	1	—
Red-backed shrike, *Lanius collurio*	0	1	—	0
Starling, *Sturnus vulgaris*	4	0	2	—
Reed bunting, *Emberiza schoeniclus*	12	16	1	0
TOTAL	192	253	9 (4.7%)	14 (5.5%)
PASSERIFORMES only	187	130	9 (4.8%)	8 (6.2%)

of serological cross-reactivity, even in the neutralization test (Filipe and Pinto 1969; Theiler and Downs 1973; Madrid and Porterfield 1974; Garea González and Filipe 1977; Calisher et al. 1989; Weingartl et al. 2003; Crill and Chang 2004; Niedrig et al. 2007). Often, several antigenically closely related flaviviruses of the same antigenic group co-occur in one area—e.g., in Central Europe USUV together with WNV, both members of Japanese encephalitis group (Weissenböck et al. 2002, 2003; Linke et al. 2007). It is therefore sometimes very difficult to decide which virus was responsible for the antibody production.

In the present study, we used neutralization with the standard topotype Egyptian strain Eg-101 of WNV (its suckling mouse brain homogenate) and carefully stored and thermally inactivated avian serum (not plasma) samples devoid of heparin, citrate, ethylenediaminetetraacetic acid (EDTA), or any stabilizing substances like merthiolate. In addition, the lot of fetal calf serum used in PRNμT was tested for antibodies against WNV in a separate assay. We estimated the results conservatively, as a 90% reduction in the number of plaques (not a 50% reduction, which is sometimes considered), and 1:20 dilution (instead of the usual 1:10) as a titer cut-off point. It is obvious that these parameters could affect the results (Buckley et al. 2006; Figuerola et al. 2007), and it is therefore advisable to interpret results of flavivirus serology with great care, especially during the serosurveys in birds and wild mammals (e.g., shot-killed game animals) where nonspecific inhibitors of viruses could occasionally oc-

TABLE 2. DETAILED COMPARISON OF RECIPROCAL PRNMT$_{90}$ TITERS AGAINST WEST NILE VIRUS (WNV) AND USUTU VIRUSES (USUV) IN WNV SEROREACTORS[a]

No.	*Species*	*Age*[b]	*Site*	*Date*	*WNV*	*USUV*
Z3	*Fulica atra*	Adult	2	25 Apr. 2006	**40**	<20
Z7	*Fulica atra*	Adult	2	25 Apr. 2006	**20–40**	<20
Z20	*Fulica atra*	Adult	2	4 May 2006	**40**	<20
Z22	*Fulica atra*	Adult	2	4 May 2006	20–40	**80**
Z32	*Fulica atra*	Adult	2	10 May 2006	20	20
N20	*Parus caeruleus*	Young	1	15 July 2006	**40**	<20
N21	*Acrocephalus scirpaceus*	Adult	1	15 July 2006	**20**	<20
N25	*Sturnus vulgaris*	Young	1	15 July 2006	**20**	<20
N62	*Remiz pendulinus*	Young	1	16 July 2006	20	NT
N97	*Sturnus vulgaris*	Young	1	18 July 2006	**20**	<20
N104	*Acrocephalus palustris*	Adult	1	18 July 2006	**160**	NT
N116	*Sylvia atricapilla*	Adult	1	18 July 2006	**20**	<20
N119	*Acrocephalus palustris*	Young	1	18 July 2006	**20**	<20
N121	*Acrocephalus schoenobaenus*	Young	1	18 July 2006	**20**	<20
N131	*Locustella luscinioides*	Adult	1	18 July 2006	**20–40**	<20
N133	*Sylvia atricapilla*	Young	1	19 July 2006	20	NT
N149	*Locustella luscinioides*	Adult	1	19 July 2006	**40**	<20
N155	*Emberiza schoeniclus*	Young	1	19 July 2006	20	NT
N209	*Alcedo atthis*	Adult	1	21 July 2006	20	NT
N217	*Acrocephalus schoenobaenus*	Adult	1	21 July 2006	20	NT
N287	*Acrocephalus scirpaceus*	Adult	1	22 July 2006	20	NT
N307	*Acrocephalus schoenobaenus*	Adult	1	23 July 2006	20	NT
N331	*Locustella luscinioides*	Young	1	23 July 2006	20	NT

[a]Boldface numbers indicate probable interpretation of the serology (WNV or USUV).
[b]Young = a hatching-year bird; adult = after-hatching-year bird; NT = not tested (very small volume of serum).

cur (e.g., Holden et al. 1965; Theiler and Downs 1973).

Previous serologic evidence of the WNV presence in the habitat of fishponds Nesyt at Sedlec was based on (1) detection of WNV antibodies in several local young (hatching-year) birds in 1985—1 Savi's warbler, 1 marsh warbler, 3 sedge warblers, 6 reed warblers, 3 bearded tits (*Panurus biarmicus*), 1 penduline tit, and 1 blue tit (Hubálek et al. 1989); and (2) seroconversion to WNV in 29% of 110 domestic ducks kept solely on the Nesyt pond in 1988, demonstrating that the virus was established in this fishpond area (Juřicová and Halouzka 1993). In a previous study by Hubálek et al. (1989), 4.3% of 704 wild birds examined in southern Moravia (most sampled in the same location on the Nesyt fishpond as in this study) during 1984–1986 had HI antibodies against WNV—a figure nearly identical to the present result (20 years later) when 5.0% of 319 birds captured on Nesyt fishpond were seropositive against WNV.

Common pochards were all seronegative in this study, as were 21 ducks of the same species in southern Spain (Figuerola et al. 2007a); also, other wild anseriform species were seronegative. However, three of 18 common coots (16.7%) were seropositive in this study, approaching results in Doñana wetlands (Spain), where 15%–50% of common coots revealed antibodies to WNV during the years 2003–2006, with a very high seroconversion rate of 17% in 2004 and 2005 (Figuerola et al. 2007b). In addition, one common coot was positive for USUV. The common coot is obviously a good indicator of the WNV presence and circulation. It is more easily accessible than anseriforms for feeding mosquitoes, because of bald spots in the plumage on the head, and long skinny legs that are exposed to mosquitoes on the shore.

Interestingly, all 30 examined individuals of the great reed warbler were negative in our survey, and similarly no WNV antibodies were found in 47 birds of this species on the Neusiedlersee in Austria (Aspöck et al. 1973), whereas other species of ecologically similar wetland warblers were positive in both areas (3 of 224 reed warblers, and 1 of 15 Savi's warbler in Austria).

Aspöck et al. (1973) examined HI antibodies in birds trapped in the reedbelt habitat on the Neusiedlersee during the autumn of 1971 (0.6% of 488 birds were seropositive to WNV), the winter of 1971 (all 125 negative), and the spring of 1972 (2.8% of 142 examined were positive). The difference between the autumn and spring seroprevalence rates indicated that at least some seropositive birds in spring had been infected with WNV in their winter stay in Africa—the individuals all included trans-Saharan migratory species: the Savi's warbler, the reed warbler, and the moustached warbler (*Acrocephalus melanopogon*). According to ringing recoveries, of the seropositive bird species found in the present study, the reed warbler, the sedge warbler, the marsh warbler, and the Savi's warbler, are trans-Saharan migrants; the blackcap is also migratory, overwintering in the Mediterranean and Africa; the common coot, the kingfisher, the penduline tit, the starling, and the reed bunting are migratory species wintering in the Mediterranean (mainly in northern Italy, but the common coot and starling also winter in western Mediterranean countries, including Spain); and the blue tit is a resident (sedentary) species although some individuals migrate from Moravia to northern Italy (Hudec 1983; Hudec and Šťastný 2005). It could mean that WNV seropositive adult birds had been infected in Africa or in southern Europe, while young (hatching-year) birds were infected in Moravia (or northeastern Europe). Maternal antibodies against WNV, transferred passively via egg yolk from seropositive females, can persist in birds on average for only 2–4 weeks (Gibbs et al. 2005; Buckley et al. 2006; Hahn et al. 2006). Identical results were described for the related mosquito-borne flavivirus of Japanese encephalitis (Buescher et al. 1959; Scherer et al. 1959). Because the hatching season of seropositive bird species of Central Europe is April to May (starling, blue tit) or May to June (the warblers: Hudec 1983), the antibodies found in young birds during the second half of July (i.e., beyond one month after hatching) were probably not maternal ones, but could have been formed after a WNV infection transmitted by mosquitoes in Moravia.

In the present study we did not find a significant difference in the seroprevalence rate between juvenile and adult birds. It is obvious that juvenile birds may be infected in the breeding grounds, whereas adult birds may be infected in the breeding grounds, during migratory stopovers, and in wintering areas.

In general, the data indicate indirectly limited WNV activity among birds in South Moravia during 2004–2006, when only 3.3% of wild birds were seropositive against WNV. This is in concordance with findings in free-living birds from some other European countries between 2001 and 2005. For instance, no or very low activity of WNV was documented in free-living birds in France both near Lyon (all 364 examined birds were seronegative: Lena et al. 2006) and in the Camargue (1.8% of 227 birds: Jourdain et al. 2007; or 4.6% of 388 passerines in 2004: E. Jourdain, pers. comm.); and in Germany (1.6% seroprevalence in 3,399 birds: Linke et al. 2007). Higher seroprevalence rates in that time period were found in some birds living in traditional enzootic areas for WNV in Europe: e.g., southern France (the Rhone delta—the Camargue), where 10.7% of 271 magpies (*Pica pica*) had antibodies to WNV (Jourdain et al. 2008) or southern Spain (the Quadalquivir delta—Doñana), where 7.3% of 534 nearly full-grown chicks of seven waterbird species had antibodies to WNV (Figuerola et al. 2007a). The only deviating study is that for Great Britain, where as much as 14.7% of 353 wild birds reacted with WNV in $PRNT_{90}$ in 2001–2002 (Buckley et al. 2003); the authors considered titers as low as 1:10 to be positive, and they did not use the topotype WNV strain, but three other WNV strains yielding widely varying results.

The detection of antibodies in one common coot (# Z22), probably attributable to USUV, is of interest. This adult migratory bird could theoretically have been infected in neighboring Austria, where this virus of African origin has appeared since 2001 (Weissenböck et al. 2002).

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PRÁCE 21

Šebesta O., Rettich F., Minář J., Halouzka J., Hubálek Z, Juřicová Z., **Rudolf I.**, Šikutová S., Gelbič I. and Reiter P. 2009. Presence of the mosquito *Anopheles hyrcanus* in South Moravia, Czech Republic. *Med. Vet. Entomol.* 23: 284–286.

Stručná charakteristika: v rámci entomologické surveillance byl nalezen komár *Anopheles hyrcanus* (vektor malárie) na místech, kde v předchozích desetiletích nebyl nikdy pozorován.

Hlavní přínos práce: *An. hyrcanus* je novým druhem pro dané území. Současně byl tento druh zachycen týmem doc. Votýpky také na jižní Moravě v letech 2005 až 2007. Dosud není jasné, zda nebyl tento komár přítomen na lokalitě již mnohem dříve (dnes odlišná metodika odchytu), nebo došlo k jeho nedávné introdukci v důsledku např. změn klimatu.

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SHORT COMMUNICATION

Presence of the mosquito *Anopheles hyrcanus* in South Moravia, Czech Republic

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Abstract. During a survey of mosquitoes in the South Moravian lowland area, the mosquito *Anopheles hyrcanus* (Pallas) (Diptera: Culicidae) was found breeding in an ancient fishpond (Nesyt). It is not clear whether this southern Palaearctic species, a known vector of malaria in Asia which has not been recorded in the Czech Republic until this year, has gone undetected in the past or whether it has recently moved into the region as a result of climate change.

Key words. *Anopheles hyrcanus*, geographic range, Central Europe, Czech Republic.

The mosquito fauna of South Moravia, in the southeastern Czech Republic, has been intensely studied in the last decade. The region is characterized by floodplain forests and meadows on the banks of the rivers Morava and Dyje, and by several fishponds. Annual flooding creates ideal breeding places for synchronous species of mosquitoes (genera *Ochlerotatus*, *Aedes*). The catastrophic floods of 1997, 2002 and 2006 resulted in an abundance of mosquitoes (Minář *et al*., 2001; Olejníček *et al*., 2003; Rettich *et al*., 2007). Regular monitoring of mosquito larvae has served as a basis for targeted mosquito control (Rettich *et al*., 2007). The role of local mosquitoes as vectors of human pathogenic viruses has been studied by several teams (Danielová *et al*., 1972, 1976; Rosický *et al*., 1980; Hubálek *et al*., 1998, 2000). Thirty-seven species of the subfamily *Culicinae* and six species of the subfamily *Anophelinae* have been recorded (Minář, 1973; Minář & Halgoš, 1997; Vaňhara & Rettich, 1998). In the genus *Anopheles*, *Anopheles messeae* Falleroni, *Anopheles claviger* (Meigen) and *Anopheles plumbeus* Stephens have been reported recently, whereas *Anopheles maculipennis s.s.* Meigen, *Anopheles atroparvus* van Thiel and *Anopheles labranchiae* Falleroni, known vectors of malaria in the region in the first half of the 20th century, have not been detected since the late 1960s (Minář & Rosický, 1975; Vaňhara, 1985, 1991; Vaňhara & Rettich, 1998; Olejníček *et al*., 2003). However, detailed studies of anophelines require specific methods, such as the collection of blood-fed females for examination of their eggs. Such techniques have not been used in the past except by Vaňhara (1985, 1991). Species records were mainly based on larval collections, sweep-net catches of adults or human-landing catches, but CDC miniature light traps supplemented with CO_2 were used in the years 2007–2008 (within the framework of the European research project EDEN [Emerging Diseases in a changing European eNvironment]). We report on the capture of a number of females of *Anopheles hyrcanus* (Pallas).

Slanisko is a nature reserve (10 ha) in the northwest Pannonian biogeographic region, on the west bank of the Nesyt fishpond near Sedlec village (48°47′ N, 16°43′ E), 176 m above sea level. This large mediaeval pond (322 ha), created in 1418, is the westernmost segment of the Lednické Rybníky National Nature Reserve fishpond system (Lednice fishponds), which includes four other medium-sized ponds. The average annual temperature and precipitation in the area are 9.3°C and 490 mm, respectively. The reserve is characterized by halophilic plants and insects. The littoral of the pond is partly covered by dense reed beds about 50 m in width.

We suspended CDC miniature light traps (BioQuip Products, Inc., Rancho Dominiquez, CA, U.S.A.) baited with CO_2 (2 kg of dry ice in a 2700-cm^3 box) 1 m above the ground in a small stand of willows adjacent to the reed beds of the pond. The traps were run from 16.00 hours to 09.30 hours mid-European time on two successive nights at 2-week intervals from spring to late autumn in 2007 and 2008. Female mosquitoes were identified according to Kramář (1958) and Becker *et al*. (2003).

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In 2007, 346 females of four genera and nine species were caught in the CO_2 traps between June and September. *Culex pipiens* L. dominated (76.9%), although a proportion of these may have been *Culex torrentium* Martini, a species that is morphologically very similar; males reared from larvae collected in the Valtice locality (near Sedlec) included both species. *Ochlerotatus cantans* (Meigen) (9.0%) (also known as *Aedes cantans*) and *Aedes vexans* (Meigen) (8.7%) were less frequent. Only two anopheline females (one *An. maculipennis s.l.*, one *An. plumbeus*) were recorded. However, local conditions for mosquitoes were unfavourable in 2007 because the water table was unusually low and the pond was artificially dried out in the summer.

In 2008 (Fig. 1), 1287 mosquito females of seven genera and 14 species were captured. *Aedes vexans* and *Oc. cantans* (including *Ochlerotatus annulipes* [Meigen], also known as *Aedes annulipes*) dominated (29.4% and 21.7%, respectively). *Culex modestus* Ficalbi (11.1%) was most abundant at the end of July and the beginning of August, and *Cx pipiens* formed 8.9% of the 2008 collection. The catch in late June included six female *An. hyrcanus* (var. *pseudopictus*), and the species was consistently present in subsequent collections, amounting to a final total of 56 females (4.3% of the total mosquito catch). Two other *Anopheles* spp., *An. maculipennis s.l.* (most probably *An. messeae*) and *An. claviger* were also collected (82 females, 6.4% of total mosquitoes). Interestingly, eggs laid by blood-fed *An. maculipennis s.l.* that had been collected in stables in the region were all *An. messeae.*

The geographic range of *An. hyrcanus s.l.* in Europe extends as far north as the Pannonian plain (Ramsdale & Snow, 2000; Becker *et al*., 2003). In countries neighbouring the Czech Republic, *An. hyrcanus* has recently been reported from Hungary (Tóth, 2003) and Slovakia (Halgoš & Benková, 2004), but not from Austria, Poland or Germany. The Sedlec locality (48°47′N) is thus the northernmost site from which it has been reported in central Europe. *Anopheles hyrcanus* is distributed below 50°N over the entire southern Palaearctic region from the Mediterranean sub-region to southeastern Asia in the Oriental region (Gutsevich *et al*., 1970; Tanaka *et al*., 1979). However, the taxonomy of *An. hyrcanus s.l.*, including the taxonomic status of *An. pseudopictus*, has remained controversial (Ramsdale, 2001). *An. hyrcanus s.l.* is an important vector of malaria in some parts of Central Asia and the Far East (Rosický & Weiser, 1952; Gutsevich *et al*., 1970).

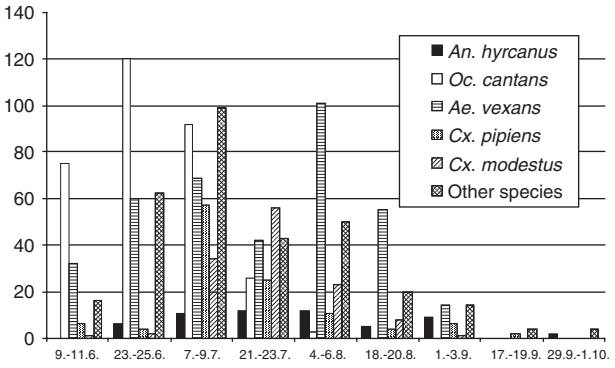

Fig. 1. Seasonal occurrence of mosquitoes at the Nesyt fishpond study site in 2008, expressed as the number of female mosquitoes caught in CDC light–CO_2 traps per 2 nights.

Larvae of the species develop in shallow water basins overgrown with vegetation, especially in reed beds and rice fields (Becker *et al*., 2003). Larvae have not been found in the surroundings of the Nesyt fishpond, but suitable breeding sites and ecological conditions are probably present. Although miniature CDC light–CO_2 traps were used in this area for the first time in 2007, no *An. hyrcanus* were caught in that year, perhaps because the pond was completely dry that summer.

It is possible that this new recording simply reflects the use of a new sampling technique, but it is also conceivable that the species has moved northwards as a consequence of the current trend in climate warming (Olejníček *et al*., 2003; Minář *et al*., 2007).

Addendum: During the review process, the presence of *Anopheles hyrcanus* in South Moravia in the years 2005–2007 was reported by Votýpka *et al*. (2008).

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PRÁCE 22

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Stručná charakteristika: v rámci evropského projektu EDEN byla provedena rozsáhlá surveillance arbovirů v komárech (vyšetřeno celkem 23 243 komárů) na vybraných lokalitách jižní Moravy. Cílem bylo pokusit se detegovat (molekulárními metodami) potažmo izolovat (inokulací sajících myší nebo na VERO buňkách) patogenní arboviry cirkulující v daném ekosystému.

Hlavní přínos práce: podařilo se izolovat 5 kmenů viru Ťahyňa, způsobujícího valtickou horečku a 1 kmen WNV (Rabensburg), způsobují západonilskou horečku. Virus Rabensburg byl vůbec poprvé izolován z mamalofilního druhu komára *Ae. rossicus*, což naznačuje možnou cirkulaci WNV (linie Rabensburg) mezi savčím hostitelem a komáry.

Příspěvek autora k dané práci: autor se podílel na sběru komárů, jejich molekulární analýze na přítomnost arbovirů a na přípravě rukopisu.

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Mosquito (Diptera: Culicidae) Surveillance for Arboviruses in an Area Endemic for West Nile (Lineage Rabensburg) and Ťahyňa Viruses in Central Europe

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ABSTRACT Six viral isolates were obtained from 23,243 female mosquitoes (examined in 513 pools) belonging to 16 species and collected along the lower reaches of the Dyje River in South Moravia (Czech Republic, central Europe) during 2006–2008: five isolates of *Orthobunyavirus* Ťahyňa (TAHV, California group, family *Bunyaviridae*: three isolations from *Aedes vexans* (Meigen), one from *Ae. sticticus* (Meigen), one from *Culex modestus* Ficalbi); and one isolation of *Flavivirus* West Nile (WNV, Japanese encephalitis group, family *Flaviviridae*)–strain Rabensburg (proposed lineage 3 of WNV) from *Ae. rossicus* (Dolbeshkin et al). All viral isolates were recovered from mosquitoes collected in 2006 (15,882 mosquitoes examined), while no virus was isolated from mosquitoes trapped in 2007 and 2008, when 1,555 and 5,806 mosquitoes were examined, respectively. The population density of local mosquitoes was very low in 2007 and 2008 because of warm and dry summer including a considerably low water table, compared with environmental conditions favorable for mosquito development in 2006. The virus isolation procedure was based on intracerebral inoculation of newborn mice. In parallel, more than one-third of the samples (183 pools consisting of 8,470 individual mosquitoes) were also examined by inoculating Vero cell cultures in Leighton tubes. However, the latter method detected only three of the six virus isolates (including WNV-Rabensburg). *Ae. rossicus* is a new potential vector for WNV-Rabensburg. This species feeds mostly on mammals including man; this raises the question whether this virus lineage is not adapted to an alternative mosquito-mammal cycle in the South-Moravian natural focus.

KEY WORDS *Flavivirus*, *Orthobunyavirus*, California group viruses, *Aedes vexans*, *Aedes rossicus*

Massive broods of mosquitoes (predominantly *Aedes* spp.) periodically occur in South Moravia (Czech Republic) along the rivers Dyje and Morava. This area has been known for a long time as a natural focus of Valtice fever, caused by Ťahyňa virus (TAHV, an *Orthobunyavirus* of the California antigenic group, family *Bunyaviridae* (Kolman et al. 1964, Rosický and Málková 1970, Danielová et al. 1972, 1976) and since 1997 also as an area endemic for West Nile-Rabensburg virus, the proposed lineage 3 of West Nile virus (WNV, a *Flavivirus* of the Japanese encephalitis virus group, family *Flaviviridae* (Hubálek et al. 1998, 2000, Bakonyi et al. 2005). This article describes the results of virus isolation attempts in local mosquitoes carried out in the years 2006, 2007, and 2008 within the framework of the EC-funded program EDEN (FP6).

Materials and Methods

Study Sites. Mosquitoes were collected for virological examination on two study sites in the district of Břeclav, South Moravia, Czech Republic. Climate of the area is relatively warm and dry; mean annual air temperature 9.1°C (January −1.7°C, July 19.2°C); mean annual precipitation 528–571 mm, with a maximum in June and a minimum in January.

Study Site 1: Nesyt. South-west banks of a large (322 ha), ancient fishpond Nesyt (48°46′34′′N, 16°42′05′′E; 176 m a.s.l.) at Sedlec near Mikulov. The Nesyt fishpond is surrounded by a wide fringe of fields (corn, maize, sugar beet), with scattered solitary trees, shrubs or their small clumps, orchards, gardens, and vineyards. A very characteristic plant community on the study site (pond) is the alliance *Phragmition communis* (with species *Phragmites communis* Trin., *Typha angustifolia* L., *T. latifolia* L., *Schoenoplectus lacustris* (L) Palla, *Glyceria maxima* (Hartm.) Holmb., *Carex*

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Table 1. Virological examination of female mosquitoes from the study sites in newborn mice, 2006–2008 (no. individuals/no. pools examined)

Study site: mosquito species	Nesyt			Soutok			Total
	2006	2007	2008	2006	2007	2008	
Ae. cinereus Meigen	185/6	3/1	84/2	573/15	2/1	—	847/25
Ae. rossicus Dolbeshkin		38/3	—	3,091/63	38/2	—	3,167/68
Ae. vexans Meigen	130/4	38/1	100/2	5,073/102	312/6	3,888/78	9,541/193
Ae. cantans (Meigen)	1,381/28	41/3	199/4	1,699/37	8/2	50/1	3,378/75
Ae. caspius (Pallas)	—	26/1	—	—	2/1	—	28/2
Ae. cataphylla Dyar	226/4	—	—	249/6	—	—	475/10
Ae. flavescens (Müller)	—	—	—	50/1	—	—	50/1
Ae. sticticus (Meigen)	141/3	16/2	—	1,536/31	26/3	—	1,719/39
Cx. modestus Ficalbi	1,467/31	—	129/3	5/1	—	—	1,601/35
Cx. pipiens L.	58/3	927/19	543/11	9/2	55/2	482/9	2,074/46
Culiseta annulata (Schrank)	—	—	103/3	—	—	—	103/3
An. claviger (Meigen)	—	—	38/1	—	—	—	38/1
An. hyrcanus Pallas	—	—	49/1	—	—	—	49/1
An. maculipennis s.l.	—	6/1	28/1	6/1	9/2	48/3	97/8
An. plumbeus Stephens	—	3/1	—	3/1	5/2	38/1	49/5
Coquill. richiardii (Ficalbi)	—	—	27/1	—	—	—	27/1
Total	3,588/79	1,098/32	1,300/29	12,294/260	457/21	4,506/92	23,243/513

riparia Curt., *Phalaris arundinacea* L.) forming dense and tall (2.8–3.6 m in the littoral zone) reed-beds. This reed belt is ≈50 m wide and covers 15% of the total pond area. Mammalian fauna of the study site consists of 33 wild species, domestic rabbit, pig, and cattle farming occurs in Sedlec village situated at the northwest bank of the fishpond. Thirty species of birds have been recorded as breeding in the reed belt, while 51 other, largely terrestrial bird species breed in close surroundings of the fishpond. Nesyt pond represents an important resting place for a great number of migrants. Twenty-four species of mosquitoes (*Culicidae*) of genera *Anopheles*, *Aedes*, *Culex*, *Culiseta*, *Coquillettidia*, and *Uranotaenia* have been recorded at the study site.

Study Site 2: Soutok. There are two collection plots within this study site: (1) 'Štrosflek' (48°39′56″N, 16°55′36″E; 154 m a.s.l.) and (2) 'Hvězda' (48°38′41″N, 16°56′07″E; 154 m a.s.l.). Both are situated in the wild game (deer, wild boar) reserve on the left, Czech bank of the Dyje River close to the Austrian village of Rabensburg lying on the opposite river bank. This floodplain forest-meadow ecosystem is periodically flooded (most frequently in spring); there are also scattered small pools with stable water, dead river arms, water channels, and periodic streams and pools in the study area, situated in an extensive plain lowland between the Rivers Dyje and Morava at their confluence. Approximately 75% of the study area is covered by forest, the rest are meadows. The vegetation of the area is characterized by the hard-wood floodplain forest alliance *Ulmenion* (leading association is *Fraxino pannonicae-Ulmetum*) and soft-wood floodplain forest alliance *Salicion albae* (leading association is *Salicetum albae*) communities. Forty species of mammals have been recorded here, as well as 104 bird species breeding in the site or nearby. However, many other avian species have been recorded as visiting this habitat during seasonal movements, especially during spring migration when the ecosystem is flooded. Twenty-four species of mosquitoes of the genera *Anopheles*, *Aedes*, *Culex*, and *Culiseta* have been recorded at the study site, many of them being seasonally very abundant.

Mosquito Collections. In 2006, mosquitoes were collected with entomological sweep nets and battery-operated aspirators over vegetation and while attempting to feed on humans at irregular intervals from May to October. In 2007 and 2008, mosquitoes were captured in Centers for Disease Control and Prevention (CDC) miniature light-CO_2 (dry ice) baited traps (BioQuip Products Inc., Rancho Dominiquez, CA) and in pigeon-baited lard-can traps (LePore et al. 2004, Deegan et al. 2005; captured mosquitoes are not in direct contact with the sentinel pigeon) regularly at 2-wk intervals from April to October, both exposed at one and 5 m (canopy level) of height. The traps were run from 1600–0900 h on two successive nights. The trapped insects were then transported to the laboratory in cooled flasks, and stored at −65°C until examination.

Virus Isolation Procedures. Mosquitoes were sexed and identified (Kramář 1958, Becker et al. 2003) on a cooled plate under stereomicroscope, and typically monospecific pools of ≈50 (10–100) females were prepared. Pools were homogenized in 1.5–2.0 ml of cooled phosphate-buffered saline pH 7.4 supplemented with 0.4% bovine serum albumin fraction V (Sigma, St. Louis, MO), penicillin (500 i.u./ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml) (PBS). The homogenates were centrifuged at 1,500 × *g* for 20 m (at 0°C), and supernatants inoculated intracerebrally (0.02 ml) in SPF suckling ICR mice (VELAZ Prague, Table 1). The mice were observed for 20 d after inoculation; the brains of dead animals were homogenized in PBS, centrifuged, and passaged intracerebrally in a new batch of suckling mice. Bacterial sterility of the suspensions was checked in meat-peptone broth (Nutrient Broth: HiMedia Labs, Mumbai, India) and thioglycollate broth (Fluid Thioglycollate Medium: HiMedia) incubated at 37°C for 5 d.

Table 2. Virological examination of mosquitoes from the study sites in Vero cell cultures, 2006–2008 (no. individuals/no. pools examined)

Study site: mosquito species	Nesyt			Soutok			Total
	2006	2007	2008	2006	2007	2008	
Ae. cinereus	17/1	—	60/1	233/5	2/1	—	312/8
Ae. rossicus	—	—	—	1,435/29	38/2	—	1,473/31
Ae. vexans	—	38/1	100/2	913/18	312/6	2391/48	3,754/75
Ae. cantans	50/1	41/3	199/4	385/8	—	—	675/16
Ae. caspius	—	26/1	—	—	—	—	26/1
Ae. cataphylla	—	—	—	24/1	—	—	24/1
Ae. sticticus	—	—	—	200/4	21/1	—	221/5
Cx. modestus	34/1	—	129/3	5/1	—	—	168/5
Cx. pipiens	7/1	927/19	529/10	—	55/2	131/2	1,649/34
Culiseta annulata	—	—	103/3	—	—	—	103/3
An. hyrcanus	—	—	49/1	—	—	—	49/1
An. maculipennis s.l.	—	6/1	—	6/1	—	—	12/2
An. plumbeus	—	—	—	—	4/1	—	4/1
Total	108/4	1,038/25	1,169/24	3,201/67	432/13	2,522/50	8,470/183

Portions of a part of the homogenates (183 pools of 8,470 individual mosquitoes, see Table 2) were also tested in parallel on Vero E6 cell cultures grown in plastic Leighton tubes (NUNC) at 37°C, using cell culture medium L-15 (Leibovitz; Sigma) with 5% (vol: vol) of heat-inactivated fetal calf serum (FCS; Sigma) and antibiotics penicillin (200 i.u./ml) and streptomycin (100 μg/ml). After the cell cultures were nearly confluent (2 d), the cultivation medium was removed, 100 μl of mosquito suspension was pipetted onto duplicate tube cultures, incubated at 37°C for 60 m, removed, and 2 ml of maintaining L-15 medium with 2% FCS and antibiotics penicillin (200 i.u./ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml) was added. The inoculated tube cultures were checked under inverse microscope for the occurrence of cytopathic effect for 7 d after inoculation. From each mosquito suspension, 200 μl aliquots were left aside, frozen and maintained at −20°C for molecular analysis of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR).

Virus Identification by Neutralization. Viral isolates were identified by the constant serum-serial virus dilution neutralization test (Lennette and Schmidt 1969). Infective mouse brain homogenates were serially 10-fold diluted from 10^{-2} to 10^{-8} in L-15 medium containing 2% heat-inactivated FCS, 30 μl of the virus dilutions were pipetted in microplates with 96 flat-bottomed wells (Sarstedt), mixed with 30 μl of normal or immune (against various arboviruses) either mouse sera prepared by three intraperitoneal doses at weekly intervals in our laboratory or mouse immune ascitic fluids (IAFs, received from the Ivanovsky Institute of Virology in Moscow), that were heat-inactivated (56°C for 30 m) and diluted 1:5 in L-15 medium. The virus-serum mixtures were incubated at 37°C for 60 m, 60 μl of the Vero E6 cell suspension (15,000 cells) in L-15 medium with 2% FCS was then added, incubated at 37°C for 4 h, and overlayed with 120 μl of 0.75% carboxymethyl cellulose in L-15 medium (Madrid and Porterfield 1969, Hubálek et al. 1979). The microplates were sealed in plastic bags, incubated at 37°C for 4–6 d according to the virus, evaluated under inverse microscope and stained with naphthalene black solution. The $\log_{10}$ neutralization indices (NI: titers with immune versus normal mouse serum) were estimated for each virus isolate, and log NI ≥2.0 values were regarded as decisive for the virus identification (Lennette and Schmidt 1969). The immune sera used in assays were prepared against the bunyaviruses Ťahyňa (TAHV, strain T16), Batai (BATV, strain Čalovo), and Sedlec (SEDV, strain AV172), flaviviruses West Nile topotype strain Eg-101 (WNV lineage 1), West Nile strain 97-103 (WNV proposed lineage three–Rabensburg virus), Usutu (USUV, strain Vienna 2001-blackbird), Central European tick-borne encephalitis virus (CEEV, strain Hypr), and orbivirus Tribeč (TRBV, strain Lipovnik 91), and the IAFs were prepared against alphavirus Sindbis (SINV), and flaviviruses Dengue-1 (DENV-1), Tyuleniy (TYUV), and Japanese encephalitis (JEV).

RNA Extraction and RT-PCR Procedure. Virus-positive mosquito pools and pools suspicious for virus (those that killed mice but with negative results in further mouse passages) were tested for viral RNA (SINV, TAHV, WNV, USUV) by virus-specific RT-PCRs. Viral RNA was extracted from 140 μl of the mosquito homogenates by using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Specific oligonucleotide primers for SINV (Kurkela et al. 2004), WNV (Huang et al. 2001), WNV lineage three Rabensburg (Bakonyi et al. 2005), and USUV (Bakonyi et al. 2004) were used for the amplifications. Diagnostic primers for the detection of TAHV in mosquitoes were designed directly for the study: forward primer TahS226f (5′-AAGCTGCTCTCGCTCGTAAG-3′) and reverse primer TahS972r (5′-GTGTGCTCCACTGAATACCT-3′). Continuous RT-PCR system encompassed the QIAGEN OneStep RT-PCR Kit (Qiagen). Each 25-μl reaction mixture contained 5 μl of 5× buffer (final $MgCl_2$ concentration 2.5 mmol/liter), 0.4 mmol/liter of each deoxynucleoside triphosphate, 20 pmol of the each primer, 1 μl enzyme mix (containing Omniscript and Sensiscript Reverse Transcriptases and HotStarTaq DNA polymerase), and 2.5 μl template RNA. Reverse transcription was carried out at 50°C for 30 m, followed by a denaturation step at 95°C for 15 m.

Table 3. Viral isolates from mosquitoes in newborn mice and Vero cell cultures

Sample no.	Mosquito sp.	Pool	Study site	Date collected	Virus identified
06-122[a]	*Cx. modestus*	32	Nesyt	1-IX-2006	TAHV
06-135	*Ae. vexans*	50	Soutok-Štrosflek	25-VII-2006	TAHV
06-154[a]	*Ae. sticticus*	50	Soutok-Hvězda	25-VII-2006	TAHV
06-157	*Ae. vexans*	50	Soutok-Hvězda	25-VII-2006	TAHV
06-222	*Ae. rossicus*	50	Soutok-Hvězda	30-VI-2006	WNV
06-250[a]	*Ae. vexans*	50	Soutok-Hvězda	26-IX-2006	TAHV

[a] Negative on Vero cell cultures.

Thereafter, the cDNA was amplified in 40 cycles of heat denaturation at 94°C for 40 s, primer annealing at 57°C for 50 s, and DNA extension at 72°C for 1 m, and the reaction was completed by a final extension for 7 m at 72°C (Bakonyi et al. 2005). The PCR reactions were performed in a PTC-200 Gradient Thermal Cycler (MJ Research, Waltham, MA). The PCR products were then separated on 2% agarose gel, stained with ethidium bromide and visualized by UV light. DNA extraction, PCR handling as well as post-PCR procedures were done in separate rooms to avoid possible cross-contamination of the samples.

Nucleotide Sequencing and Sequence Analysis. The partial nucleic acid sequences of the WNV isolate 06-222 and of an earlier isolate (99-222, previously identified as WNV lineage 3, Rabensburg; Bakonyi et al. 2005) were determined within this study. The nucleic acids of the strains' putative structural protein coding regions were amplified in RT-PCR assays, resulting in overlapping amplification products (Bakonyi et al. 2005). The nucleotide sequences of the products were determined in both directions, using fluorescence-based sequencing amplifications (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Stafford, TX). Sequences were read in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and were identified by the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The overlapping nucleotide sequences were aligned, continuous sequences were compiled, and they were compared with the complete genome sequence of the WNV Rabensburg strain 97-103 (GenBank accession number AY652464), using the Align Plus four (Scientific and Educational Software), ClustalX 1.81, and BioEdit 4.8.6 programs. The putative amino acid sequences of the structural protein (C, M, and E) coding region of the precursor polypeptide were deduced from the nucleotide sequences, and were aligned with that of the 97-103 strain.

Results

Virus Isolations from Mosquitoes. The mosquitoes examined virologically in the years 2006, 2007, and 2008 are listed in Tables 1 and 2, and the results in Table 3. In 2006, six viral isolates were obtained by intracerebral inoculation of suckling mice out of a total of 15,882 mosquitoes (339 pools) examined, while no virus was isolated in 2007 and 2008 when 1,555 mosquitoes (53 pools) and 5,806 mosquitoes (121 pools) were tested, respectively. Five mosquito suspensions killed the newborn mice very rapidly, within 3–6 d post-intracerebral inoculation (AST = 3.5–5.1 d), while the sample 06-222 caused death of newborn mice much slower, within 8–18 d (AST = 10.8 d). However, at the first and second passage, the survival time of the mice decreased to 8.2 and 7.0 d, respectively (Table 4), and at the third passage it was further reduced to 6.0 d with the range 5–8 d. When using Vero cells, only three of the six virus strains isolated by the suckling mice intracerebrally assay could be recovered (Table 3).

Identification of Virus Isolates. Neutralization $\log_{10}$ indices (NI) of immune mouse serum prepared against TAHV and tested with strains 06-122, 06-135, 06-154, 06-157, and 06-250 (Table 3), were >2.0, whereas they were <0.8 with immune sera or IAFs raised against all other arboviruses tested (BATV, SINV, WNV, JEV, DENV-1, CEEV, TYUV), indicating that these five virus isolates were identified as TAHV. However, isolate 06-222 reacted with immune sera and IAFs with following NI (in order with decreasing values): WNV-Rabensburg 3.5, WNV-Eg101 3.0,

Table 4. Fatality rate and avg survival time of newborn mice (SM) after intracerebral inoculation with particular viral isolates

Isolate no.	SM passage 0 (original suspension)		SM passage 1		SM passage 2		Virus identified
	FR[a]	AST[b] (range)	FR[a]	AST[b] (range)	FR[a]	AST[b] (range)	
06-122	12/12	3.5 (3–4)	11/11	3.0 (3–3)	NT	NT	TAHV
06-135	11/11	4.3 (4–5)	10/10	3.0 (3–3)	NT	NT	TAHV
06-154	22/23[c]	4.2 (3–6)	9/9	3.0 (3–3)	NT	NT	TAHV
06-157	22/22[c]	4.2 (3–6)	10/10	3.1 (3–4)	NT	NT	TAHV
06-222	9/12	10.8 (8–18)	18/18	8.2 (6–12)	8/8	7.0 (6–11)	WNV
06-250	10/11	5.1 (4–6)	NT	NT	NT	NT	TAHV

[a] Fatality rate: no. of mice killed/no. of mice inoculated; [b] average survival time (d); [c] including a re-isolation attempt.
NT, not tested.

Table 5. Nucleotide substitutions of three WNV–Rabensburg strains, which were isolated from mosquitoes collected in 1997, 1999, and 2006 in the same geographic location

Region	5′UTR	preM			M	E					NS1	
Position	84	582	609	720	885	1330	1599	2016	2148	2247	2613	2949
97-103	c	a	c	c	c	a	t	t	c	t	g	c
99-222	·	·	t	a	t	g*	c	c	t	c	a	t
06-222	t	g*	·	a	·	·	·	·	·	·	·	·

Positions refer to the complete genome sequence of WNV Rabensburg strain 97–103 (AY652464). Coding regions are indicated in the top line. Nucleotide substitutions resulting in amino acid changes are marked with *.

USUV 1.5, DENV-1 1.0, CEEV 0.8, SEDV 0.8, SINV 0.3, BATV 0.3, TAHV <0.3, TRBV <0.3. Consequently, this isolate proved to be a WNV, possibly belonging to WNV lineage 3–Rabensburg (because of the highest NI value).

Strain 06-222 has further been characterized by a low virulence for adult mice: it did not kill 6-wk old ICR mice inoculated intraperitoneally (i.p.), subcutaneously (s.c.), or even intracerebrally (four mice tested per each route), which is in contrast to Eg-101 topotype strain of WNV (lineage 1) that kills adult mice by intracerebral administration (Melnick et al. 1951). However, strain 06-222 killed suckling mice when given as infective 5% mouse brain suspension at either route (s.c., i.p., i.c.; at least eight newborn mice were tested per each route).

Virus RNA Detection in Mosquito Suspensions. A total of 27 positive or suspicious mosquito pools were tested using RT-PCR. Viral RNA was detected in six of them: the mosquito homogenates 06-122, 06-135, 06-154, 06-157, and 06-250 were found to contain TAHV RNA, and 06-222 revealed an amplification product indicative of WNV.

Sequence Comparisons of WNV Rabensburg Strains. The nucleotide sequences of the 06-222 and 99-222 strains were determined between nucleotide positions 23 and 3114, referred to the WNV Rabensburg isolate 97-103. These sequences cover the coding regions of the putative structural proteins C, M, and E. The nucleotide substitutions of the three strains are summarized in Table 5. Within the investigated region, 12 nucleotide substitutions were identified, most of them transitions. One nucleotide change in strain 99-222 resulted in an amino acid substitution within the putative E protein (Thr_{412} to Ala), and another change resulted in an amino acid substitution within the putative preM protein (Ile_{162} to Met). Nucleotide sequences identified in this study were submitted to GenBank under accession numbers GQ421358 and GQ421359.

Discussion

Whereas Ťahyňa virus has been isolated in South Moravia repeatedly and frequently (e.g., Kolman et al. 1964, Rosický and Málková 1970, Danielová et al. 1976, Hubálek et al. 2000), WNV was first found in this area only in 1997 (Hubálek et al. 1998). Here we report the recovery of the third isolate (06-222) of WNV lineage 3 (Rabensburg) from mosquitoes in the Czech area 'Soutok' at the confluence of the rivers Dyje and Morava and forming, at the same time, the conjunction of frontiers among three countries: Czechland (Czech Republic), Austria, and Slovakia. The first two strains of WNV-Rabensburg were isolated previously from *Cx. pipiens* mosquitoes in 1997 (97-103) and in 1999 (99-222) in the same locality (Hubálek et al. 1998, 2000). It is obvious that WNV-Rabensburg is persisting in this natural focus. We can only speculate whether the WNV strain isolated from *Ae. cantans* mosquitoes collected at Malacky, West Slovakia (air distance ≈30 km from the Czech 'Soutok') in 1972 (Labuda et al. 1974) could not have been also the Rabensburg lineage.

Local circulation of WNV in South Moravia was first indicated in 1985 by detecting specific antibodies in 4.3% of 704 free-living wetland birds, including 17 young (hatching year) wetland passerines of seven species, on Nesyt fishpond (Hubálek et al. 1989). This enzootic focus was confirmed 3 yr later during a serosurvey of 110 sentinel domestic ducklings kept on this pond over the summer season, when WNV antibodies appeared in 29% of the birds (Juřicová and Halouzka 1993). In 1997, the first five human West Nile fever cases were observed in South Moravia (Hubálek et al. 1999), in parallel with the first isolation of WNV from mosquitoes in this region (Hubálek et al. 1998).

All three present WNV-Rabensburg strains have in common a peripheral mouse virulence lower than that of the topotype Egyptian WNV strain Eg-101 (Melnick et al. 1951). They do not kill adult laboratory mice at either route, even when given intracranially. It might be a common feature of this lineage, making it a potential candidate for a WNV vaccine. WNV strains with a decreased neuroinvasiveness have been reported (Pogodina et al. 1983, Halevy et al. 1994). This attenuation, even within WNV lineage 1, could be caused by glycosylation, mutation or other changes of the viral envelope protein (Halevy et al. 1994, Adams et al. 1995, Berthet et al. 1997, Chambers et al. 1998).

In 2006, minimum infection rate (MIR, expressed as the mean number of virus isolates per 1,000 mosquitoes tested) for TAHV and its principal vector *Ae. vexans* was 0.58 (1:1,734), which is very similar to that found in 1997 when it was 0.60 (1:1,670) in the same area (Hubálek et al. 2000). MIR for TAHV and the other virus-positive mosquito species *Ae. sticticus* and *Cx. modestus* were also very closely related, 0.60 (1:1,677) and 0.68 (1:1472), respectively. However, no

TAHV isolates were recovered from *Ae. vexans* in 2007 (350 individuals examined) and 2008 (as much as 3,988 individuals tested). For WNV-Rabensburg and its potential new vector *Ae. rossicus*, the MIR was 0.32 (1:3,091) in 2006, whereas no WNV isolate was recovered from a total of 2,074 *Cx. pipiens* and 1,601 *Cx. modestus*, principal vectors of WNV, in 2006–2008. Previously, MIR for WNV-Rabensburg in *Cx. pipiens* was as high as 4.31 (1:232) in 1997 but low, 0.28 (1:3,546), in 1999 (Hubálek et al. 2000).

The population density of local mosquitoes was very low in 2007 and 2008, because of warm and dry summer including a considerably low water table, compared with environmental conditions favorable for mosquito development in 2006. This may be the reason why no viruses were isolated from mosquitoes in 2007 and 2008 rather than the change in the capture method that was because of the implementation of the EDEN project standardized techniques.

Ae. rossicus may be considered a new potential vector for WNV. Interestingly, this species feeds mostly on mammals including man (Kramář 1958) whereas a majority of competent mosquito vectors of WNV are ornithophilic. This raises the question whether the WNV-Rabensburg lineage might be adapted to an alternative mosquito-mammal cycle in the South-Moravian natural focus.

The three WNV-Rabensburg isolates show very little nucleotide sequence diversity (0.1–0.3%), which indicates that practically the same virus strain has been circulating in the region during 9 yr, and the structural proteins have not been exposed to considerable selective pressure. The background of the stable endemic maintenance of the WNV-Rabensburg lineage needs further investigations.

More than one-third of the samples (183 pools consisting of 8,470 mosquitoes) were also examined by inoculating Vero cell cultures in Leighton tubes in parallel to mouse inoculation: this method detected only three of the six virus strains (including WNV). The isolation assay on Vero E6 cells seems therefore to be (at least in our hands) significantly less sensitive than the suckling mouse intracerebral assay. In contrast, specific RT-PCRs detected RNAs of TAHV and WNV in all suspensions that were virus-positive by the suckling mouse inoculation assay.

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PRÁCE 23

Šebesta O., Halouzka J., Hubálek Z., Juřicová Z., **Rudolf I.**, Šikutová S., Svobodová P., Reiter P. 2010. Mosquito (Diptera: *Culicidae*) fauna in an area endemic for West Nile virus. *J. Vector Ecology*, 35: 156–162.

Stručná charakteristika: dílem metodická práce si kladla za cíl porovnat lokality (Nesyt, Pohansko) a druhy pastí (CDC past doplněna CO_2 a past s holubem jako návnadou, tzv. bird-baited trap) při sběru komárů v rámci projektu EDEN. V roce 2007 a 2008 bylo nasbíráno celkem 9268 komárů náležejících k 22 druhům.

Hlavní přínos práce: podařilo se prokázat rozdíly v početnosti komárů a jejich druhovém složení v návaznosti na použitý druh pastí a také studovanou lokalitu.

Příspěvek autora k dané práci: autor se podílel na extenzivním sběru komárů v rámci projektu EDEN a částečně na jejich zpracování a také na přípravě rukopisu.

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Mosquito (Diptera: Culicidae) fauna in an area endemic for West Nile virus

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ABSTRACT: Mosquito collections with CDC light traps using dry ice and pigeon-baited traps were carried out in south Moravia (Czech Republic) from April to October in 2007 and 2008 at two study sites. In 2007, 11 two-day captures were carried out in two-week intervals, and 1,490 female mosquitoes of nine species were caught. In 2008, 15 two-day trappings of mosquitoes were carried out: 6,778 females of 22 species of mosquitoes were trapped. The results showed marked differences in abundance and species composition of mosquitoes between both study sites and between the trapping methods. In the floodplain forest ecosystem of the Soutok study area, *Aedes vexans* predominated. The species composition in the Nesyt study site was more varied and the most common species was *Culex pipiens*. At the latter study site, *Anopheles hyrcanus* (var. *pseudopictus*) and *Uranotaenia unguiculata,* mosquito species with largely southern Eurasian distribution, were repeatedly demonstrated. The largest capture of mosquitoes was in traps with CO_2 placed at a height 1 m above the ground. The capture of mosquitoes in the pigeon-baited traps as well as in the traps with CO_2 placed in the canopy of trees was markedly lower in both study sites, with the predominant species being *Culex pipiens*. ***Journal of Vector Ecology* 35 (1): 156-162. 2010.**

Keyword Index: *Anopheles, Aedes, Culex*, climate, ecology, mosquitoes.

INTRODUCTION

Special attention has been directed toward mosquitoes in south Moravia within the Czech Republic as a result of the frequent flooding of their extensive breeding sites in flood-plain forests along the lower courses of the Morava and Dyje rivers (Kramář and Weiser 1951). Numerous entomological studies have been carried out here (Kramář 1958, Palička 1967, Vaňhara and Rettich 1998, Minář et al. 2001, Olejníček et al. 2004, Rettich et al. 2007). Regular monitoring of mosquito breeding sites, primarily focused on protecting the human population from this pestilent insect, has also taken place in the region since 1995 (Šebesta, unpublished data).

A second reason for the increased interest in mosquitoes of south Moravia is the relatively warm climate of this region. The lowlands near the lower courses of the Morava and Dyje form one of the warmest regions of the Czech Republic and, due to their position in proximity to the Pannonian lowlands and the lowlands of Lower Austria, serve as a gateway for thermophilic species of plants and animals. Several interesting species of mosquitoes have been discovered here. In the middle of the 20th century, for example, the occurrence of *Anopheles atroparvus* and *An. labranchiae* (Havlík and Rosický 1949, 1952, Rosický and Havlík 1951, Minář and Rosický 1975), the incidence of which was not reported in other parts of the Czech Republic, was recorded here. Other interesting findings arose in the 1970s and 1980s, when the incidence of *Ae. nigrinus* (Vaňhara 1987), and the Mediterranean species *Uranotaenia unguiculata* (Ryba et al. 1974) and *Culex martinii* (Vaňhara 1981, 1986), was noted here for the first time in the Czech Republic. The latest species found is *An. hyrcanus* (Votýpka et al. 2008, Šebesta et al. 2009). These species had also not been found previously at other locations in the Czech Republic. Of the 45 species of mosquitoes whose occurrence within the entire Czech Republic has been reported (Minář and Halgoš 1997, Országh et al. 2006, Rettich et al. 2007), 37 species in total have been detected in south Moravia (Vaňhara 1991, Vaňhara and Rettich 1998, Rettich et. al. 2007, Šebesta et al. 2009).

Great attention is also devoted to local mosquitoes as potential vectors of pathogens, from which the Tahyna, Batai, Lednice, and West Nile viruses have been recorded (Danielová et al. 1972, Rosický et al. 1980, Hubálek et al. 1998, 2000). Up until the middle of the last century, an endemic incidence of malaria was noted in this region (Havlík and Rosický 1952).

As part of the European project EDEN (2005–2009), mosquito fauna has been studied since 2006 in the south Moravian endemic region of the "Rabensburg" genomic lineage of West Nile virus (Hubálek et al. 1998, 2000, Bákonyi et al. 2005). The aim of this study was to analyze species composition of local mosquito fauna, compare it with previous reports of other authors, and collect material for subsequent arbovirological studies.

MATERIALS AND METHODS

The two selected study sites, where the presence of the West Nile virus had been documented, are about 20 km distant from one another. They constitute two distinct, yet

Figure 1. Map of study sites in the Czech Republic.

typical, habitats for the monitored region (Figure 1).

The Soutok area (48°37' – 48°44'N, 16°53' – 16°59'E; 151–153 m above sea level) is part of an extensive complex of floodplain forests and irregularly inundated meadows located between the Morava and Dyje Rivers and in close proximity to their confluence ("*soutok*" in Czech), relatively unaffected by human activities. Spring floods of various amounts are an almost yearly event and are caused by groundwater and seepage water. The region also often tends to be flooded by overflowing rivers. In the summer months, overflows occur irregularly and sometimes repeatedly in a single year. The main breeding sites of *Aedes* species are found here. In this location, two localities were selected. The results from both, however, are processed together. In each of the two locations, the West Nile virus (Rabensburg lineage) was recently isolated from *Cx. pipiens* mosquitoes (Hubálek et al. 1998, 2000, Bakonyi et al. 2005).

Nesyt Fishpond (48°47'N, 16°43'E, 176 m a.s.l.) is located near the village of Sedlec. It is part of a complex of five fishponds forming the Lednické Rybníky National Nature Reserve. Nesyt was established in 1418, and with an area of 322 ha, it is the largest Moravian fishpond. Its banks are bordered with a dense, almost impenetrable growth of reeds (*Phragmites communis*), which in some places reaches a width of several tens of meters. The mosquito trapping site is comprised of a cluster of trees (willows) and shrubs growing on the edge of the waterfront vegetation and is bordered by a meadow with the Slanisko Nature Reserve, characterized by the appearance of halophilic plants and insects (e.g., *Scorzonera parviflora, Tripolium pannonicum, Bucculatrix maritima, Coleophora halophilella)*. At Nesyt, circulation of the West Nile virus was documented indirectly years ago by the detection of specific antibodies in local domestic ducks (Juřicová and Halouzka 1993). The location is outside the flooded area, and the condition of the water there is stable.

The region of south Moravia is characterized by a relatively dry and warm climate with an average daily temperature of 9.3° C and an average annual precipitation of 490 mm. From a meteorological perspective, the conditions in the two years of this study (2007, 2008) were different. The winters were warm, and no snow cover was formed in south Moravia. The mean January 2007 temperature was +4.2° C (the warmest January within the last 50 years; difference from the average 1961-1990 is +6.1° C). In January 2008 the mean temperature was +2.1° C (difference +4.0° C from the average). Mean temperatures in February 2007 and 2008 were +4.4° C (difference +4.1° C) and 3.2° C (difference +2.9° C), respectively (Figure 2). Snowfall was low even in the mountains in the Czech Republic, and thus the spring floods did not arrive. The flow rate of water in the Morava and Dyje Rivers was below average for nearly the entire year, and thus neither river overflowed. The larval site at the Soutok location was inundated only by groundwater and seepage water for only a short period and to a small extent.

Trapping of mosquito adults was conducted from the beginning of April to the end of October. Two types of traps were used: CDC miniature light traps with CO_2 (BioQuip Products, Inc., Rancho Dominiquez, CA, U.S.A., supplemented with dry ice, and lard-can traps baited with a live pigeon (LePore et al. 2004, Deegan et al. 2005). Both types of traps were hung in parallel at heights of 1 m and

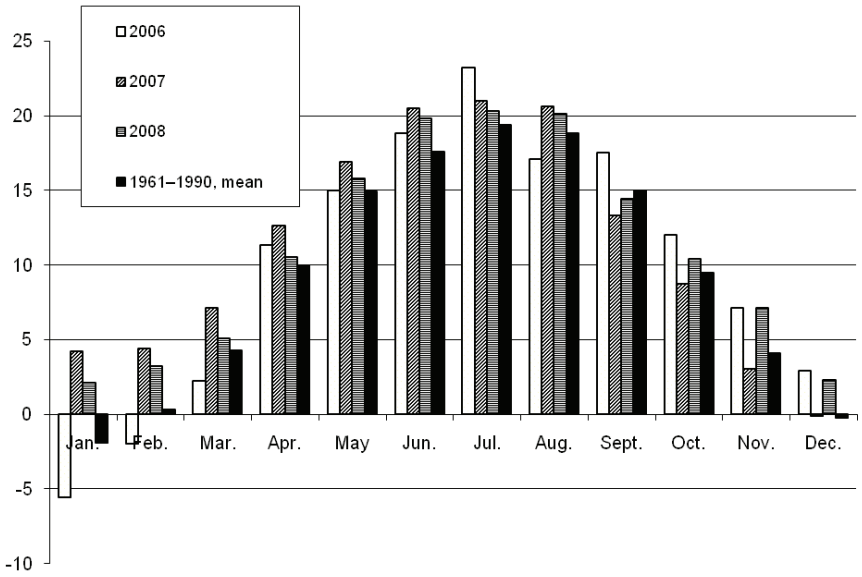

Figure 2. Mean monthly air temperature (° C) in the study area, 2006-2008, compared with a long-term average (Velké Pavlovice; data from Czech Hydrometeorological Institute in Brno).

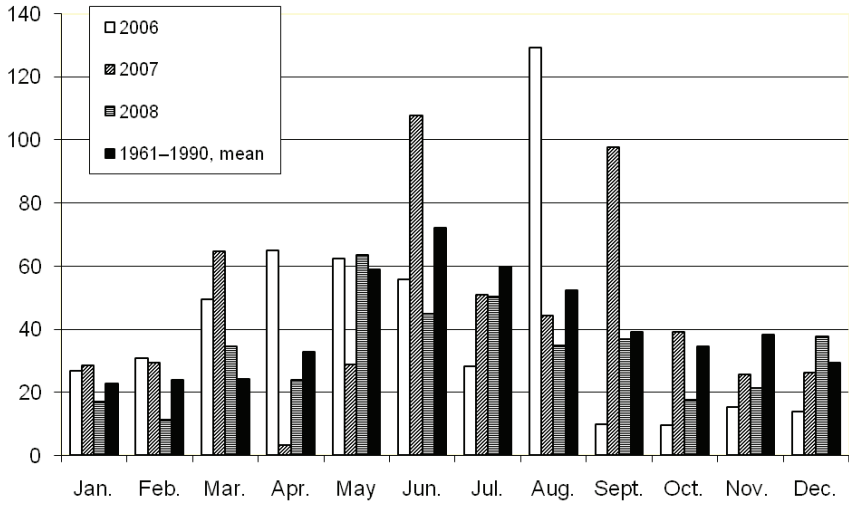

Figure 3. Monthly sum of precipitation (mm) in the study area, 2006-2008, compared with the long-term average (Velké Pavlovice; data from Czech Hydrometeorological Institute in Brno).

Table 1. Species composition of mosquitoes at study site Nesyt.

Species	Number collected	Percent of collection
Culex pipiens/torrentium	1,514	51.9
Aedes vexans	430	14.8
Aedes cantans	393	13.5
Aedes cinereus	83	2.8
Aedes sticticus	19	0.7
An. maculipennis s.l.	42	1.4
An.claviger	50	1.7
Cs. annulata	105	3.6
Cq. richiardii	29	1.0
Ae. flavescens	9	0.3
Ae. cataphylla	7	0.2
Cx. modestus	136	4.7
Cs. morsitans	2.0	0.1
An. hyrcanus	57	2
Other species	39	1.3
Total	2,915	

Table 2. Species composition of mosquitoes at study site Soutok.

Species	Number collected	Percent of collection
Ae. vexans	4,618	86.3
Ae. sticticus	337	6.3
Cx. pipiens/torrentium	239	4.5
An. maculipennis s.l.	34	0.6
An. plumbeus	45	0.8
Ae. cantans	19	0.4
Ae. rossicus	34	0.6
Other species	27	0.5
Total	5,353	

5 m; the horizontal distance between individual traps was about 25 m. The traps were distributed around 16:00 (EET) and were left exposed overnight. Mosquitoes were collected in the morning around 09:00, transported in a refrigerating bag (at about 0° C), and stored in the laboratory at –60° C until examination. Identification of females was conducted according to Kramář (1958) and Becker et al. (2003), and isolated males were not included in the overall results. In parallel with these trapping techniques, control collections were done of mosquito males (hypopygium morphology) and fed anopheline females (for oviposition) and larvae and pupae to make exact species identification of mosquitoes possible. A paired t-test was used to statistically compare the data.

RESULTS

A total of 8,268 female mosquitoes of 22 species, belonging to six genera, was caught in the traps during 2007–2008 (Tables 1 and 2). One additional species (*Ae. dorsalis*) was collected only with an entomological net.

At the Nesyt location, a total of 2,915 female mosquitoes was caught during the two years. The relative overall abundance was 14.0 females/trap/night. The mosquitoes belonged to 17 species, the dominant being *Cx. pipiens*, with 1,514 captured females, representing 51.9% of the total number of captured specimens. A summary of the mosquitoes captured at Nesyt is shown in Table 1. The collection of five females of *Ur. unguiculata* and, in particular, 57 females of *An. hyrcanus* (var. *pseudopictus*) (2.0%) is interesting. At the Nesyt location, this species was the most abundant of the *Anopheles* genus (Šebesta et al. 2009). In the Soutok region, 5,353 females were caught in 2007 and 2008 combined. The relative overall abundance of mosquitoes was 12.87 females/trap/night. The mosquitoes belonged to 13 species, the dominant being *Ae. vexans* with 4,618 females (86.3%). A summary of species captured at this location is shown in Table 2.

In both years, capture in CDC light–CO_2 traps was

Table 3. Total female mosquitoes captured in different traps, 2007-2008.

	CDC light–CO_2 traps		Pigeon-baited traps		Total
	1 m height	5 m height	1 m height	5 m height	
Anopheles claviger	53	0	0	0	53
An. hyrcanus	56	1	0	0	57
An. maculipennis s. l.[1]	74	2	0	0	76
An. plumbeus	36	9	3	0	48
Aedes cinereus	82	1	0	0	83
Ae. rossicus	34	3	0	0	37
Ae. vexans	4,988	60	2	1	5,051
Ae. cantans[2]	398	9	1	4	412
Ae. caspius	25	3	0	0	28
Ae. cataphylla	15	0	0	0	15
Ae. excrucians	4	0	0	0	4
Ae. flavescens	8	1	0	0	9
Ae. sticticus	350	4	2	0	356
Culex modestus	126	10	2	0	138
Cx. pipiens[3]	451	863	198	241	1,753
Culiseta annulata	102	6	0	0	108
Cs. morsitans	1	1	0	0	2
Coquillettidia richiardi	29	3	1	0	33
Uranotaenia unguiculata	5	0	0	0	5
Total	6,837	976	209	246	8,268

[1] *An. maculipennis* and *An. messeae*.
[2] Together with *Ae. annulipes.*
[3] Together with Cx. *torrentium.*

considerably higher (25.3 per trap per night) and all detected species were found therein (Table 3). *Ae. vexans* was the most represented, while *Cx. pipiens, Ae. cantans*, *Ae. sticticus*, and *Cx. modestus* were markedly less so and other species represented fewer than 1% of the total number of captured females. Only 455 specimens (1.4 females/trap/night) of eight mosquito species were caught in pigeon-baited traps, with *Cx. pipiens* the dominant species collected (Table 3).

A markedly higher capture of mosquitoes was recorded in traps placed at a height of 1 m. In total, 7,046 females were caught (22.3 trap/night). The dominant species was *Ae. vexans*, with lesser numbers of *Cx. pipiens*. At a height of 5 m ("canopy"), the occurrence of 1,222 female mosquitoes (3.9 per trap per night) was recorded, with *Cx. pipiens* dominant and lesser numbers of *Ae. vexans* (Table 3). We tested the statistical significance of differences in mosquito yields with traps situated at the two levels (1 m and 5 m), using a paired t-test and omitting those collection days when no mosquitoes were caught in the compared pair of traps. The light-CO_2 traps at ground level caught an overall average (both years, all study sites) of 88.07 mosquitoes, while those in the canopy captured only 7.93 individuals, a highly significant (P = 0.0004) difference. However, the average number of *Cx. pipens* was 5.38 at ground level, but 10.14 at the canopy level, a significant (P = 0.005) difference. It was also significant when both years 2007 and 2008 were treated separately (P = 0.017 and P = 0.05, respectively). In the pigeon-baited traps, the overall average was 3.53 at ground level, and 4.07 at canopy level for all mosquitoes (both values do not differ significantly, P = 0.20), and for *Cx. pipiens* the averages were 3.36 and 4.10, respectively. This difference is also not statistically significant (P = 0.12).

DISCUSSION

The recent emergence of a few important mosquito-borne viruses in Europe (West Nile *Flavivirus*, Chikungunya *Alphavirus*) has increased the interest of medical entomologists in monitoring mosquitoes in endemic areas. Most European teams have used trapping methods similar to those in the present study, especially the CDC miniature light traps with CO_2 and bird-baited traps (Savage et al. 1999, Esteves et al. 2004, Romi et al. 2004, Balenghien et al. 2006, Ponçon et al. 2007, Aranda et al. 2009).

Forty-five species of mosquitoes were recorded in the Czech Republic, and 37 of them were also found in southern Moravia (Vaňhara 1991, Minář and Halgoš 1997, Vaňhara

Table 4. List of all mosquito species found in the Czech Republic, with their previous reports from South Moravia and the present study.

Species	S. Moravia	This study	Species	S. Moravia	This study
Anopheles atroparvus van Thiel	+[1]		*Ae. pullatus* (Coquillett)		
An. claviger (Meigen)	+	+	*Ae. punctor* (Kirby)	+	
An. hyrcanus (Pallas)	+[1]	+	*Ae. refiki* (Medschid)	+	
An. labranchiae Falleroni	+[1]		*Ae. riparius* (Dyar & Knab)		
An. maculipennis (Meigen)	+	+	*Ae. rossicus* Dolbeskin,		
An. messeae Falleroni	+	+	Gorickaja & Mitrofanova	+	+
An. plumbeus Stephens	+	+	*Ae. rusticus* (Rossi)		
Aedes annulipes (Meigen)	+	+	*Ae. sticticus* (Meigen)	+	+
Ae. cantans (Meigen)	+	+	*Ae. vexans* (Meigen)	+	+
Ae. caspius (Pallas)	+	+	*Coquillettidia richiardii* (Ficalbi)	+	+
Ae. cataphylla (Dyar)	+	+	*Culex hortensis* Ficalbi		
Ae. cinereus Meigen	+	+	*Cx. martinii* Medschid	+[1]	
Ae. communis (De Geer)	+		*Cx. modestus* Ficalbi	+	+
Ae. diantaeus (Howard, Dyar & Knab)			*Cx. pipiens* Linnaeus	+	+
Ae. dorsalis (Meigen)	+	+	*Cx. territans* Walker	+	
Ae. excrucians (Walker)	+	+	*Cx. torrentium* Martini	+	+
Ae. flavescens (Muller)	+	+	*Culiseta alaskaensis* (Ludlow)	+	
Ae. geminus Peus	+		*Cs. annulata* (Schrank)	+	+
Ae. geniculatus Olivier	+		*Cs. glaphyroptera* (Schiner)		
Ae. intrudens (Dyar)	+		*Cs. morsitans* (Theobald)	+	+
Ae. leucomelas (Meigen)	+		*Cs. ochroptera* (Peus)		
Ae. nigrinus (Eckstein)	+[1]		*Cs. subochrea* (Edwards)	+	
Ae. pulcritarsis (Rondani)			*Uranotaenia unguiculata* Edwards	+[1]	+

[1]Within the Czech Republic, only reported from southern Moravia.

and Rettich 1998, Országh et al. 2006, Rettich at al. 2007, Šebesta et al. 2009) (Table 4). In this study, 23 species were recorded. Females of *Ae. cantans* and *Ae. annulipes,* and *Cx. pipiens* and *Cx. torrentium,* were not always distinguishable with certainty and were regarded tentatively in this study as being either *Ae. cantans* or *Cx. pipiens*. Using the oviposition identification technique, *Anopheles maculipennis* s.l. was represented by two species, *viz An. messeae* and *An. maculipennis* s.s. *Aedes dorsalis* was only collected with entomologic nets.

We have not found any mosquito of the species *Ae. communis* that was reported by other authors from South Moravia. On the other hand, *Ur. unguiculata* was rarely reported by previous authors (Ryba et al. 1974), while it appeared in three samplings in this study. *An. hyrcanus* was first found here in 2005 (Votýpka et al. 2008). That study, however, was not published until the end of 2008 and thus was not known to us at the time of our study (Šebesta et al. 2009). The nearest finding of *An. hyrcanus* until this time was reported in Slovakia (Halgoš and Benková 2004).

The results of this study were affected by unusual meteorological conditions in the years 2007-2008, i.e., warm winter weather without snow cover associated with the resulting absence of floods. In this regard, it is interesting to compare the incidence of mosquitoes in this year with the results of a study from 2006 (Rettich et al. 2007), when, in addition to destructive spring floods, two local floods in June and August also affected the Soutok area. All of these events were followed by mosquito calamities. In that year, an extensive study of mosquito larvae was conducted: during April floods a large amount of larvae of spring species, especially *Ae. cataphylla* (20.4% of all collected larvae), *Ae. cantans/annulipes* (19.6%), and *Ae. intrudens* (7.1%) was discovered. At almost the same time as larvae of the spring species, larvae of species more typical of the summer season also appeared (*Ae. sticticus:* 39.0%, *Ae. vexans:* 8.3%, *Ae. cinereus/rossicus:* 4.2%). During the June and August floods of 2006, *Ae. vexans* (38.2% in June and 57.4 % in August), *Ae. sticticus* (30.3% and 34.6%, respectively), and *Ae. cinereus/rossicus* (26.1% and 7.9%, respectively) larvae predominated. In our study, the occurrence of female mosquitoes of spring species was detected only rarely; mosquito activity increased

over the course of June, and the dominant species was *Ae. vexans*. It is also interesting that mosquitoes collected with entomological nets in the same habitats in 2006 yielded two arbovirus strains (Tahyna and West Nile), while no virus was recovered from mosquitoes collected during the present study in 2007 and 2008 (Hubálek et al. 2010).

A marked difference was recorded between the two study sites in the species composition of mosquito fauna. Fewer mosquito species were detected in the Soutok area and there was dominance of flood-water species *Ae. vexans* and *Ae. sticticus*, while at the Nesyt location the species composition was more varied. The results of mosquito collections were affected in both years by weather, resulting in a low incidence of pest mosquito species. This was reflected particularly in the Soutok area, where the number and size of periodic pools was markedly reduced as compared to normal.

The capture of mosquitoes in both types of traps varied greatly in terms of both quantity and species composition. Capture yield was markedly higher in CDC mini-light traps with CO_2, and all species of mosquitoes detected in this study were recorded in these traps (except for *Ae. dorsalis*). The capture efficiency of mosquitoes in pigeon-baited traps was very low but selective: *Cx. pipiens* was dominant in these traps. The height of the trap also had a crucial impact on the capture of mosquitoes. The entry of mosquitoes in traps placed 1 m above ground was almost seven times greater than into traps placed in the canopy 5 m above ground. The difference in species composition also was remarkable. At 1 m, all species of mosquitoes were detected in a composition corresponding with their incidence (with the exception of *Cx. pipiens*), while at 5 m *Cx. pipiens* clearly predominated and was caught significantly more frequently than in the ground traps. With pigeon-baited traps, we did not find significant differences in the all-mosquito or *Cx. pipiens* yield between the traps situated at different levels.

In conclusion, the study confirmed species richness of mosquito fauna in South Moravia, the region of occurrence of mosquito-borne diseases of humans including Ťahyňa bunyavirus and occasionally West Nile flavivirus infections (Rosický et al. 1980, Hubálek et al. 2000). In addition, two species of mosquitoes not occurring elsewhere in Czechland (a short geographic term for the Czech Republic) were repeatedly detected: *An. hyrcanus* and *Ur. unguiculata,* both southern faunistic elements. We also found that *Cx. pipiens* predominated at the canopy level with no difference between the trap type.

Acknowledgments

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PRÁCE 24

Šebesta O., **Rudolf I.**, Betášová L., Peško J., Hubálek Z. 2012. An invasive mosquito species *Aedes albopictus* found in the Czech Republic. *EuroSurveill.*17(43). pii: 20301.

Stručná charakteristika: tzv. tygří komár *Aedes albopictus* patří mezi invazivní druhy komárů, které dnes představují zdravotní riziko dokonce i pro obyvatele Evropy. Tento druh komára, původně s endemickým výskytem v Asii, se stal postrachem hlavně jako přenašeč dvou závažných onemocnění – horečky dengue a chikungunya. Byl dosud zaznamenán ve více než 19 zemích Evropy a dokonce byl vektorem u autochtonních infekcí horeček Dengue a Chikungunya ve Francii a Chorvatsku. Protože *Ae. albopictus* je nejčastěji introdukován importem ojetých pneumatik, importem tropické rostliny ('Lucky bamboo') nebo pozemní dopravou, zaměřili jsme se na příjezdové cesty z jižní Evropy a na několika odpočívadlech pro automobilovou dopravu jsme umístili tzv. ovitraps pro záchyt vajíček, případně larev *Ae. albopictus*.

Hlavní přínos práce: ve dvou zářijových termínech se nám na odpočívadle v Mikulově podařilo nalézt 17 larev *Ae. albopictus*, což zdůrazňuje riziko introdukce potažmo usídlení tohoto invazivního druhu dokonce i ve střední Evropě.

Příspěvek autora k dané práci: autor se podílel na designu studie, surveillance komárů v terénu (instalace a odečítání pastí) a přípravě rukopisu.

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An invasive mosquito species *Aedes albopictus* found in the Czech Republic, 2012

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Between July and September 2012, seventeen larvae of the invasive mosquito species *Aedes (Stegomyia) albopictus* (Skuse) were discovered using 60 ovitraps at four study sites alongside two main road exits in South Moravia, Czech Republic. This is the first report of imported *Ae. albopictus* in the Czech Republic. The findings highlight the need for a regular surveillance programme to monitor this invasive species throughout western and central Europe.

Background

Of the invasive mosquitoes discovered in Europe recently, the Asian tiger mosquito *Aedes albopictus* (Skuse) represents the major threat to public health. Historically, this species originated in South-East Asia, but it has spread to the Americas, parts of Africa, northern Australia, and 19 European countries (Albania, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, France, Germany, Greece, Italy, Malta, Monaco, Montenegro, the Netherlands, San Marino, Serbia, Slovenia, Spain, Switzerland, Vatican City State) during the last decades. The species is now widely established and reportedly a nuisance mosquito in Italy, parts of France and Spain [1]. *Ae. albopictus* is globally an important vector of human pathogens such as chikungunya and dengue viruses as well as filarial nematodes represented by *Dirofilaria* spp., and an experimentally proven vector of eastern equine encephalitis, Venezuelan equine encephalitis, La Crosse encephalitis, Japanese encephalitis, West Nile and several other viruses [2,3].

Its eggs are frequently transported via used tire trade or by importation of lucky bamboo [2]. However, the most important mode of long-distance dispersal of *Ae. albopictus* in Europe in the last decade seems to be transportation by ground vehicles (i.e. lorries, cars, caravans) from southern Europe [4,5].

While two frequently used main roads connecting the Czech Republic with southern European countries cross the border in South Moravia, no systematic surveillance of invasive mosquito species has been conducted until present. This led us to periodically monitor invasive mosquito species at this so-called 'Moravian entrance gate' using ovitrap installations.

Trapping of mosquitoes

To monitor the presence of *Ae. albopictus* we used traditional ovitraps [6]. These were constructed from a dark blue 800 ml plastic cup and supplemented with 500 ml of dechlorinated tap water and a floating wooden tongue depressor paddle wrapped into rough cotton fabric that was in contact with the water line to ensure *Ae. albopictus* oviposition. Ovitraps were placed on shrubs, columns or public lighting in close proximity to parking spaces about 50 cm above the ground. Wooden paddles and water were periodically replaced (every 7 days) and transported in closed containers to the laboratory. The paddles were incubated at 25°C in humid atmosphere for three days and then kept immersed below the water surface at 25°C for another 12 days. Additionally, water from the ovitrap containers was incubated in the laboratory at 25°C for one week. Both components were daily examined for the presence of hatching eggs or larvae. Larvae and adults reared from larvae were morphologically identified according to recent entomological keys [6,7].

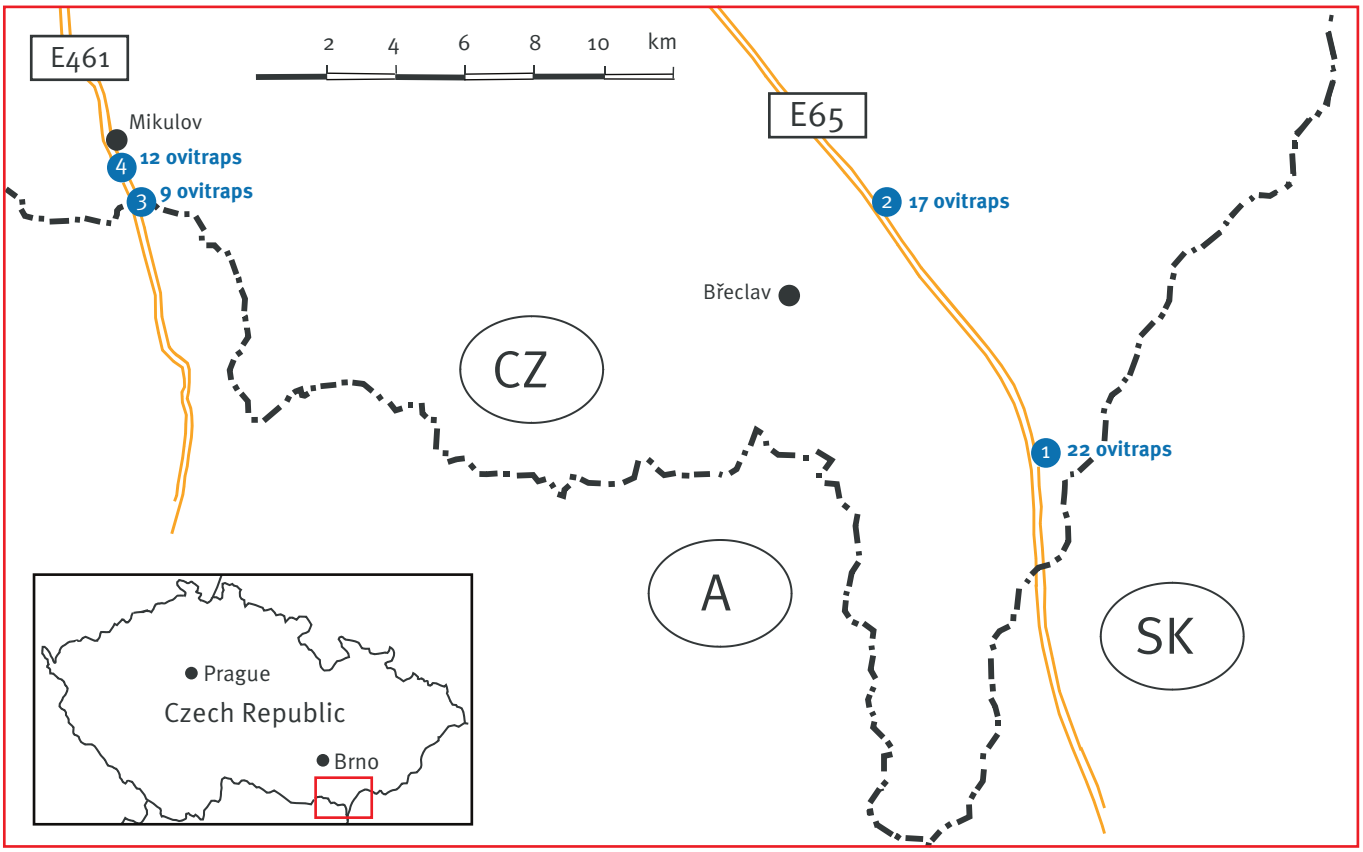
Study sites and findings

Several ovitraps were placed at four study sites (parking lots) in close proximity to exits of two main roads respectively connecting Austria and Slovakia with the Czech Republic (Figure). A total of 60 ovitraps were installed between the beginning of July and the end of September 2012.

The first two ovitrap sites (study sites 1 and 2) were situated near the main road E65, a transit route for goods to the Czech Republic from Slovakia and Hungary as well as from Balkan countries (Romania, Bulgaria, Croatia, Serbia, Greece). Individual and collective transport between western (e.g. Germany, Belgium, the Netherlands), central and southern Europe also operates through this main road. Study site 1 (22 ovitraps) was in Lanžhot (N 48°43,554´, E016°59,041´, 155 m above sea level (a.s.l.)), at a one km distance from Slovakia. The location is used for refreshment and

Figure

Locations (n=4) of ovitraps (n=60) for invasive mosquito monitoring, South Moravia, Czech Republic, July–September 2012



Location where ovitraps were placed, the number in the circle indicates the site number for the purpose of the study.

A: Austria; CZ: Czech Republic; SK: Slovakia.

Mosquito traps (ovitraps) were placed at four locations near the two main roads E461 and E65 which are respectively shown on the map in yellow.

refueling, with a parking capacity of about 100 spaces. Study site 2 (17 ovitraps) was at Ladná (N 48°48,669´, E016°53,600‘, 177 m a.s.l.) and situated approximately 16 km north of the first study site alongside the same main road. The site serves mainly as a refueling and rest area with a parking capacity of about 40 spaces. Two additional ovitrap sites (study sites 3 and 4) were chosen beside main road E461, where this road enters the Czech Republic from Austria. The main road E461 is frequently used for transit of goods from southern Europe (Italy, Slovenia, Croatia, Serbia, Montenegro, Macedonia, Albania) to the Czech Republic. Study site 3 (9 ovitraps) was Mikulov II (N 48°47,424´, E016°38,154´, 198 m a.s.l.), a former customs' house now solely intended for refreshment. It is located on the Czech–Austrian border and has a parking capacity of about 10 spaces. Study site 4 (12 ovitraps) was Mikulov I (N 48°47,845´, E016°37,970´, 207 m a.s.l.), at the periphery of the town of Mikulov about 1.2 km north of study site 3. It serves a rest and refueling purpose and has a parking capacity of about 20 spaces.

From study site 4, we found 16 larvae of *Ae. albopictus*. Eight larvae in stage IV were euthanised for identification while the remaining eight were left to rear to adult stage (five females and three males) and also subsequently identified. Interestingly, all mosquito larvae developed from ovitraps set up within two subsequent intervals (20 August and 27 August 2012). Furthermore, one larva of *Ae. albopictus* developed from an ovitrap situated at the study site 3, on 10 September 2012, while no deposited eggs were detected in the study sites 1 and 2.

Conclusion

South Moravia is owing to its mild climate the most favourable habitat for breeding of mosquitoes within the Czech Republic [8]. Massive broods of mosquitoes (predominantly *Aedes* spp.) periodically occur here along the rivers Dyje and Morava. This area has been known for a long time as a natural focus of several mosquito-borne viruses: mainly Ťahyňa virus, the etiologic agent of Valtice fever, and since 1997 also

West Nile virus lineage 3 – Rabensburg [9,10]. Many mosquito species occurring in the Czech Republic were only recorded in this region, e.g. *Anopheles atroparvus, An. hyrcanus, An. labranchiae, Aedes nigrinus, Uranotaenia unguiculata, Culex martinii* [11,12]. We should take this region into consideration when searching for a suitable habitat for possible introduction and subsequent establishment of invasive mosquito species in central Europe. Our findings suggest that *Ae. albopictus* may be able to complete its developmental cycle in this region, and in case of a mild winter might also survive in the stadium of eggs [13]. Our results also indicate that ovitraps are a suitable tool for monitoring invasive mosquitoes on parking lots alongside main roads where alternative egg depositing water is likely less available.

In conclusion, we provide the first evidence of import of *Ae. albopictus* in the Czech Republic. Interestingly, *Ae. albopictus* has not yet been reported from the neighbouring central-European countries Austria, Slovakia, Hungary or Poland.

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PRÁCE 25

Bocková E., **Rudolf I.**, Kočišová A., Betášová L.,Venclíková K., Mendel J., Hubálek Z. 2013. *Dirofilaria repens* Microfilariae in *Aedes vexans* Mosquitoes in Slovakia. *Parasitol. Res.* 112: 3465–3470.

Stručná charakteristika: mnohobuněční parazité *Dirofilaria immitis* a *D. repens* přenášejí závažné onemocnění psů. Dirofilárie jsou přenášeny komáry a i člověk se jako příležitostný hostitel může nakazit mikrofilariemi, které u člověka způsobují kožní či oční formu dirofilariózy (*D. repens*), u psů převážně formu kardio-pulmonární (*D. immitis*). Cílem práce bylo potvrdit přítomnost dirofilárií v komářích vektorech v oblasti s endemickým výskytem psí dirofilariózy.

Hlavní přínos práce: poprvé byly na Slovensku detegovány dirofilarie (*D. repens*) v komárech *Ae. vexans*. Práce tak doplňuje eko-epidemiologická data týkající se výskytu dirofilariózy na Slovensku.

Příspěvek autora k dané práci: autor se podílel na designu studie, molekulární analýze komárů na dirofilárie a na přípravě rukopisu.

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ORIGINAL PAPER

Dirofilaria repens microfilariae in *Aedes vexans* mosquitoes in Slovakia

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Abstract In this study, we screened field-caught mosquitoes for presence of *Dirofilaria* spp. by using a polymerase chain reaction (PCR) assay. Potential occurrence of *Dirofilaria repens* and *Dirofilaria immitis* microfilariae was examined in 3,600 mosquitoes of eight species (*Aedes vexans*, *Aedes cinereus*, *Aedes rossicus*, *Culex pipiens*, *Culiseta annulata*, *Ochlerotatus sticticus*, *Ochlerotatus cantans* and *Ochlerotatus caspius*) collected from five locations in two districts (Kosice and Trebisov) of Eastern Slovakia, endemic region of canine dirofilariasis. Collection of mosquitoes was performed between May and August 2012 in premises known to be inhabited by *Dirofilaria*-infected dogs. PCR assays were performed on 72 pools, each pool containing 50 mosquitoes of the same species, collected on the same location. Each pool was examined separately for the presence of *D. immitis* and *D. repens*, respectively. A positive finding of *D. repens* was recorded in one pool of *A. vexans* mosquitoes collected in Košické Olšany village. Minimum infection rate in *A. vexans* was 1:1,750, i.e. 0.57 per 1,000 mosquitoes. The identity of *D. repens* was confirmed by direct sequencing of PCR product which has shown 100 % homology with sequence attributed to *D. repens* (GenBank accession number AJ271614). This study represents the first molecular evidence of *D. repens* microfilariae in mosquitoes in Slovakia and highlights a need for better surveillance of zoonotic dirofilariasis in central Europe.

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Introduction

Two filarial species represent causative agents of dirofilariasis of dogs and foxes in Slovakia: *Dirofilaria repens* (Railliet and Henry 1911) which is localised in the hypodermis of the host and causes the so-called subcutaneous form of dirofilariasis with the presence of nodular lesions and eczematous dermatitis (Rocconi et al. 2012) and *Dirofilaria immitis* (Leidy 1856), the agent of the cardiopulmonary form of the disease. Both filarial species have zoonotic potential and, despite the fact that a human represents only an occasional (dead-end) host and that microfilariae are not able to complete their development in a human, the number of infections in humans is rising, and *D. repens* is the most important causative agent of human dirofilariasis in Europe (McCall et al. 2008; Ondriska et al. 2010).

Mosquitoes represent an essential part of the dirofilarial life cycle and means of dispersion. They function not only as vectors but also as essential secondary hosts in which microfilariae develop to L3 larvae. Along with humidity, temperature is one of the most important environmental factors that regulate larval development of *D. repens* in mosquitoes. Temperature dictates the time requirements for the development of microfilariae to infective larvae (Sassnau and Genchi 2013). Their development in mosquitoes can last 8–10 days at 28–30 °C, 11–12 days at 24 °C and 16–20 days at 22 °C (Cancrini et al. 1988). L3 microfilariae then migrate to the mosquito's proboscis, and from there, they are later inoculated to a new host during blood feeding. Transmission of dirofilariasis is dependent upon the presence of sufficient numbers of infected, microfilaraemic dogs, susceptible mosquitoes and a suitable climate to permit extrinsic incubation of parasite in the mosquito vector (Medlock et al. 2007; Genchi et al. 2009).

When assessing the role and importance of mosquitoes in the epidemiology of *Dirofilaria* spp., it is necessary to consider the bionomics as such and the vector's capacity.

Important attributes in the bionomics of mosquitoes in filarial transmission include the following: the vector's behaviour on the basis of which it searches for competent hosts, its ability to disperse from the place of reproduction, the vector's geographical distribution, the vector's activity time horizon, the number of generations per year and the vector's population size and seasonal occurrence. The interactions between animal/human, mosquito and nematode biology contribute to the clinical spectrum and geographical distribution of *Dirofilaria* (Genchi et al. 2009). A vector's capacity relates to the potential for pathogen transmission via the insect population and includes the flying range of the insect, the host and the environmental variable parameters, including vector's occurrence, vector's survival, intensity of bite and transmission, preferences and occurrence of the host (Saegerman 2008).

To the best of the author's knowledge, no studies have been carried out regarding mosquitoes as vectors of dirofilariasis in Slovakia. Entomological and molecular studies have been performed in this study to determine potential mosquito species involved in circulation of these zoonotic microfilariae in endemic region.

Materials and methods

Study area

The research was concentrated on four locations situated in the area of the Košická Basin (Panovce, Gynov, Beniakovce, Košické Olšany) and one location in Michalany (District of Trebisov), located in the Eastern Slovak Lowland (Fig. 1). The Košická Basin lies in the south-eastern part of Slovakia. In the west, it borders with the Slovak Karst and the Slovak Ore Mountains, and in the north, with the Sarisska Highlands and Ondavska Highlands; in the east, it is connected with the Slanske Mountains, and in the south, it forms the border with Hungary. The total area of the basin is 1,153 km^2; from the geomorphologic point of view, the territory is mostly of a plane type, and from the hydrological point of view, it contains the basins of Bodva, Hornad, Torysa and Ida Rivers. The basin has a warm and moderately dry climate. The average annual rainfall ranges between 600 and 850 mm; the air humidity is 60–70 %. Almost the entire area has an early onset of spring, summers are rather long (52–60 days) with high average daily temperatures (18–20 °C), and winters are short and mild with average daily temperatures between −3 and −6 °C (Slovak Hydrometeorological Institute 2011), with a low number of days with snow cover. The long-term average annual air temperature is 8.7 °C. The area of Eastern Slovak Lowland is situated near the borders with Ukraine and Hungary. In the north up to the north-east, it is surrounded by the Vihorlat Mountains; in the north, by the Beskidian Piedmont; in the north-west and west, by the Slanske Mountains; and in the south-west, by the Zemplín Mountains. The total area is 2,500 km^2 with the altitude of 94–200 m above sea level. The area has a fan-pattern network of rivers comprising the Bodrog, Ondava, Latorica, Laborec, Uh and Topľa Rivers. The region has a mild and dry climate. The average annual rainfall is 600–750 mm. In winter months, the temperatures range between −2 and −4 °C. Summers are long (52–70 days) with average temperatures of 17–20 °C. The long-term average annual air temperature is 9–10 °C.

Mosquito trapping

Mosquitoes were sampled using CO_2-baited CDC light traps which were exposed from 5–6 p.m. to 7–8 a.m. of the following day. Collections were done in each site every week during April to August. Mosquitoes were collected from traps each morning within 30 min of dawn. During transportation from the field, the collected individuals were kept on dry ice. After transport to the laboratory at the Department of

Fig. 1 Territory of Slovakia, with administrative districts and study sites (*1* Panovce, *2* Gynov, *3* Beniakovce, *4* Kosicke Olsany, *5* Michalany)

Parasitology, mosquitoes were knocked down by placing trap containers in a −18 °C freezer for 15–30 min and subsequently separated by species and sex. Mosquitoes were identified using available identification keys (Kramar 1958; Becker et al. 2010).

Set of biological material for the PCR analysis

For the PCR analysis, we used 3,600 adult female mosquitoes which were divided, based on the species diagnostics, into 72 pools, each pool containing 50 individuals of the same species, collected on the same location. Each pool was examined separately for the presence of *D. immitis* and *D. repens*.

Homogenization of mosquitoes

The collected mosquitoes were mechanically disrupted using a ceramic blender in 500 μL of phosphate-buffered saline under sterile conditions.

Genomic DNA isolation

The total genomic DNA was extracted from 100 μL of the mosquito homogenate with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

PCR procedure Primers were designed to amplify approximately 200-bp region of mitochondrial cytochromoxidase subunit I gene of *Dirofilaria* spp. parasites (Rishniw et al. 2006). PCR amplification was performed with two sets of primers: DI COI-F1 (5′-AGT GTA GAG GGT CAG CCT GAG TTA -3′) and DI COI-R1 (5′- ACA GGC ACT GAC AAT ACC AAT-3′) for detection of *D. immitis* and DR COI-F1 (5′- AGT GTT GAT GGT CAA CCT GAA TTA-3′) and DR COI-R1 (5′- GCC AAA ACA GGA ACA GAT AAA ACT-3′) for detection of *D. repens*. Primers used in our study are routinely employed in molecular diagnostics and genotyping of *D. immitis* and *D. repens* in clinical samples (dog blood) as well as in mosquito vectors. Each reaction tube contained 75 mmol/L Tris–HCl (pH 8.8), 20 mmol/L $(NH_4)_2SO_4$, 0.001 % Tween 20, 2.5 mmol/L $MgCl_2$, 200 mmol/L mixture of dNTPs, 2.5 U Taq purple DNA polymerase (Top-Bio, Czech Republic) and 25 pmol of respective primer pair. PCR reaction was performed in PTC-200 Gradient Thermal Cycler (MJ Research, USA) under the following conditions: initial denaturating step at 94 °C for 2 min, followed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s consisting of 32 cycles and final extension at 72 °C for 7 min. The PCR products were then separated on 1.5 % agarose gel, stained with GelRed (Biotium, USA) and visualised by UV light. DNA extraction, PCR handling as well as post-PCR procedures were done in separate rooms to avoid possible cross-contamination of the samples. Specific PCR product was further characterised by sequence analysis.

Sequence analysis of PCR product

The PCR product was purified by means of precipitation in PEG/Mg/NaAc (26 % polyethylene glycol, 6.5 mM $MgCl_2 \cdot 6H_2O$, 0.6 M $NaAc \cdot 3H_2O$). Direct sequencing of purified PCR product was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) according to the manufacturer's instructions and purified with EtOH/EDTA precipitation. The sequencing was performed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). PCR amplicon was multiple sequenced from both directions to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR, Inc., USA) and also checked manually. The FASTA format and BLAST programme (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches.

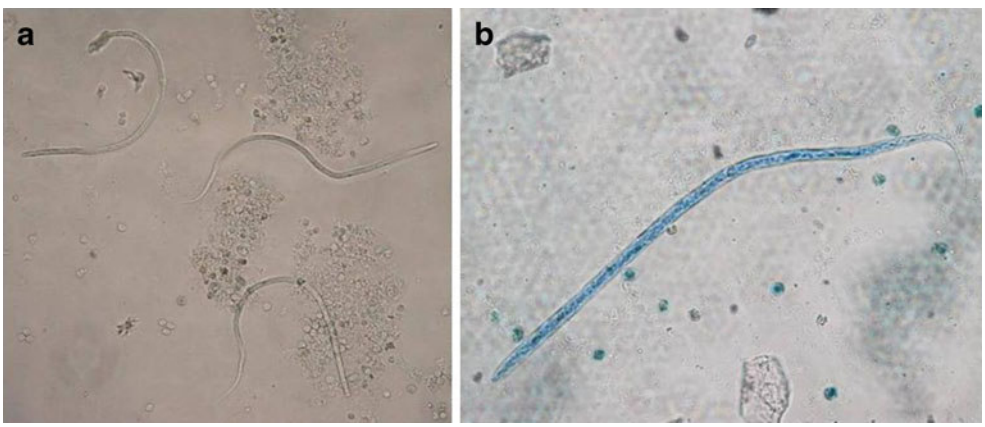

Fig. 2 *D. repens* microfilariae from a dog living in endemic area after concentration with the Knott's test (**a**) (×200) and after Diff-Quick staining (**b**) (×400)

Table 1 Locations, species and numbers of examined mosquitoes

Location	Mosquito species	Number of examined mosquitoes/ number of examined pools
Panovce	*A. cinereus*	600/12
	A. rossicus	50/1
	A. vexans	100/2
	C. pipiens	50/1
	O. cantans	250/5
	O. sticticus	150/3
Gynov	*A. vexans*	150/3
	C. annulata	50/1
	C. pipiens	50/1
	O. sticticus	50/1
Košické Olšany	*A. vexans*	600/12
	C. pipiens	150/3
Beniakovce	*A. vexans*	300/6
	C. pipiens	300/6
	O. caspius	100/2
Michalany	*A. vexans*	600/12
	C. pipiens	50/1
Total		3,600/72

Results

Molecular detection of microfilariae (Fig. 2) in vectors was carried out using 3,600 mosquitoes of eight species (*Aedes vexans*, *Aedes cinereus*, *Aedes rossicus*, *Culex pipiens*, *Culiseta annulata*, *Ochlerotatus sticticus*, *Ochlerotatus cantans* and *Ochlerotatus caspius*) (Table 1). The PCR analysis confirmed the presence of *D. repens* DNA in one pool (no. 10) of *A. vexans* mosquitoes (Fig. 3) collected on the location in Košické Olšany village (Fig. 1). The identity of positive specimen was confirmed by direct sequencing of PCR product which has shown 100 % homology with sequence attributed to *D. repens* (GenBank accession number AJ271614).

Discussion

Previous research on dirofilariasis in carnivores in Slovakia has provided important information on causative agents of the disease and prevalence in dogs in different areas and districts (Miterpakova et al. 2008, 2009, 2010, 2012; Iglodyova et al. 2012a, b). Dirofilariasis has become a problem which occurs each year, and the number of canine cases is constantly growing. In spite of the fact that it is a zoonotic disease, in Slovakia, there is still lack of information on current and competent vectors. Available literature sources report more than 70 mosquito species of the Culicidae family (genera: *Culex*, *Aedes*, *Ochlerotatus*, *Anopheles*, *Armigeres*, *Coquilettidia* and *Mansonia*) that are able to transmit dirofilariae (Cancrini et al. 1995; Pampiglione and Rivasi 2000; Vezzani and Carbajo 2006; Vezzani et al. 2011). Many of these mosquito species occur in Slovakia as well (Orszagh et al. 2001; Jalili et al. 2000), and some of them are common. For the initial stage of the research, we have chosen the

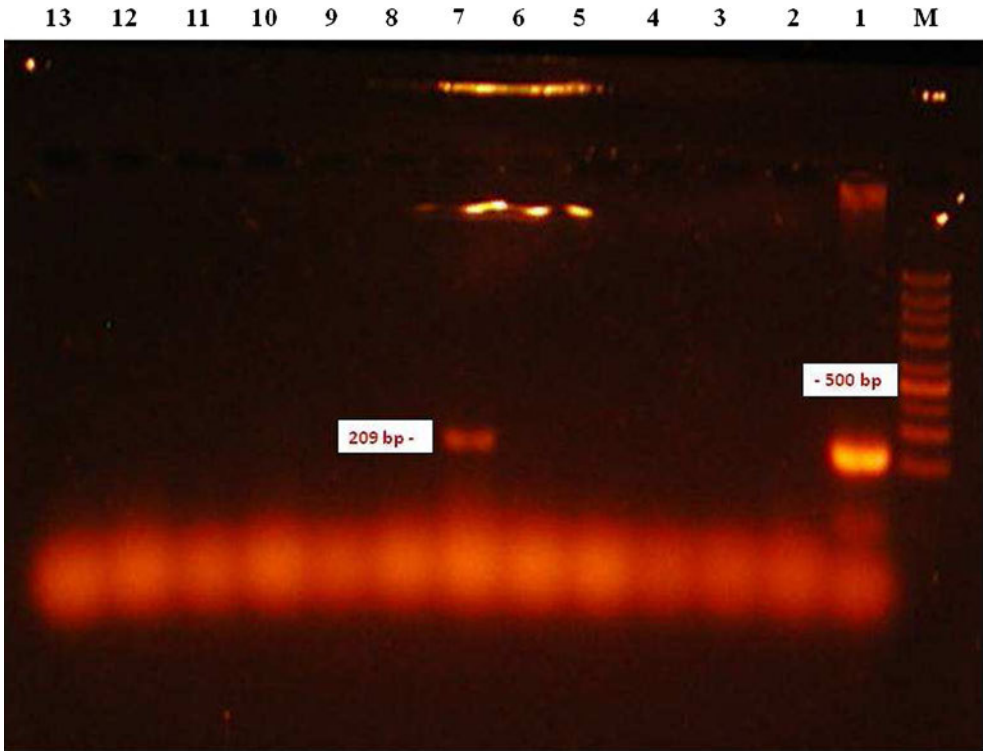

Fig. 3 Gel electrophoresis of the amplified product of the *COI* gene of *D. repens*. *Lane M* 1,500–200 bp marker; *1* positive control; *2–6*, *8–13* negative samples; *7* positive sample

mosquitoes collected at five locations in Eastern Slovakia. Selection of individual locations was based on their geographical position (Košická Basin and Eastern Slovak Lowland), local and climatic conditions, habitats suitable for life of vectors and the information on infection-positive findings in definite hosts. The Kosice town district and the Kosice vicinity district lie in the Košická Basin and have very favourable climatic conditions for vector development. By our existing research on this territory, we have established the presence of 16 mosquito species (Bockova and Kocisova 2011). Results of the researches on canine dirofilariasis within the monitored territory indicate that the prevalence increased from 6.4 % in years 2007–2010 to 11.6 % in 2011 (Iglodyova et al. 2012b). At Košické Olšany, where we confirmed the presence of microfilariae in *A. vexans*, no research focused on detection of microfilaraemia in dogs has been carried until now. Michalany village is situated in one of the endemic areas of dirofilariasis in the district of Trebisov that belongs to the warmest areas in Slovakia. Prevalence of canine dirofilariasis in this district reaches 54.4 % (Iglodyova et al. 2012a).

Batches of mosquitoes for PCR analysis were chosen on the basis of theoretical information which suggests that *A. vexans*, *O. caspius* and *C. pipiens* (Latrofa et al. 2012; Yildirim et al. 2011) are the potential vectors of dirofilariae. Mosquitoes *A. vexans*, *C. pipiens* and *Aedes (Stegomyia) albopictus* are regarded to be the most important transmitting agents of *D. repens* and *D. immitis* in Europe. By the PCR analysis and subsequent sequencing, we have proved the presence of DNA of *D. repens* microfilariae in *A. vexans* mosquitoes. In similar trials carried out in north-east Italy (Latrofa et al. 2012), the authors report positive findings of *D. repens* microfilariae in *C. pipiens* and *D. immitis* in *A. vexans*, *O. caspius* and *C. pipiens*. Similar results were achieved in Turkey (Yildirim et al. 2011), where the authors state that the main vector of *D. immitis* is *A. vexans* and *C. pipiens*.

It is interesting to point out that in Slovakia, *D. immitis* in dogs has so far occurred only in co-infection with *D. repens* (seven cases, 2.1 % prevalence) (Miterpakova et al. 2008), while in Turkey or in Italy, it occurs alone in a prevalence between 2 and 30 % (Yildirim et al. 2011; Latrofa et al. 2012).

A. vexans and *C. pipiens* mosquitoes are among the most common mosquito species in Slovakia. *A. vexans* has several attributes of an ideal vector, especially its wide geographical distribution, short development cycle (in suitable conditions lasting 1–3 weeks), polycyclicity and ability to form multiple populations, especially after floods, ability of females to fly to distances more than 15 km away from the reproduction site and wide host preference.

C. pipiens is originally an ornitophilic mosquito (Kramar 1958; Becker et al. 2010) but has now adopted endophagic and anthropophagic behaviour in central and north Europe where it now also searches for human blood outdoors, as it happens in southern parts of the continent. This pattern also overlaps with the spread of canine *D. immitis* and *D. repens* infection in central and north-eastern countries (e.g. south Switzerland, Germany, Czechland, Hungary, Serbia and Slovakia) (Tasic et al. 2008; Genchi et al. 2009; Pantchev et al. 2009). In some parts of Eastern Slovakia (Kosice and Kosice vicinity), we very often encounter feeding on humans and domestic animals. Its role as a vector is primarily connected with the transmission of avian plasmodia, Sindbis alphavirus (Berezin et al. 1972) and West Nile *Flavivirus* (Anderson and Main 2006; Hubálek 2008).

Both these mosquito species are most abundant during the hottest months of the year, which increases the probability of spreading temperature-dependent pathogens, for example, dirofilariae. Our conclusion from this study corresponds to the finding by Iglodyova (personal information), which states that the largest number of microfilaraemic dogs occurs in the period between spring and summer and between summer and autumn, i.e. when the first generation or winter-surviving females emerge and are at maximum abundance.

Of the confirmed vectors of *Dirofilaria* spp. occurring in Slovakia, the most likely potential vectors include *Anopheles maculipennis* s.l., which occurs frequently in the Slovak lowlands, as well as *O. caspius* and *A. cinereus*. They could also include *Anopheles hyrcanus*, *Ochlerotatus geniculatus* and *Coquilettidia richiardii*, which, however, are only sporadically collected in the monitored areas.

Eastern Slovakia has often proved to have exceptionally favourable conditions for disease transmission via vectors. Until the 1950s, it was an endemic area for malaria; in the last 10 years, it has been shown to be a canine babesiosis focus, and dirofilariasis is now spreading in this area as well.

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PRÁCE 26

Bakonyi T., Kolodziejek J., **Rudolf I.**, Bercic R.L., Nowotny N., Hubálek Z. 2013. Partial genetic characterization of Sedlec virus (Orthobunyavirus, *Bunyaviridae*). *Infect. Genet. Evol*. 19: 244–249.

Stručná charakteristika: virus Sedlec byl izolován v roce 1984 z krve rákosníka obecného (*Acrocephalus scirpaceus*) na jižní Moravě. Na základě prvotních fyzikálně-chemických studií byl zařazen mezi bunyaviry. Cílem práce bylo sekvenovat důležité úseky genomu viru a na základě nich zařadit virus do systému.

Hlavní přínos práce: byla provedena fylogenetická analýza viru Sedlec (S a L segment), která tento virus začlenila do rodu *Orthobunyavirus*, patrně nové séroskupiny. Izolace nových virů a jejich další výzkum z hlediska patogenity je nezbytný pro stanovení možných zdravotních rizik pro populaci. Např. nedávno popsaný virus Schmallenberg, který je geneticky velmi podobný viru Sedlec, způsobuje závažnou veterinární nákazu především u ovcí a koz v západní Evropě.

Příspěvek autora k dané práci: autor se podílel na hodnocení fylogenetického postavení nového viru v systému bunyavirů a také na přípravě rukopisu.

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Partial genetic characterization of Sedlec virus (*Orthobunyavirus*, *Bunyaviridae*)

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ABSTRACT

Sedlec virus (SEDV) was isolated from the blood of a reed warbler (*Acrocephalus scirpaceus*) in July 1984 in South Moravia, Czech Republic. In this study first genetic data of SEDV are presented which allow an estimate on its phylogenetic and taxonomic positioning within the genus *Orthobunyavirus*. The phylogenetic analysis of a 369 nt long stretch within the S segment (nucleocapsid protein gene and non-structural S protein gene) indicates genetic relatedness of SEDV to Leanyer virus and Simbu group viruses, while the phylogenetic tree based on 1796 nt long sequences of the L segment (RNA-dependent RNA polymerase gene) demonstrates genetic relationship of SEDV to two yet unclassified orthobunyaviruses: I612045 virus (isolated in India in 1961) and Oyo virus (isolated in Nigeria in 1964). Considering the genetic distances and the phylogenetic analyses, SEDV might represent a novel serogroup of the *Orthobunyavirus* genus.

1. Introduction

The *Orthobunyavirus* genus of the *Bunyaviridae* family currently comprises 48 distinct virus species (King et al., 2012); however, several further virus strains are candidate members of the genus. Altogether more than 170 named viruses are considered belonging to this genus. Based on antigenic relatedness, orthobunyaviruses are divided into 18 serogroups. These viruses are usually transmitted by mosquito or ceratopogonid vectors and are distributed worldwide, although most of them were isolated in tropical and subtropical areas (Elliott, 1997). In Europe seven orthobunyaviruses were detected so far (Lundström, 1999; Hubálek, 2008). Tahyna virus (TAHV), Inkoo virus (INKV) and Snowshoe Hare virus (SSHV) are members of the California group. TAHV is a human pathogen, detected in central- and southern Europe, while INKV occurs in northern Europe and no disease was associated with it so far. SSHV was isolated in eastern Europe (Russia). Batai (syn. Calovo) virus (belonging to the Bunyamwera group) was detected in central, southern and northern European countries, but its medical and veterinary relevance remains unclear. Lednice virus of the Turlock serogroup was detected in central Europe, without known human infections. Recently a novel orthobunyavirus, named Schmallenberg virus, emerged in western Europe and caused significant disease outbreaks in domesticated ruminants, predominantly in cattle and sheep (Hoffmann et al., 2012; Beer et al., 2013). The virus has shown the closest genetic relationship to Simbu group orthobunyaviruses (Hoffmann et al., 2012; Goller et al., 2012).

During an investigation of wild birds for the presence of viruses, an agent was isolated from a pool of blood samples from four young (hatching-year), asymptomatic reed warblers (*Acrocephalus scirpaceus*), collected on 30 July 1984 in the reed-bed littoral of Nesyt fishpond in South Moravia, Czech Republic (48°468′ N, 16°43′ E). The new virus was named Sedlec (to be read 'Sedlets', but not 'Sedlek') virus, SEDV (Hubálek et al., 1989, 1990), and it was included in the International Catalogue of Arboviruses (Karabatsos, 1985). The morphological and physico-chemical characteristics of the virus indicated that it is a bunyavirus, although complement-fixing antigen did not react with immune mouse sera or ascitic fluids prepared against a wide variety of bunyaviruses. Pathogenicity studies revealed a moderate virulence in mice (it killed suckling and adult mice when given intracerebrally, but not when inoculated intraperitoneally). A subsequent serological survey at the same geographic site in 1988 detected neutralizing antibodies against SEDV in 23% of wetland passerines, indicating local circulation of the virus in the area (Hubálek et al., 1990).

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The aim of this study was to genetically characterize SEDV and reveal its relationship to the emerging Schmallenberg virus and to other orthobunyaviruses.

2. Materials and methods

The SEDV prototype strain AV 172 has been propagated intracerebrally in suckling mice. Centrifuged homogenate of the 4th passage of SEDV in suckling mouse brains was used in this study. Viral RNA was extracted with the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic regions of SEDV were amplified in a continuous RT-PCR system using the QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Degenerated oligonucleotide primers were designed on consensus sequences of relatively conserved genome regions, based on multiple alignments of available orthobunyavirus S, M and L genome segments. Primers were designed with the help of the Primer Designer 4 program (Scientific and Educational Software, version 4.10), synthesized by Life Technologies, Ltd. (Paisley, Scotland, UK), and used in 0.8 μM concentrations in the RT-PCRs. The sequences of the primersgenerating specific products are shown in Table 1. The thermal profile of the RT-PCRs was 50 °C for 30 min, 95 °C for 15 min, 40 × [94 °C for 40 s, 50 °C for 50 s, and 72 °C for 1 min], and 72 °C for 7 min. If amplification products of the previously calculated sizes were observed after agarose gel electrophoresis, they were excised from the gel, DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and fluorescence-based direct sequencing was performed in both directions (described in Bakonyi et al., 2004). The nucleotide sequences were identified by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), overlapping sequences were compiled, and deposited in GenBank database under accession numbers KC978768 and KC978769. For phylogenetic analyses, SEDV sequences were aligned with all corresponding in GenBank available orthobunyavirus sequences, which included representatives of all major serogroups (Table 2). Multiple sequence alignments were performed using BioEdit Sequence Alignment Editor (version 7.0.9.0, http://www.mbio.ncsu.edu/bioedit/bioedit.html) and verified by the ClustalX program (version 2.0.10, Larkin et al., 2007). Phylogenetic analyses were performed on the alignmentsusing a Maximum Likelihood method based on the Tamura–Nei model in MEGA5 (Tamura et al., 2011), and were repeated by both Neighbor-Joining method in MEGA5 (Tamura et al., 2011) and modified Neighbor-Joining method in ClustalX (Thompson et al., 1997). The stability of the trees was

Table 1
Oligonucleotide primers used for the amplification of genomic regions of Sedlec virus.

Name	Target segment	Primer sequence (5′ to 3′)	Product size
BunyS 137f	S	ARAAGAAGGCCAAGGATNGT	446 bp
BunyS 582r		CATCCAYTSYTCAGCAGTTW	
BunyL 3243f	L	TGTCHAAATGGAGTGCYCAR	637 bp
BunyL 3879r		GCTAAATCTTCATAWGGCCC	
BunyL 2218f		TATWTGGTTCCCWGGWARDG	1105 bp
SedL 3322r		CTCATCAGGTAGCAATAGTG	
SedL 3767f		CTTACTGCTGTTGGCGATTG	293 bp
BunyL 4059r		AMRTTCCCWGMTTCYAADCC	

f: forward (genomic), r: reverse (complementary) primer.

Table 2
Bunyavirus nucleic acid sequences included in phylogenetic analyses.

Virus name	GenBank accession number	
	S segment	L segment
Aino	M22011	HE795087
Akabane	AB000852	AB190458
Anhembi	JN572064	JN572062
Anopheles A	FJ660415	na
Anopheles B	FJ660417	na
Apeu	DQ188952	na
Batai	X73464	na
Batama	FJ660420	na
Bruconha	DQ188953	na
Bunyamwera	NC_001927	X14383
Buttonwillow	AF362398	na
Bwamba	EU564827	na
Cache Valley	GU018037	na
Cachoeira Porteira	JN968592	JN968590
Caraparu	DQ188976	EF122411
Chatanga	EU486163	HQ734818
Douglas	AF362393	HE795090
Facey's Paddock	AF362400	na
Guaroa	X73466	JN801039
Hantaan	NC_005218	NC_005222
Iaco	JN572067	JN572065
I612045	HM621780	HM627179
Ingwavuma	AM709782	na
Inkoo	U47137	EU789573
Itaqui	DQ188951	na
Jamestown Canyon	EF681848	HM007358
Jatobal	AF312382	na
Kaikalur	AF362394	na
Kairi	X73467	na
La Crosse	NC_004110	GU206122
Leanyer	HM627177	HM627178
Lumbo	X73468	na
M'Poko	AM711133	na
Macaua	JN572070	JN572068
Madrid	DQ188957	na
Main Drain	X73469	na
Marituba	DQ188949	na
Melao	U12802	na
Mermet	AF362399	na
Nola	AM711134	na
Northway	X37470	na
Nyando	AM709781	na
Oropouche	NC_005777	AF484424
Ossa	DQ188954	na
Oya	AB075611	na
Oyo	HM639778	HM639780
Peaton	AF362401	HE795093
Pongola	EU564828	na
Sabo	AF362396	HE795096
Sango	AF362402	HE795099
Sathuperi	AF362403	HE795102
Schmallenberg	HE649914	HE649912
Sedlec	KC978768	KC978769
Serra do Navio	U47140	na
Shamonda	AF362404	HE795105
Shuni	AF362405	na
Simbu	AF362397	HE795108
Snowshoe Hare	EU294510	EU203678
Sororoca	JN572073	JN572071
Tacaimua	FJ660416	na
Tahyna	U47142	HM036210
Taiassui	JN572076	JN572074
Tensaw	FJ943507	FJ943510
Tete	FJ660419	na
Tinaroo	AB000819	na
Trivittatus	U12803	na
Tucunduba	JN572079	JN572077
Vinces	DQ188958	na
Wyeomyia	FJ235921	JN801038
Yaba-7	AF362392	na
Zungarococha	na	JN157805

na: Not available.

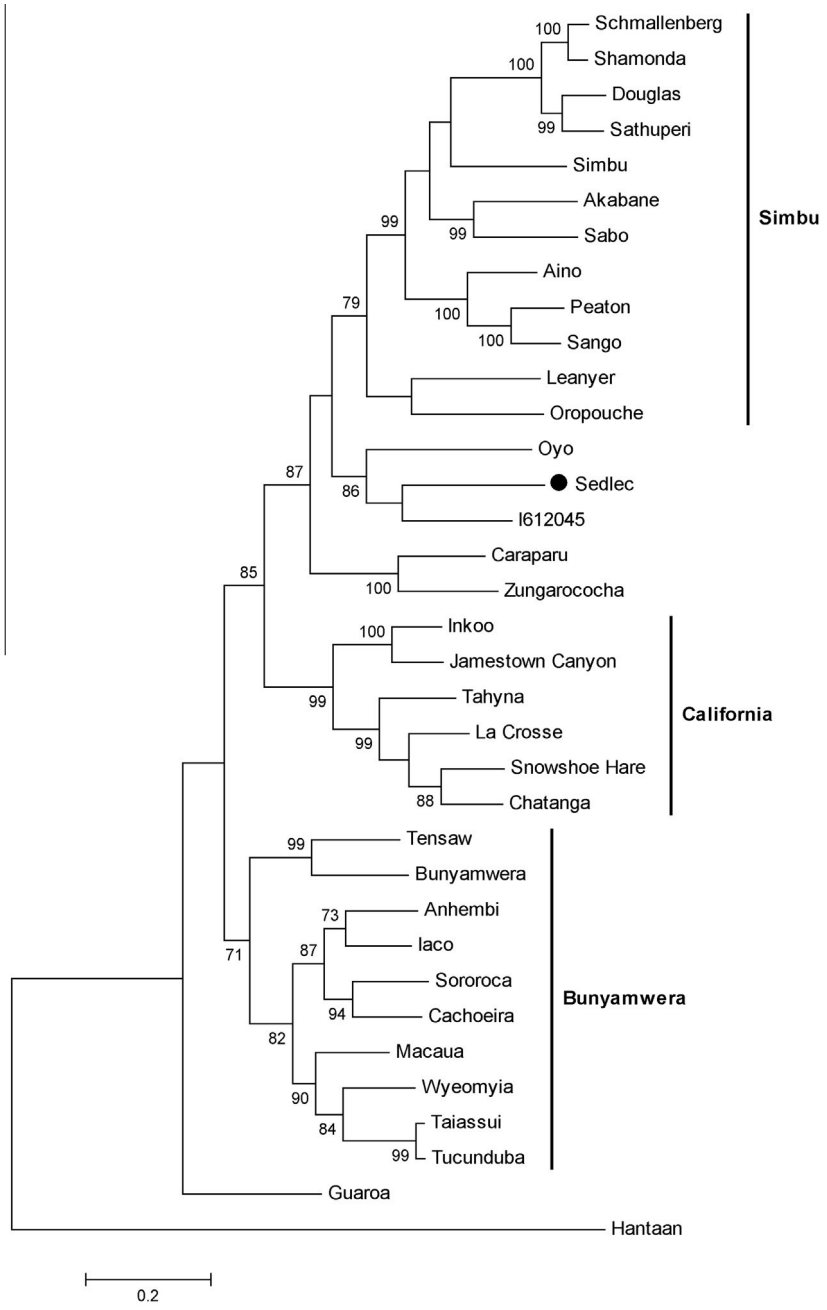

Fig. 1. Phylogram demonstrating the genetic relationships of orthobunyavirus nucleotide sequences in a partial S segment (nucleoprotein gene and non-structural protein NS-S gene) region. GenBank accession numbers are provided in Table 2. Hantaan virus was used as outgroup to root the tree. Sedlec virus sequence described in this paper is marked with a black dot. Bootstrap values >70% are displayed at nodes. The main serogroups are indicated with vertical bars on the right. The horizontal bar on the left represents the genetic distance.

tested by bootstrap resampling analysis of 1000 replicates. The probable relationship between SEDV and other known orthobunyaviruses was displayed in phylograms. The corresponding sequences of the small and large segments of Hantaan virus (genus *Hantavirus*, GenBank accession numbers NC_005218 and NC_005222) were used as outgroups to root the trees.

3. Results

SEDV specific genome sequences were amplified of the S and L segments (Figs. 1 and 2), while primers targeting the M segment failed to produce virus-specific amplification products. In the S segment, a 369 nucleotide long sequence was determined which exhibited the highest similarities (up to 79%) to Simbu group orthobunyaviruses and Leanyer virus. Degenerated consensus primers targeting the L segment (BunyL 3243f-3879r) amplified a SEDV specific product. Its nucleotide sequence was determined, and sequence-specific primers were designed (SedL 3322r and SedL 3767f), which were combined with degenerated consensus primers (BunyL 2218f and BunyL 4059r, respectively). Further specific amplification products were obtained, sequenced, and the overlapping sequences were aligned and compiled to a 1796 nucleotide long consensus sequence. In the L segment, BLAST search indicated the highest similarities of SEDV (up to 70%) to I612045 virus, Leanyer virus, Oyo virus and Simbu group viruses, respectively.

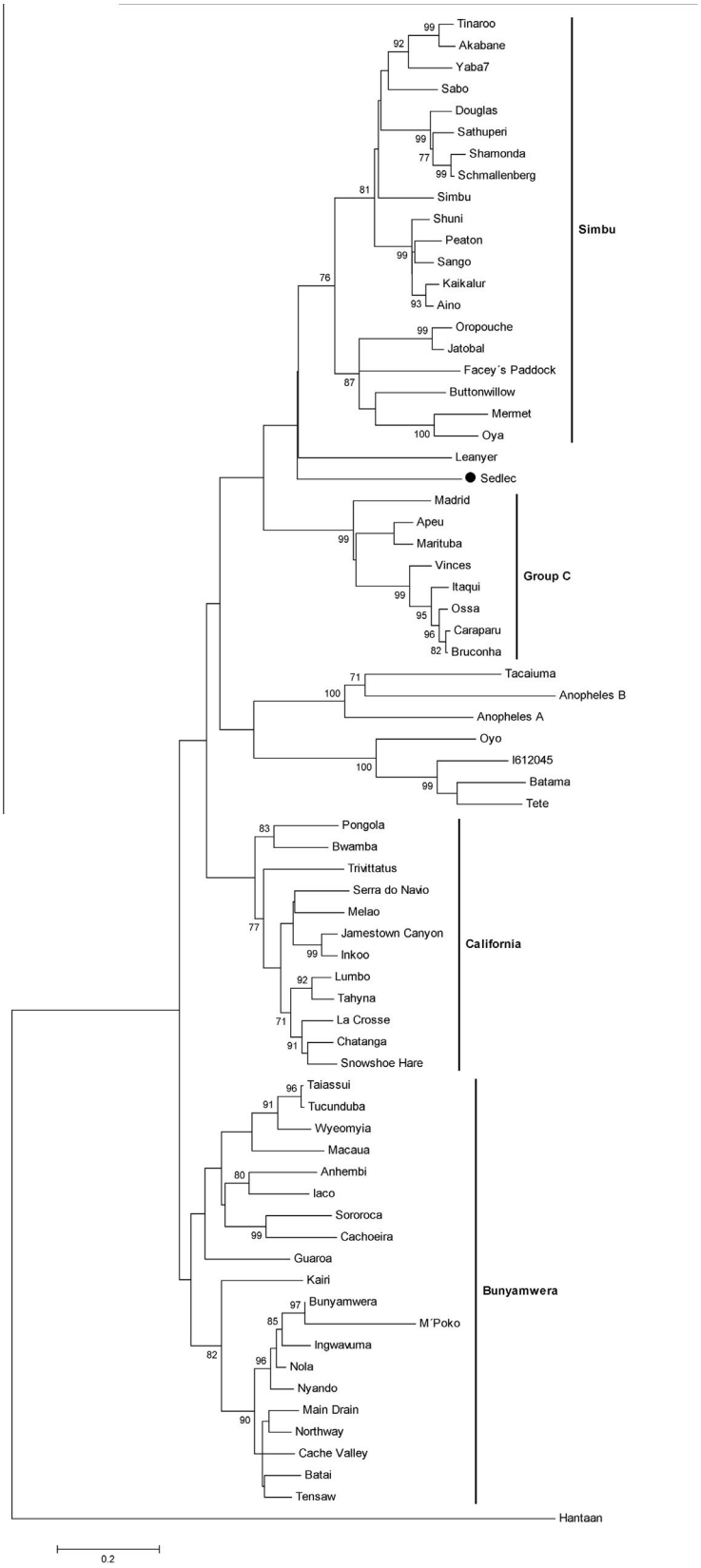

Fig. 2. Phylogram demonstrating the genetic relationships of orthobunyavirus nucleotide sequences in a partial L segment (RNA-dependent RNA polymerase gene) region. GenBank accession numbers are provided in Table 2. Hantaan virus was used as outgroup to root the tree. Sedlec virus sequence described in this paper is marked with a black dot. Bootstrap values >70% are displayed at nodes. The main serogroups are indicated with vertical bars on the right. The horizontal bar on the left represents the genetic distance.

The putative partial amino acid sequence of the S segment (consisting of 123 amino acids) showed the highest (70%) similarity to the Simbu virus partial nucleocapsid protein, and in alternative reading frame (62 aa) to the La Crosse virus non-structural protein NS-S (58% similarity). The putative partial amino acid sequence of the L segment (598 aa) showed the highest identity (68%) to the Leanyer virus RNA-dependent RNA polymerase.

The genetic relationships between SEDV and other orthobunyaviruses were inferred with phylogenetic analyses involving SEDV and 68 other orthobunyaviruses (S segment partial sequences) and 33 other orthobunyaviruses (L segment partial sequences), respectively. The phylogram based on the S segment (nucleoprotein and non-structural protein NS-S genes, partial sequences, Fig. 1) indicates that SEDV represents a separate branch, with Leanyer virus and Simbu group viruses being the closest relatives. In the L segment (RdRp gene, partial sequences, Fig. 2), SEDV is forming together with I612045 and Oyo viruses a distinct subclade, which shares a putative common ancestor with Simbu group viruses and Leanyer virus.

4. Discussion

The unexpected emergence of Schmallenberg virus in Europe in 2011 directed our attention to the genetic characterization of SEDV, which was isolated in Europe 27 years before, however its genetic relationship to other bunyaviruses including Schmallenberg virus remained unclear. The partial genome sequences of SEDV, however, unambiguously demonstrate that this virus is distinct from Schmallenberg virus and from all so far genetically characterized orthobunyaviruses. Phylogenetic studies indicated a genetic relatedness of SEDV to Simbu group viruses: according to the S segment phylogeny SEDV occupies an own distinct branch, close to Leanyer virus and Simbu group viruses. Leanyer virus was isolated from *Anopheles meraukensis* mosquitoes collected in Leanyer, Northern Territory of Australia in 1974 (strain AusN16701, Doherty et al., 1977) and from *Culicoides marksi*, also in the Northern Territory of Australia (Standfast et al., 1984). It has never been associated with any illness; however, neutralizing antibodies to Leanyer virus were detected in cattle in the Northern Territory and in Queensland, Australia (Doherty et al., 1977). Detailed data on the genetic and phylogenic characteristics of Leanyer virus were provided recently (Savji et al., 2011). This virus did not show serological cross-reactivity with other orthobunyaviruses in haemagglutination-inhibition tests and in virus-neutralization tests. The genetic characterization also indicated significant differences between Leanyer virus and Simbu group viruses. Therefore the authors of the study suggested that Leanyer virus is a distinct species within the orthobunyaviruses, closely related to Simbu group viruses, but possibly representing a new antigenic complex (Savji et al., 2011). The genetic distance between SEDV and its closest relatives is longer than the distance between Leanyer virus and Simbu group viruses.

In the L segment phylogeny, SEDV formed a group together with the unclassified orthobunyaviruses I612045 virus and Oyo virus. The GenBank records of I612045 virus (accession numbers HM627179–HM627181) show that this virus was identified in India in 1961, while Oyo virus (accession numbers HM639778–HM639780) was collected in Nigeria in 1964. Unfortunately, further details of these viruses were not found in literature. Interestingly, in the L segment phylogeny I612045 and Oyo viruses cluster close to Simbu group viruses and Leanyer virus, while in the S segment tree these viruses cluster together with Tete groupviruses (Tete and Batama viruses).

Thus, based on the phylogenetic analyses, SEDV virus could not be classified into one of the known orthobunyavirus serogroups.

SEDV antigens were tested for cross reactivity in complement fixation tests with immune sera or ascitic fluids of several bunyaviruses, including Simbu virus and Tete virus (Hubálek et al., 1989). Similar to Leanyer virus, serological cross reactions were not detected between SEDV and any other orthobunyavirus. The serological data on I612045 and Oyo viruses are not available in the literature yet. Further studies on the antigenic relationships of these four viruses may support the hypothesis on another, possible novel serogroup of orthobunyaviruses. These four related viruses were isolated in four different continents between 1961 and 1984. Although medical or veterinary importance has not been attributed to them so far, their geographic distribution indicates that presumably orthobunyaviruses related to Simbu group viruses may circulate covertly in vertebrate hosts and arthropod vectors in Europe and in other continents. It is possible that the vector of SEDV might be a ceratopogonid, in analogy with viruses of the related Simbu serogroup. Due to their segmented genomes, orthobunyaviruses may be involved in genetic reassortment events (Yanase et al., 2010, 2012; Elliott and Blakqori, 2011). If an emerging, pathogenic virus (e.g., Schmallenberg virus) and a locally circulating, related orphan orthobunyavirus simultaneously infect cells of a host or a vector, significant viral characters (i.e., host spectrum, pathogenicity, antigenic properties) may change in reassortant progeny viruses, with possible considerable veterinary or medical consequences. Due to globalisation, the intensity of international transportation and trade of goods, as well as travelling between countries and continents dramatically increased within the last decades. Therefore the risk for the introduction of exotic viruses became also higher. In several European countries the recent emergence of exotic arboviruses (e.g., Bluetongue virus, West Nile virus, Usutu virus, Chikungunya virus, Dengue virus, and Schmallenberg virus) urged the public health and veterinary authorities to establish vector- and arbovirus-surveillance and monitoring systems. Such surveys may reveal further details on the distribution and prevalence of SEDV and other known or yet unknown bunyaviruses in Europe. Genetic and antigenic characterizations of these viruses may contribute to our knowledge on the diversity, ecology and risk assessment of emerging bunyaviruses.

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PRÁCE 27

Hubálek Z., Ludvíková E., Jahn P., Treml F., **Rudolf I.**, Svobodová P., Šikutová S., Betášová L., Bíreš J., Mojžíš M., Tinák M., Boldižár M., Citsoňová G., Staššíková Z. 2013. West Nile virus equine serosurvey in the Czech and Slovak Republics. *Vector-borne and Zoonotic Dis*. 13: 733–738.

Stručná charakteristika: WNV způsobuje velmi závažné klinické onemocnění u koní. Cílem práce bylo provést séropřehled koní na protilátky k WNV u českých a slovenských koní s cílem zjistit možnou cirkulaci viru v regionu včetně zhodnocení případných zdravotních rizik i pro místní obyvatele.

Hlavní přínos práce: byly poprvé nalezeny protilátky k WNV u koní na Slovensku, kteří nevycestovali. Práce naznačuje aktivní cirkulaci WNV na jižním Slovensku, kam se WNV patrně rozšířil ze sousedního Maďarska, kde v roce 2008 vypukla rozsáhlá epidemie západonilské horečky.

Příspěvek autora k dané práci: autor se podílel na sběru sér koní v terénu, inaktivaci sér, hodnocení neutralizačního testu a přípravě rukopisu.

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West Nile Virus Equine Serosurvey in the Czech and Slovak Republics

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Abstract

A serological survey for West Nile virus (WNV) infection involved 395 horses from 43 administrative districts of the Czech Republic (163 animals) and 29 districts of Slovakia (232 animals), sampled between 2008 and 2011. Using a plaque-reduction neutralization microtest, antibodies to WNV were not detected in any horse from the Czech Republic, whereas 19 nonvaccinated horses from Slovakia had specific antibodies to WNV (no cross-reactions were observed with tick-borne encephalitis and Usutu flaviviruses in those animals). The seropositivity rate of nonvaccinated horses in Slovakia was 8.3% (95% confidence interval [CI] 4.7–11.9%), and autochthonous local infection with WNV occurred at least in 11, *i.e.*, 4.8% (95% CI 2.0–7.6%) of the animals. All seropositive horses lived in six lowland districts of southern Slovakia; overall, 15.1% (95% CI 8.8–21.4%) of 126 nonvaccinated horses were seropositive in those districts, situated relatively closely to the border with Hungary, *i.e.*, the country where WNV disease cases have been reported in birds, horses and humans since 2003.

Key Words: *Flavivirus*—Mosquito-borne virus—West Nile virus—Neutralizing antibodies—Horses.

Introduction

West Nile virus (WNV, a *Flavivirus* of the Japanese encephalitis antigenic group, family *Flaviviridae*) circulates in natural foci between birds and bird-feeding mosquitoes largely of the genus *Culex* (e.g., *Cx. pipiens* and *Cx. modestus* in Europe). Humans and horses are regarded as "dead-end" hosts of WNV because of the low and short viremia produced. However, equids are very susceptible to WNV infection, which can be responsible for encephalomyelitis in a fraction of infected animals, and lethality in horses can occur (Cantile et al. 2000, Salazar et al. 2004, Venter et al. 2009). Horses also seroconvert rapidly upon WNV infection, and WNV antibodies can be easily detected in serological tests, facilitating the assessment of the epidemiological situation (surveillance) of WNV activity in particular areas.

WNV has recently re-emerged and spread in Europe, including central Europe (Hubálek and Halouzka 1999, Autorino et al. 2002, Durand et al. 2002, Zeller and Schuffenecker 2004, Angelini et al. 2010, Monaco et al. 2010, Papa et al. 2010, Sirbu et al. 2011). For instance, in the Czech Republic (Czechland, for short), West Nile fever was diagnosed in five persons in south Moravia in 1997, and the virus was also isolated from mosquitoes in both Czechland (Hubálek et al. 1999) and Slovakia (Labuda et al. 1974). However, serological surveys in humans and other vertebrates (Hubálek et al. 1999) have not yet detected a remarkable WNV activity in these countries. On the other hand, significant WNV activity involving cases in birds and horses has been demonstrated in adjacent southern countries—Hungary and Austria—in the last years (Bakonyi et al. 2006, Kutasi et al. 2011, Wodak et al. 2011). The aim of our study was to investigate indirectly for the first time whether WNV circulates among horses in Czechland or Slovakia, using a serosurvey. Signs of WNV circulation in horses (cases, seroconversion) might be an early indicator before the identification of human cases (Chevalier et al. 2011).

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Materials and Methods

Serum samples

Equine blood samples were randomly collected from 43 out of 77 administrative districts in Czechland (163 horses) and from 29 out of 72 administrative districts in Slovakia (232 horses) between the years 2008 and 2011 (Figs. 1 and 2). A census of horse populations, conducted by the Ministries of Agriculture of the Czech and Slovak Republics, estimated approximately 80,000 and 15,000 individuals, respectively. In general, criteria for selection of animals were lowland regions with presence of abundant mosquito populations (and a potential risk of mosquito-borne infections). None of the sampled horses had moved from the stable locality during the last summer/autumn season at least. The age of examined animals was between 1 and 30 years. The median age of Czech animals was 7 (range, 1–23) years, and that of Slovak horses 9 (range, 1–30) years. Among the Czech animals, there were 75 males (stallions, geldings) and 84 females (mares); the figures for Slovak horses were 96 and 110, respectively. The blood sera were stored at −20°C.

Viruses

Three flaviviruses were used for the neutralization test: (1) WNV strain Eg-101 - Egyptian topotype of WNV, lineage 1, passaged 15 times in suckling mouse brain (SMB), homogenized in phosphate-buffered saline (PBS; pH 7.2) with 0.4% bovine serum albumin fraction V (BSA) and antibiotics, and cleared by centrifugation at $1500 \times g$ for 15 min (+4°C). (2) Tick-borne encephalitis virus (TBEV) strain Hypr, passaged 10 times in mouse brain, then 55 times in HeLa cells, and finally once in SMB; infectious SMB was homogenized in PBS with 0.4% BSA and antibiotics, and centrifuged. (3) Usutu virus (USUV) strain Vienna 939 passaged three times in Vero cells and once in SMB, homogenized in PBS with 0.4% of BSA and antibiotics, and cleared by centrifugation.

Plaque-reduction neutralization microtest

The method described by Madrid and Porterfield (1974) was adapted for use in 96-well (flat-bottomed) microplates for cell cultures (Hubálek et al. 1979, Hubálek et al. 2008). Briefly, 30 μL of thermally inactivated (at 56°C for 30 min) sera diluted 1:10 (screening) in Leibowitz L-15 medium with antibiotics were mixed with 30 μL of WNV in L-15 medium with 3% fetal calf serum (FCS) for cell culture (Sigma), containing about 30 plaque-forming units (PFU). The serum–virus mixture was incubated at 37°C for 60 min; then 60 μL of a Vero E6 cells (grown at 37°C for 3–4 days) suspension in L-15 with 3% FCS and antibiotics were added to each test well (about 20,000 cells per well). After an incubation at 37°C for 4 h, 120 μL of overlay (1.5% carboxymethylcellulose sodium salt in L-15 supplemented with 3% FCS and antibiotics) was added to each well. The microplates were covered with lids, sealed in small plastic bags, and incubated at 37°C. The cells were checked for plaques and cytopathic effect under an inverted microscope after 3 and 4 days, and then stained with 0.1% Naphthalene Black on the fifth day. Control sera (positive and negative) were included in each run of the test. The micro-plaque-reduction neutralization microtest (PRNT) was validated earlier using positive and negative equine (Weissenböck et al. 2003), other mammalian (including human), and avian sera; this test is used routinely in our laboratory for detection of neutralizing antibodies to WNV, TBEV, and USUV.

Serum samples that neutralized WNV with a 90% or greater reduction of PFU numbers at the 1:10 dilution during screening were titrated in duplicate by two-fold dilutions in L-15 medium, and the dilutions corresponding to 90% reduction of PFU were regarded as the antibody titers ($PRNT_{90}$). Sera

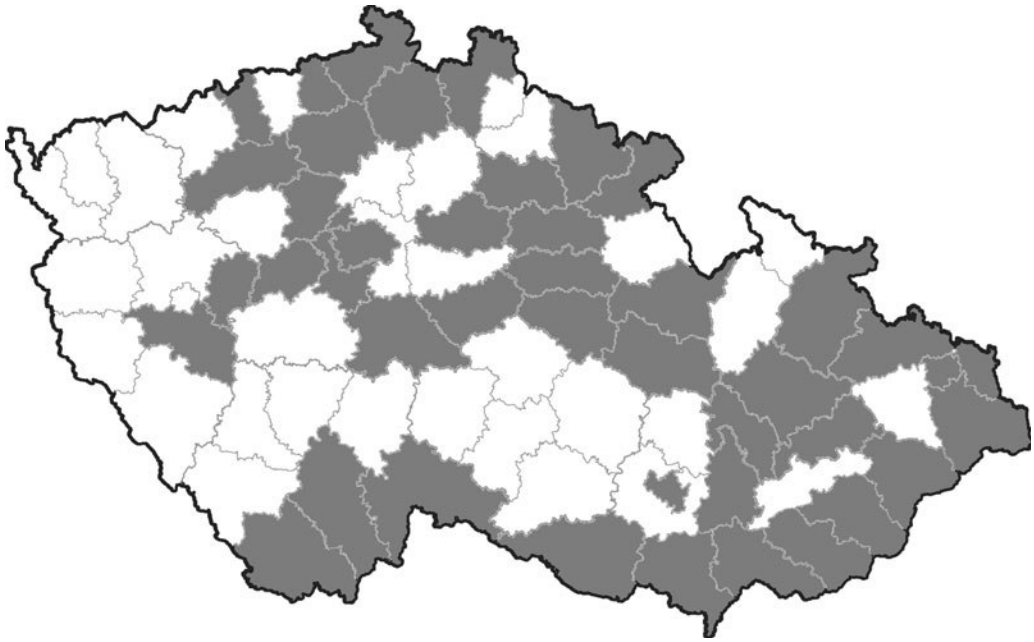

FIG. 1. Map of Czechland (Czech Republic), with administrative districts; the districts where horses were examined are given in gray.

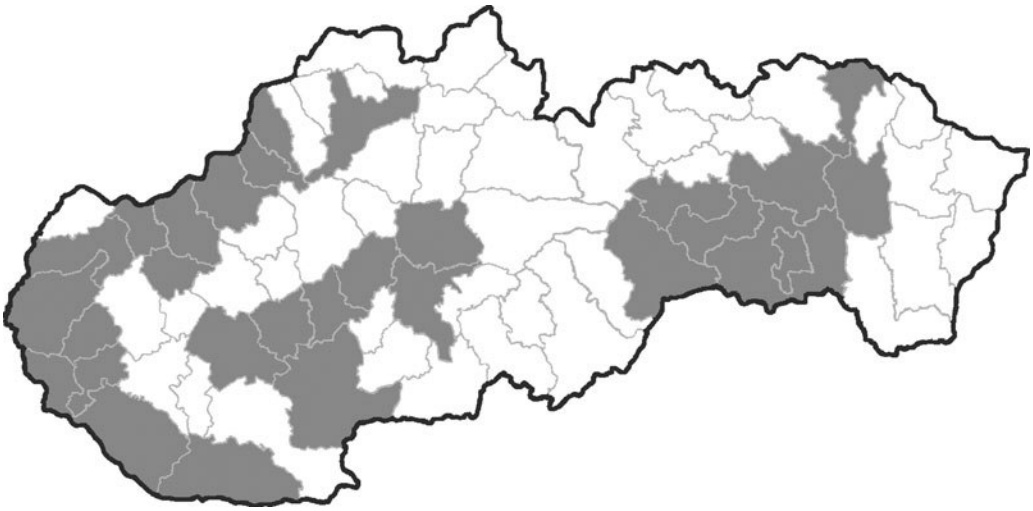

FIG. 2. Map of Slovakia, with administrative districts; the districts where horses were examined are given in gray.

were considered positive if they had a neutralizing activity at dilutions superior to 1:20.

The sera reacting with WNV were also tested against other flaviviruses occurring in central Europe—TBEV and USUV. The $PRNT_{90}$ assay for these viruses was carried out in the same way as for WNV.

Results

Antibodies neutralizing WNV were not detected in any of the 163 examined horses from Czechland, whereas 22 of 232 examined horses from Slovakia revealed specific antibodies to WNV, with the antibody titers ranging from 1:40 to 1:640 (Table 1); they were all seronegative with TBEV (the $PRNT_{90}$ titer against TBEV was less than 1:10 in all cases), whereas three of them gave a very low-titer (1:10) reaction with USUV (nos. 20, 27, and 108). WNV-seropositive animals were between 2 and 12 years old, and consisted of 10 males (stallions or geldings) and 12 mares.

The history of each WNV-seropositive horse was checked. No marked clinical signs compatible with WNV disease (high fever and/or neurological abnormalities) were recorded in these

Table 1. $PRNT_{90}$ Reciprocal Titers of Antibodies against West Nile Virus in Equine-Specific Seroreactors, and Their History

Horse no.	*District*	*Sex*	*Age (years)*	*Date collected*	*WNV titer*	*Origin (country)*	*Past stay in WNV-endemic countries*	*WNV vaccine*
20	Dun.Streda	M	5	Sep. 2010	160	US	Russia	**Yes**
23	Dun.Streda	F	3	Sep. 2010	320	US	Russia	**Yes**
26	Komárno	F	4	Sep. 2010	640	Slovakia	—	No
27	Komárno	M	4	Sep. 2010	320	Slovakia	—	No
30	Komárno	M	8	Sep. 2010	40	Hungary	Hungary, Italy	No
46	Bratislava	M	7	Oct. 2010	160	Italy	Hungary, Austria	No
51	Bratislava	M	12	Oct. 2010	320	Germany	Austria, Hungary	**Yes**
63	Bratislava	M	11	Oct. 2010	40	Slovakia	—	No
67	Bratislava	M	2	Oct. 2010	80	Slovakia	—	No
107	Levice	F	10	Mar. 2011	80	Slovakia	—	No
108	Levice	F	6	Mar. 2011	320	Slovakia	—	No
121	Senec	M	7	Apr. 2011	320	Czechland	—	No
KP3	Komárno	F	6	Aug. 2011	40	Slovakia	Hungary (2011)	No
KP4	Komárno	F	12	Aug. 2011	40	Slovakia	—	No
KP7	Komárno	F	11	Aug. 2011	80	Slovakia	Hungary	No
KP9	Komárno	F	12	Aug. 2011	40	Slovakia	Hungary	No
KP22	Komárno	M	8	Aug. 2011	40	Slovakia	—	No
KP24	Komárno	F	8	Aug. 2011	640	Slovakia	—	No
KP41	Pezinok	F	8	Aug. 2011	80	Slovakia	—	No
SVU20	Senica	F	18	Mar. 2011	80	Italy	Hungary	No
SVU100	Holíč	F	10	Aug. 2011	320	US	—	No
SVU118	Pezinok	M	7	Jul. 2011	40	Czechland	Hungary, Austria	No

All tested animals were asymptomatic, and seronegative for tick-borne encephalitis virus (the $PRNT_{90}$ titer with TBEV was <10) and Usutu virus.

WNV, West Nile virus; M, male; F, female; PRNT, plaque-reduction neutralization test; TBEV, tick-borne encephalitis virus.

seroreactors in the past. They were born in Slovakia (13), Czechland (2), Italy (2), and Hungary (1), and four originated from the United States and Germany. However, three seropositive horses had been immunized with WNV vaccine (no other seroreactor was vaccinated in the past). The latter three seroreactors therefore were excluded from the Slovak prevalence study, giving a seroprevalence rate in nonvaccinated animals of 19/229, *i.e.*, 8.3% (95% CI 4.7–11.9%). All WNV-seropositive horses only lived in six districts of southern Slovakia (Komárno, Levice, Senec, Bratislava, Pezinok, and Senica), situated exclusively in a lowland part of the country below 200 meters above sea level (Fig. 3). The overall prevalence of antibodies neutralizing WNV was 15.1% (95% confidence interval [CI] 8.8–21.4%) in 126 nonvaccinated horses examined from those six affected districts, and the difference in seroprevalence rate based on local infection with WNV (11 animals) between the six positive districts in southern Slovakia and all other Slovakian districts was statistically significant ($\chi^2 = 7.59$; $p = 0.006$).

When the 11 autochthonous horse infections with WNV were analyzed for age factor, it was found that their average age was 7.4 (median 8) years versus 10.5 (median 10) years in all seronegative Slovak horses, but the difference was statistically insignificant (Mann–Whitney test, $p = 0.143$). The seropositivity rate in the age group 1–4 years was 8.6% ($n = 35$), in the group 5–8 years 8.2% ($n = 61$), 9–12 years 6.5% ($n = 46$), and in the horses older than 12 years 0.0% ($n = 64$).

Discussion

Out of 22 WNV-seropositive horses in Slovakia, at least 11 (*i.e.*, 4.8% of 229 nonvaccinated animals; 95% CI 2.0–7.6%; five males, six females) revealed autochthonous (local) infection with WNV (they were born in Slovakia or Czechland and did not travel to WNV-endemic countries), confirming circulation of WNV in southern Slovakia, whereas in eight other animals it cannot be excluded with certainty that they could have been infected in the country where they were born or had lived for a certain period (*i.e.*, Italy, Hungary, United States). The remaining three seroreactors developed immunity after a previous WNV vaccination.

Detection of specific antibodies neutralizing WNV in local horses in Slovakia (for the first time in the country) has indicated enzootic transmission of the virus. Although no equine serosurvey for WNV was carried out previously in Slovakia, it is probable that WNV activity in southern Slovakia started only a few years ago. For instance, one 2-year-old animal (no. 67 in Table 1) was found to be positive (and stayed in Slovakia), indicating that WNV had circulated in the last 2 years preceding the sampling (*cf.* also other young horses nos. 26 and 27). The decreasing trend of seropositivity along the age gradient also indicates a recent WNV activity in southern Slovakia, possibly reflecting an expansion from the WNV endemic area in northwestern Hungary. There is no marked geomorphological or climatological barrier between these two regions.

The WNV lineages 1 and 2 were detected in Hungary recently (Bakonyi et al. 2006, Kutasi et al. 2011). However, it is impossible to differentiate infections caused by individual genomic lineages of WNV using a neutralization test. Thus we do not know which WNV lineage occurs in southern Slovakia at present.

PRNT is regarded a "gold standard" in flavivirus serology and also used for confirmation of other serological tests [enzyme-linked immunosorbent assay (ELISA), hemagglutination-inhibition test] because it is well known that flaviviruses present a high degree of serological cross-reactivity, sometimes even in the neutralization test (Madrid and Porterfield 1974, Calisher et al. 1989, Niedrig et al. 2007). Often several antigenically similar flaviviruses of the same or related flavivirus group might co-occur in one area. Therefore, we examined WNV seroreactors also against TBEV and USUV (*i.e.*, the flaviviruses occurring in central Europe).

In a similar Central European study, sera of 350 horses from eastern Austria were examined for WNV antibodies in 2002 and all were found negative, except for four seropositives out of 35 horses (11.4%) that were transported from Hungary (the country of their origin) via Austria to Germany; these animals had no obvious clinical signs when examined at the border (Weissenböck et al. 2003). A recent study demonstrated WNV-neutralizing antibodies in 3.4% of 2098 horses in

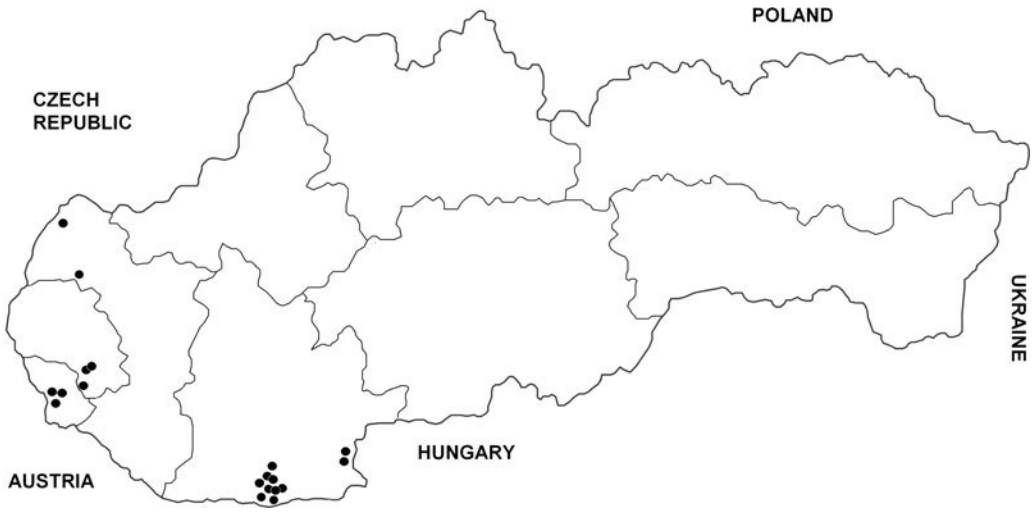

FIG. 3. Distribution of horses with antibodies neutralizing WNV in Slovakia, 2010–2011. (Three animals immunized with a WNV vaccine have not been included here.)

western Croatia (Barbic et al. 2012). In Spain, WNV antibodies were detected in 8.3% of 157 feral horses from the Guadalquivir marshes (NP Doñana) in 2005 (Jiménez-Clavero et al. 2007). In southern France (Camargue, a WNV endemic zone), overall 8.5% seropositive horses were detected in 2000 (Durand et al. 2002) and 5.3% in 2001 (Leblond et al. 2005). The seropositivity rate (in terms of neutralizing antibodies to WNV) found in Slovak horses in this study (8.3%) is very similar to that observed in Spain and southern France. However, equine seroprevalence rates for WNV in hyperendemic areas can sometimes be as high as 34%—Danube delta in Romania (Savuta et al. 2007), 22%—Volga delta in southern Russia (Lvov et al. 2005), or even 78%—Ferlo area in Senegal (Chevalier et al. 2006). Selective serosurveys for WNV in nonvaccinated, local horses obviously present a very useful indicator of the virus activity in an area, and a predictor for potential risk of occurrence of human cases or epidemics of West Nile fever (Mattar et al. 2005, Corrigan et al. 2006, Jiménez-Clavero et al. 2007, Epp et al. 2008, Angelini et al. 2010).

It would be interesting to continue monitoring horses to obtain information on the timing of WNV circulation in Slovakia, and in particular to detect or isolate the virus following determination of its origin.

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Author Disclosure statement

No competing financial interests exist.

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PRÁCE 28

Hubálek Z., **Rudolf I.**, Čapek M., Bakonyi T., Betášová L., Nowotny N. 2012. Usutu Virus in Blackbirds (*Turdus merula*), Czech Republic, 2011-2012. *Transbound. Emerg. Dis.* 61: 273–276.

Stručná charakteristika: USUV je původně africký arbovirus přenášený komáry rodu *Culex*, obratlovčími hostiteli amplifikátory jsou ptáci a pro některé zástupce řádu *Passeriformes* (zejména kosa černého) je USUV patogenní a způsobuje encefalitidu, myokarditidu nebo hepatitidu. Podle posledních dat může být USUV patogenní také pro člověka. Studii inicioval nález mrtvého kosa v Brně Pisárkách.

Hlavní přínos práce: jde o první izolaci tohoto arboviru u obratlovců (ptáků) na našem území a naznačuje možnou introdukci ze sousedního Rakouska, kde je virus etablován již od roku 2001.

Příspěvek autora k dané práci: autor se podílel na molekulární analýze viru Usutu v kosech a také na přípravě rukopisu.

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Transboundary and Emerging Diseases

SHORT COMMUNICATION

Usutu Virus in Blackbirds (*Turdus merula*), Czech Republic, 2011–2012

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Keywords:
Usutu virus; blackbird; *Turdus merula*; Czechland

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Summary

The central European lineage of Usutu virus was isolated from a blackbird (*Turdus merula*), which was found dead in the city of Brno, Czech Republic, in 2011. The virus RNA was detected in two other dead blackbirds in Brno during 2012.

Introduction

Usutu virus (USUV), a *Flavivirus* of the Japanese encephalitis serogroup (family *Flaviviridae*), was first isolated by B. M. McIntosh from *Culex neavei* mosquitoes in South Africa in 1959 (Karabatsos, 1985). Arthropod vectors of USUV are largely ornithophilic mosquitoes, especially *Culex* spp., while amplifying vertebrate hosts are birds. The virus is highly pathogenic for certain passeriform birds (e.g. *Turdus* spp.) causing in them encephalitis, myocarditis and hepatitis. On the other hand, pathogenicity of USUV for other avian species is limited (Chvala et al., 2005, 2006).

Surprisingly, this African arbovirus emerged in central Europe (in and around Vienna, Austria) in 2001 and caused significant wild bird mortality, predominantly in blackbirds (*Turdus merula*), but also in various other species of birds kept in aviaries (Weissenböck et al., 2002, 2003; Bakonyi et al., 2004; Chvala et al., 2004, 2007). Later, the epornitic also occurred in neighbouring countries: Hungary (Bakonyi et al., 2007), Italy (Manarolla et al., 2010), Switzerland (Steinmetz et al., 2011) and Germany (Jöst et al., 2011; Becker et al., 2012), essentially in the lowland river valley ecosystem. Present geographic distribution of USUV includes Africa (Morocco, Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Cote d'Ivoire: Nikolay et al., 2011) and Europe (including Spain: Busquets et al., 2008; Vázquez et al., 2011); antibodies to USUV were also detected in migratory birds in Czechland and Poland (Hubálek et al., 2008a,b) and the UK (Buckley et al., 2003).

Material and Methods

On 25 May 2011, an adult, well-nourished (105 g) male blackbird (*Turdus merula*) was found dead in the Brno town area (Brno – Pisárky; 49°19′N, 16°58′E), South Moravia, Czechland (Czech Republic). Organ samples were examined for the presence of viruses by isolation attempts on outbred (ICR) SPF mice: centrifuged 10% suspensions of heart and brain in PBS with 0.4% bovine serum albumin, and antibiotics were inoculated intracerebrally into 8

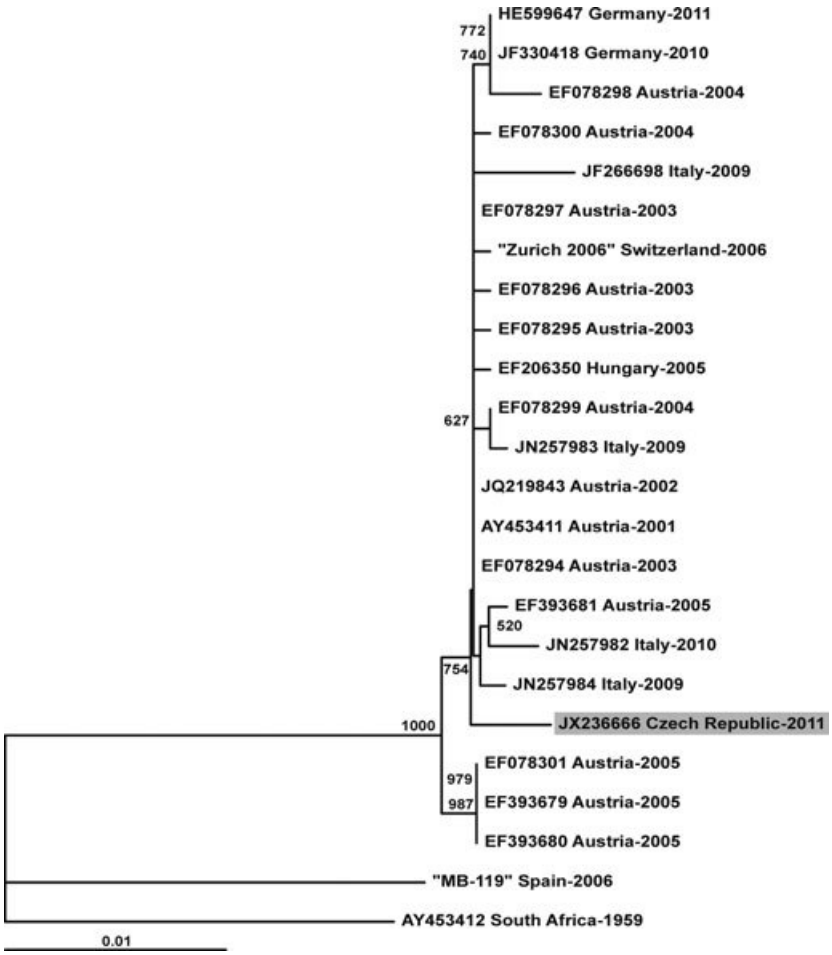

Fig. 1. Phylogram demonstrating the genetic relationships of Usutu virus (USUV) nucleotide sequences in the partial E glycoprotein gene region. Sequences are indicated by codes containing the GenBank accession number or the name of the virus strain, the country of origin and the year of sample collection. The Czech sequences described in this paper are highlighted by grey background. Bootstrap values $\geq$ 500 (50%) are displayed. The bar on the left represents the genetic distance.

and 9 newborn mice, respectively. The brain and heart homogenates of the blackbird were additionally tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of flavivirus-specific RNA. RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). Generic flavivirus primers (Scaramozzino et al., 2001) as well as USUV-specific diagnostic primers (Usna129f, 5′-AGGACCATTGGTTAGGAAGA-3′ and Usna663r, 5′-GGCTTGACAACACAATCATC-3′) and West Nile virus-specific primers (Papa et al., 2011) were employed. A continuous RT-PCR system using the QIAGEN OneStep RT-PCR Kit (Qiagen) was applied on the RNA extracts.

The homogenized brain tissue of the dead mice was also tested for the presence of USUV RNA by amplifying two different genomic regions of USUV, that is, one stretch covering a partial envelope (E) protein and the non-structural 1 (NS1) protein coding regions (between nt. positions 1179 and 3017, referring to the complete USUV genome sequence with the GenBank accession number NC_006551), and another genome stretch covering a partial non-structural 5 (NS5) protein coding region and the 3′untranslated region (UTR) (between nt. positions 10128 and 10828) (Bakonyi et al., 2004).

The above PCR amplification products were sequenced in both directions as described previously (Bakonyi et al., 2004). The compiled nucleotide sequences of the Czech USUV were aligned and compared with other USUV strains. A phylogenetic tree was established using a modified neighbour-joining method (ClustalX; Thompson et al., 1997). The stability of the tree was tested by bootstrap analysis of 1000 replicates.

Results and Discussion

At necropsy, no gross lesions were observed on the internal organs of the blackbird. All mice inoculated with the organ suspensions of the bird died within 4–6 days postinoculation; the average survival time was 4.9 and 5.7 days for the heart and brain suspension, respectively. Both organ homogenates of the blackbird were strongly positive for flavivirus and USUV RNA, respectively, but negative for West Nile virus RNA. USUV RNA was also identified by specific PCR assays in the brain homogenates of the inoculated mice.

The E and NS1 sequence of the Czech USUV isolate differed in five of 1839 investigated nucleotides ($C_{1311}T$, $T_{1332}C$, $A_{1413}T$, $T_{2322}C$, and $T_{2419}C$) as compared with the first European USUV isolate Vienna 2001-blackbird (GenBank acc.no. NC_006551; 99.7% identity). The nucleotide substitutions did not alter the putative amino acid sequence. Similarly, high identity rates were found to other central European USUV isolates. The sequence of the partial NS5 protein coding region including the 3′UTR was found 100% identical to the sequence of the USUV strain that emerged in Hungary in 2005 (GenBank acc.no. EF206350; Bakonyi et al., 2007). A neighbour-joining phylogram based on the partial E protein coding region is shown in Fig. 1. The partial nucleotide sequences of the blackbird-derived Czech USUV isolate were submitted to GenBank under the accession numbers JX236666 and JX236667.

Our study reveals the presence of USUV in Czechland, adding another country to the area of USUV distribution. The genetic comparisons indicate that practically the same virus strain has been circulating in central Europe since its introduction. However, while in other countries such as Austria, Italy, Switzerland and Germany significant wild bird (predominantly blackbird) mortality was observed, in Czechland and Hungary, only sporadic cases have been diagnosed so far. Furthermore, it is interesting to note that the bird described here was found dead at the end of May, that is, very early in the USUV transmission season, and that the infection occurred again in an urban area, as it happened also in Vienna, Budapest, and Zurich, respectively (Weissenböck et al., 2002; Bakonyi et al., 2007; Steinmetz et al., 2011). During the preparation of this manuscript (2012), USUV RNA was detected by RT-PCR in another blackbird which was found dead in the Brno area, which may be an indication for a possible USUV epidemic in future years in this area in case of favourable climatic and ecological conditions. The occurrence of USUV in a region in which also other flaviviruses such as West Nile virus lineage 3 (Rabensburg virus; Bakonyi et al., 2005; Hubálek et al., 2010), tick-borne encephalitis virus and possibly also West Nile virus lineage 2 (Bakonyi et al., 2006; Wodak et al., 2011) are circulating simultaneously is – due to the high rate of cross-reactions among these viruses – a further challenge for a correct serological diagnosis of these infections.

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Note Added in Proof

Another (third) blackbird (young, 65 g) that had been found dead in Brno on 9 August 2012, was positive (PCR) for USUV.

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PRÁCE 29

Rudolf I., Šebesta O., Mendel J., Betášová L., Bocková E., Jedličková P., Venclíková K., Blažejová H., Šikutová S., Hubálek Z. 2014. **Zoonotic *Dirofilaria repens* (Nematoda: Filarioidea) in *Aedes vexans* mosquitoes, Czech Republic. *Parasitol. Res.* 113: 4663-4667.**

Stručná charakteristika: dirofilárióza patří mezi tzv. emergentní zoonózy. Cílem práce bylo potvrdit přítomnost patogenních dirofilárií (*D. immitis* a *D. repens*) v komářích vektorech v oblasti jižní Moravy, kde bylo v roce 2005 diagnostikováno několik autochtonních případů psí dirofilariózy.

Hlavní přínos práce: poprvé na našem území se podařilo detegovat patogenní dirofilarie (*D. repens*) v komárech *Ae. vexans*, což bylo startovacím impulsem pro další sledování možných případů onemocnění u psů nebo dokonce člověka ('One health approach').

Příspěvek autora k dané práci: autor se podílel na designu studie, molekulární analýze komárů na dirofilárie a na přípravě rukopisu.

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SHORT COMMUNICATION

Zoonotic *Dirofilaria repens* (Nematoda: Filarioidea) in *Aedes vexans* mosquitoes, Czech Republic

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Abstract The surveillance of vectors for arthropod-borne pathogens is nowadays an important tool in surveillance programmes throughout Europe. Whereas many studies have been performed to screen arthropods for viruses or bacterial pathogens, only limited information is available concerning the geographical distribution and vector range of pathogenic filariae in Central Europe. To consider the prevalence of filarial parasites in mosquito vectors, we performed a molecular survey of mosquitoes for filarial DNA. Mosquito collection was conducted at six study sites in the South Moravian region (Czech Republic) close to the borders with Slovakia and Austria from 2009 to 2011. Molecular screening of mosquitoes was conducted using conventional PCR with primers designed to amplify the mitochondrial cytochromoxidase subunit I gene as well as the partial 5.8S ribosomal RNA gene. A total of 13,222 mosquitoes belonging to six species were captured and distributed into 237 pools with different numbers of individuals. Overall, four pools were positive for *Dirofilaria repens* (a minimum infection rate 0.03 %) at two study sites (both natural and urban). Another filarial parasite detected during a study into *Aedes vexans* mosquitoes revealed the closest homology to *Setaria* spp. We detected specific *D. repens* DNA in *Ae. vexans* mosquitoes for the first time in the Czech Republic and confirmed the circulation of *Dirofilaria* spp. in a natural focus of infection providing an epidemiological link between autochthonous canine cases and mosquito vectors in the area studied.

Keywords *Aedes vexans* · Mosquito vectors · *Dirofilaria repens* · Dogs · Zoonotic dirofilariosis · *Setaria* spp.

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Introduction

Dirofilariae are important arthropod-borne parasitic helminths of dogs and other carnivores that also can be transmitted to humans. *Dirofilaria immitis*, responsible for heartworm disease, is widespread around the world, whereas *Dirofilaria repens*, the etiological agent of subcutaneous or ocular infections, has a geographical distribution restricted to the Old World. Both *Dirofilaria* species are zoonotic, and the human infections caused by *D. repens* are increasing in Europe (Pampiglione and Rivasi 2000).

Various mosquito species of the genera *Aedes*, *Anopheles*, *Culex* and *Ochlerotatus* take part in the transmission of dirofilariae in Europe (Pampiglione et al. 1995).

In the Czech Republic, canine dirofilarial infection has been diagnosed in dogs coming from endemic areas in the past and as such was considered an imported infection. Nevertheless, in 2005, microfilariae were detected in seven (9 %) out of 77 dogs from the Břeclav area, close to Slovak border that had never travelled abroad. The results of acid phosphatase staining as well as PCR confirmed *D. repens* infection (Svobodová et al. 2006).

However, the role of particular vectors in the transmission cycles of pathogenic filarial species and their geographical distribution remain largely unknown. The aim of the study was to conduct molecular screening of mosquitoes for the presence of zoonotic filarial parasites in an area endemic for canine dirofilariosis, considered a real public health hazard.

Materials and methods

Study sites

Mosquito collections were carried out at six sites located in the basin of the River Dyje between 2009 and 2011 (Fig. 1). The Sedlec site (48°47′ N, 16°42′ E, 169 m a.s.l.) is situated on the edge of the Nesyt fishpond. Nesyt is a part of the Lednice Pond system and, with an area of 322 ha, is the largest pond in Moravia. The site consists of a group of bushes and low trees, mostly willows (*Salix fragilis*), growing on the edge between the pond's embankment vegetation and a meadow. The bank of the pond is densely vegetated, mostly by reeds (*Phragmites australis*). The meadow is part of the Slanisko National Natural Reserve and is characterised by the occurrence of halophilic flora and fauna (e.g. *Scorzonera parviflora*, *Trifolium pannonicum* and *Spergularia salina*). The Lednice site (48°47′ N, 16°49′ E, 162 m a.s.l.) is situated on the edge of Mlýnský Pond, which is also part of the Lednice Pond system. Mlýnský Pond has an area of 107 ha. The site consists of dense reed vegetation (*P. australis*) and a group of bushes and low trees (*S. fragilis*). The Křivé Jezero site (48°51′ N, 16°49′ E, 160 m a.s.l.) is situated within the nature preserve of the same name and is only minimally influenced by human activity. It lies in the valley of the Dyje immediately below the dam of the last lake of the Nové Mlýny reservoir system. The Kančí Obora site (48°46′ N, 16°52′ E, 154 m a.s.l.) is located approximately 14 km from the Křivé Jezero site, downstream following the Dyje. This site is frequently visited by tourists and local residents to walk their dogs. The collection site is situated approximately 500 m from the district town of Břeclav. The Soutok site (48°39′ N, 16°58′ E, 147 m a.s.l.) is situated close to the confluence of the rivers Morava and Dyje and is remote from all residential areas (9 km from the town of Lanžhot). Its distance from the Kančí Obora site is approximately 15 km. The sites Křivé Jezero (Curved Lake), Kančí Obora (Boar's Forest) and Soutok (Confluence) are composed mainly of alluvial forest with mixed tree species (*Salix* spp., *Populus* spp., *Quercus robur*, *Fraxinus angustifolia*, *Tilia cordata*, and *Carpinus betulus*) and wet meadows (*Alopecurus pratensis*, *Poa pratensis*, and *Carex praecox*). The Lanžhot site (48°43′ N, 16°58′ E, 151 m a.s.l.) consists of a farmstead with houses and several small stables. Farm animals are stabled here, in particular horses. It is situated on the edge of an alluvial forest (the Soutok Game Preserve) about 800 m from the town of Lanžhot (Šebesta et al. 2012).

Mosquito trapping and identification

To trap female mosquitoes, we used EVS light traps (BioQuip Products, Inc., Rancho Dominiquez, CA, USA) supplemented

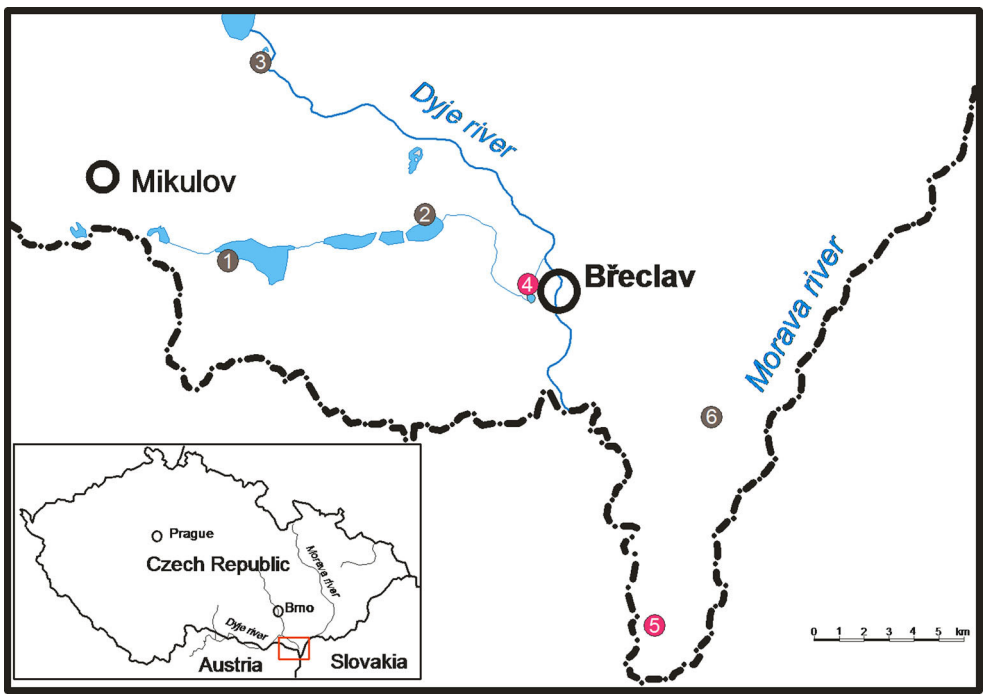

Fig. 1 Locations of six study sites for mosquito trapping, South Moravia, Czech Republic. Mosquito trapping sites (*1* Sedlec, *2* Lednice, *3* Křivé Jezero, *4* Kančí Obora, *5* Soutok, *6* Lanžhot). Areas with positive findings are coloured with red

by dry ice and situated in a protected place at a height of 1 m. The exposure was through the night from 16:00 to 8:00 Central European Summer Time. Captured mosquitoes were transported in closed and chilled containers to laboratory where they were classified and stored for further processing in freezers at a temperature of −60 °C. Mosquito species were morphologically identified using the determination key by Becker et al. (2010).

Set of biological material for the PCR analysis

For the PCR analysis, we used 13,222 adult female mosquitoes which were divided, based on species determination, into 237 pools (each pool containing predominantly 50 individuals of the same species, collected at the same location). Each pool was examined separately for the presence of *D. immitis* and *D. repens*, respectively.

Homogenisation of mosquitoes and DNA isolation

The collected mosquitoes were mechanically disrupted using a ceramic mortar in 500 μl of phosphate-buffered saline solution with 0.4 % of foetal bovine albumin under sterile conditions. The total genomic DNA was extracted from 100 μl of the mosquito homogenate with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Molecular screening for *Dirofilaria* spp.

Primers were designed to amplify approximately 200 bp region of mitochondrial cytochromoxidase subunit I (COI) gene of *Dirofilaria* spp. parasites (Rishniw et al. 2006). PCR amplification was performed with two sets of primers: DI COI-F1 (5′-AGT GTA GAG GGT CAG CCT GAG TTA-3′) and DI COI-R1 (5′-ACA GGC ACT GAC AAT ACC AAT-3′) for detection of *D. immitis*; DR COI-F1 (5′-AGT GTT GAT GGT CAA CCT GAA TTA-3′) and DR COI-R1 (5′- GCC AAA ACA GGA ACA GAT AAA ACT-3′) for the detection of *D. repens*.

For sequencing, primers targeting the *D. repens* 5.8S ribosomal DNA, DIDR-F1 (5′-AGT GCG AAT TGC AGA CGC ATT GAG-3′) and DIDR-R1 (5′-AGC GGG TAA TCA CGA CTG AGT TGA-3′) were applied on PCR positive samples. The primers used in our study are routinely employed in molecular diagnostics and the genotyping of *D. immitis* and *D. repens* in clinical samples (dog blood) as well as in mosquito vectors. PCR amplification as well as all post-PCR procedures were performed according to Rishniw et al. (2006). The processing of mosquito pools, DNA extraction, PCR handling (preparation of mastermix, PCR reaction) and post-PCR procedures (agarose gel electrophoresis) were carried out in separate rooms to avoid cross-contamination of the samples.

Sequence analysis of PCR products

The PCR product was purified by precipitation with PEG/Mg/NaAc (26 % polyethylene glycol, 6.5 mM $MgCl_2 \cdot 6H_2O$, 0.6 M $NaAc \cdot 3H_2O$). Direct sequencing of the purified PCR product was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) according to the manufacturer's instructions and purified with EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). PCR amplicons were bidirectionally sequenced to ensure high-quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR Inc., USA) and also checked manually. The FASTA format and BLAST program (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches.

Results and discussion

A total of 13,222 female mosquitoes were collected from 2009 to 2011 at six study sites. The most abundant mosquito species

Table 1 Summary details of *Dirofilaria* spp. positive mosquito pools (Collection site: 1 Sedlec, 2 Lednice, 3 Křivé Jezero, 4 Kančí Obora, 5 Soutok, 6 Lanžhot)

Indication of pool	Filarial species	GenBank accession no.	Max. % identity to GenBank entry (accession no.)[a]	Collection site	Collection date	Mosquito species	Pool size (no. mosquitoes)
03-2011	*D. repens*	KM108773	97	4	5 October 2011	*Ae. vexans*	50
41-2010	*D. repens*	KM108774	98	4	15 July 2010	*Ae. vexans*	50
46-2010	*D. repens*	KM108775	99	4	28 July 2010	*Ae. vexans*	50
59-2010	*D. repens*	NA	99	5	16 July 2010	*Ae. vexans*	50

NA not assigned (short sequence)

[a] As of 24 July 2014

in the collection was *Aedes vexans* (*n*=12,042), followed by *Culex modestus* (*n*=537), *Aedes sticticus* (*n*=282), *Culex pipiens* (*n*=193), *Aedes rossicus* (*n*=150) and *Anopheles maculipennis* sensu lato (*n*=18). A total of 237 mosquito pools were examined by PCR targeting mitochondrial cytochromoxidase subunit I gene RNA segment. Whereas all pools from the 2009 collection were negative, four pools from *Ae. vexans* (three specimens collected in July 2010 and in October 2011 at the Kančí Obora study site and one specimen in August 2010 at the Soutok locality) showed specific *Dirofilaria* COI PCR amplification (Table 1) with a minimum infection rate of 0.03 %. The identity of all positive specimens has been confirmed by sequencing of the 5.8S ribosomal RNA gene PCR product, which has shown 97 to 99 % similarity with sequences attributed to *D. repens* (GenBank: AY693808). Interestingly, no *D. immitis* parasite was found in the course of the study. The remaining two filarioid DNA sequences demonstrated the presence of a parasite of the genus *Setaria* spp. (samples collected from *Ae. vexans* at Kančí Obora in July 2010). Two further COI PCR products were confirmed by direct sequencing of the 5.8S ribosomal RNA gene with the same primers as for *Dirofilaria* spp. which revealed the closest match to *Setaria* spp. (GenBank: EF196088).

The first record of *Dirofilaria repens* in the Czech Republic has added another country to the list of *Dirofilaria* spp. endemic areas. The South Moravia region has long been recognised as favourable place for the mass breeding of mosquitoes (the local mosquito fauna involves 30 species of the genera *Anopheles*, *Aedes*, *Ochlerotatus*, *Culex*, *Culiseta*, *Coquillettidia* and *Uranotaenia*) (Šebesta et al. 2012). The mosquito *Ae. vexans* has many of the attributes of an ideal vector species. It is widely distributed, can become very abundant (mainly after flooding), often at the same time when pathogen activity is at its peak, and it feeds readily on humans and domestic animals including dogs (Becker et al. 2010). Detection of *D. repens* in *Ae. vexans* mosquito, which is very abundant in summer season (the development from hatching of the first instars to emergence of the adults lasts 1 week in ideal circumstances) might also pose an epidemiological risk for the contracting of human disease in the area. Taking into account the long flying distance (migrations of up to 15 km), this species becomes suitable for local transmission and the spread of zoonotic dirofilariosis into new endemic foci. Outside the traditional Mediterranean distribution area, dirofilarial worms have also recently been described in mosquitoes from Slovakia (Bocková et al. 2013), Austria (Silbermayr et al. 2014) and Germany (Czaijka et al. 2014; Kronefeld et al. 2014), the more so as in two of the four cases (Bocková et al. 2013; Czajka et al. 2014) *Ae. vexans* was involved.

Interestingly, three of the four positive *Dirofilaria* spp. pools were detected at the Kančí Obora site, which is situated close to an inhabited area and frequently used for leisure activities including dog walking. Despite the simultaneous detection of *D. repens* in mosquitoes and dogs in an area, no human cases have been recorded so far in contrast to neighbouring Germany (Tappe et al. 2014), Slovakia (Hrckova et al. 2013) and Poland (Cielecka et al. 2012). However, the possible emergence of human disease (particularly skin and ocular forms) cannot be excluded the coming seasons.

Detection of another filarial parasite of the genus *Setaria* confirmed observations from Germany, where *Setaria tundra* was documented in field-collected mosquitoes of the species *Ae. vexans* (Kronefeld et al. 2014). However, the pathogenic potential of *Setaria* for free-living animals (e.g. cervids) still remains unknown.

We detected *D. repens* DNA in *Ae. vexans* mosquitoes for the first time in the Czech Republic and confirmed the circulation of *Dirofilaria* spp. in a natural focus of infection where autochthonous canine cases and infected mosquito vectors occur. The additional finds of *Setaria* spp. highlight the need for continuing surveillance of mosquito-borne filarial diseases in Central (and Western) Europe. The co-occurrence of the vector and the disease in dogs might anticipate an early appearance of the first human cases of zoonotic dirofilariasis in the area. Public health authorities should therefore be aware of the increased risk to residents due to the circulation of zoonotic filariae.

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PRÁCE 30

Rudolf I., Bakonyi T., Šebesta O., Peško J., Venclíková K., Mendel J., Betášová L., Blažejová H., Straková P., Nowotny N., Hubálek Z. 2014. West Nile virus lineage 2 isolated from *Culex modestus* mosquitoes in the Czech Republic, 2013: expansion of the European WNV endemic area to the North? *EuroSurveill.* 19(31):pii=20867.

Stručná charakteristika: po prvním nálezu WNV viru Rabensburg v komárech na jižní Moravě v roce 1997 a 1999 jsme se zaměřili na intenzivní surveillance komárů na přítomnost patogenních arbovirů včetně WNV. V rámci evropských projektů EDEN a EDENext jsme molekulárně nebo formou izolačních pokusů vyšetřili desítky tisíc komárů.

Hlavní přínos práce: poprvé se nám na našem území podařilo izolovat z komárů *Cx. modestus* patogenní WNV linii 2, která je původcem mnoha recentních epidemií západonilské horečky v Evropě a která se tímto stává i možným zdravotním rizikem pro obyvatele České republiky.

Příspěvek autora k dané práci: autor se podílel na designu studie, extenzivním sběru komárů v průběhu několika sezón, molekulárních analýzách včetně jejich vyhodnocení a na přípravě rukopisu.

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West Nile virus lineage 2 isolated from *Culex modestus* mosquitoes in the Czech Republic, 2013: expansion of the European WNV endemic area to the North?

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We report the detection and isolation of four almost identical strains of West Nile virus (WNV) lineage 2 from *Culex modestus* mosquitoes collected at three fish ponds in South Moravia, Czech Republic, during August 2013. Phylogenetic analysis demonstrated that the Czech WNV strains isolated are closely related to Austrian, Italian and Serbian strains reported in 2008, 2011 and 2012, respectively. Our findings show the current northernmost range of lineage 2 WNV in Europe.

In South Moravia in the Czech Republic, surveillance activities for mosquitoes and mosquito-borne pathogens have been carried out for several decades, but until our findings in 2013 presented here, WNV lineage 2 (WNV-2) had not been detected.

Background

WNV is a mosquito-borne virus (genus *Flavivirus*; family *Flaviviridae*) that is widely distributed in Africa, the Middle East, Asia and southern Europe [1] and was recently introduced in the Americas [2]. WNV circulates in natural foci between birds (as amplifying hosts) and bird-feeding mosquitoes, in Europe principally *Culex pipiens* and *Cx. modestus* [3]. Humans and horses are considered accidental dead-end hosts. Most individuals infected with WNV are asymptomatic. Symptoms may develop in 20–40% of people with WNV infection, most frequently characterised as influenza-like symptoms, (West Nile fever (WNF)). Less than 1% of infected individuals develop severe neuroinvasive disease, which can be classified into three main clinical syndromes: West Nile meningitis, West Nile encephalitis and acute flaccid paralysis [4].

Several human and/or equine WNF outbreaks have occurred in the last decades in Europe, for example, in Romania (1996), Italy (1998) and Russia (1999) [1]. From 2008 onwards, an unexpected explosive spread of WNV-2, which resulted in several hundreds of human neuroinvasive cases, has been documented in Hungary, Greece and Serbia [5-7].

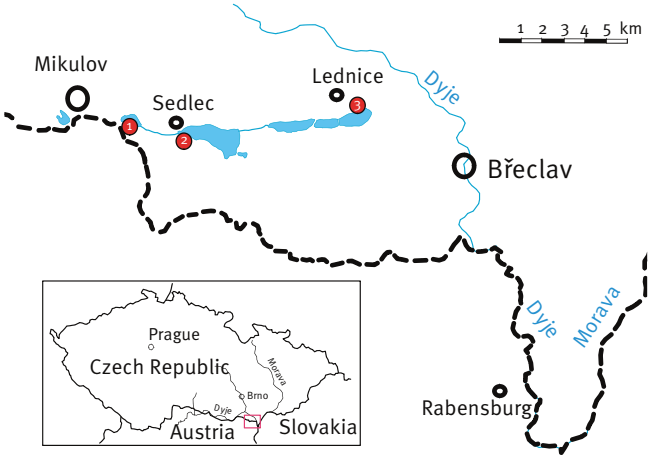
In the Czech Republic, three identical strains of WNV (proposed genomic lineage 3: Rabensburg) were isolated from *Cx. pipiens* and *Aedes rossicus* mosquitoes in 1997, 1999 and 2006 [8,9]. Although neutralising antibodies against WNV have been found rarely in humans in the Czech Republic, two confirmed cases of WNF in humans were reported after heavy floods in 1997 [10]. In addition, WNV-specific antibodies have been detected in resident wild bird species [11]. The above rare traces of WNV infections in the Czech Republic before 2008 were most likely due to WNV lineage 1. Sera collected from 163 horses, originating from 43 out of 77 administrative districts of the Czech Republic between 2008 and 2011, all proved negative for WNV antibodies [12]. Because of the rapidly changing epidemiological situation regarding WNF in Europe, we decided to perform virological surveillance of mosquitoes for WNV and related pathogenic flaviviruses (e.g. Usutu virus) to investigate the epidemiological relevance of WNF in the Czech Republic.

Study site

In this study, mosquitoes were collected within reed belts (*Phragmition communis* alliance) of the fish ponds 'Nesyt' (48 ° 46'35''N, 16 ° 42'05''E; 176 m above sea level (a.s.l.)) and 'Nový' (48 ° 46'57''N, 16 ° 40'13''E; 177 m a.s.l.) at Mikulov, and the fish pond 'Mlýnský' at Lednice (48 ° 47'19''N, 16 ° 49'2''E; 175 m a.s.l.) during July and August 2013 (Figure 1). The climate at the ponds is relatively warm and dry: the mean annual air temperature is 9.1 °C (January –1.8 °C, July 19.2 °C); the mean annual precipitation is 571 mm

Figure 1

Locations of three study sites for *Culex modestus* trapping, South Moravia, Czech Republic, July–August 2013



Fish ponds:
1 Nový
2 Nesyt
3 Mlýnský

(range: 284–919 mm) (data purchased from the Czech Hydrometeorological Institute). A total of 30 species of birds have been recorded breeding in the reed belts; 51 other avian species breed in the close surroundings of the ponds and an additional 54 wild wetland and terrestrial bird species visit this habitat during their seasonal movements. Mosquitoes in South Moravia comprise 30 species of the genera *Anopheles, Aedes, Ochlerotatus, Culex, Culiseta, Coquillettidia* and *Uranotaenia* [13].

Mosquito collection, molecular screening and virus isolation attempts

Mosquitoes were captured using CDC minilight-CO_2-baited traps (EVS CO_2 Mosquito Trap, BioQuip Products, Inc., United States) placed at a height of approximately 1 m above the ground. The traps were run on two successive nights at two-week intervals. The caught insects were transported to the laboratory of the Institute of Vertebrate Biology, Brno, Czech Republic, in cooled flasks (4 to 8 °C) and stored at −65 °C until examination. They were identified under a stereomicroscope and monospecific pools consisting of 50 *Cx. modestus* females were homogenised in 1.5 ml cooled phosphate buffered saline pH 7.4 supplemented with 0.4% bovine serum albumin (Sigma) and antibiotics (PBS-BSA) and centrifuged.

Viral RNA was extracted from 140 µl mosquito homogenates using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Oligonucleotide primers targeting the NS5 region of flaviviruses were used for screening [14]. If samples were positive, a set of WNV-specific primers were used in continuous reverse transcription (RT)-PCRs for amplification of overlapping genome fragments that covered the entire genome sequences of the detected viruses [15]. Amplification products were sequenced directly (Microsynth, Balgach, Switzerland), sequences were aligned and compiled, and identified by basic local alignment search tool (BLAST) search against the GenBank database. The WNV sequences were aligned with 25 complete or nearly complete lineage 2 WNV sequences deposited in GenBank database. Phylogenetic and molecular evolutionary analyses were conducted using neighbor-joining and maximum likelihood algorithms (MEGA version 6 [16], with 1,000 replicates for bootstrap testing) and inferred genetic relationships were shown in a phylogram.

Mosquito homogenates of WNV PCR-positive samples (20 µl) were inoculated intracerebrally into specified pathogen-free suckling ICR mice (SM). The brains of SM that succumbed to the infection were homogenised in PBS-BSA, centrifuged and passaged (intracerebrally) in a new batch of SM. Bacterial sterility of the suspensions was checked in meat-peptone and thioglycollate broths incubated at 37 °C [9].

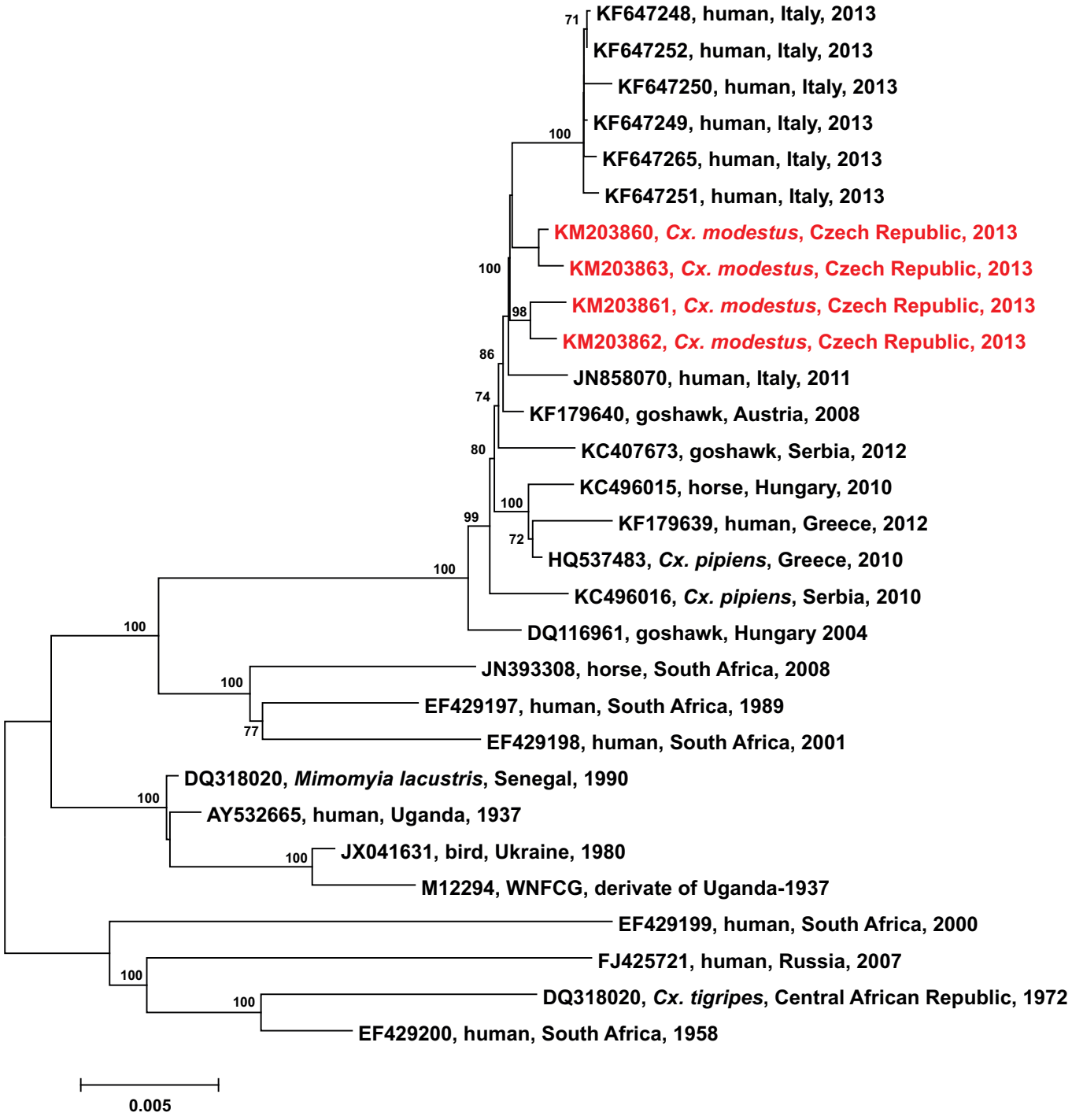
West Nile virus prevalence in *Culex modestus* mosquitoes

A total of 32,500 female *Cx. modestus* mosquitoes in 650 pools were examined for flaviviruses by RT-PCR. RNA of lineage 2 WNV was detected in four pools of insects collected in August 2013: number 13-104 (collected at Nový fish pond), number 13-329 (collected at Nesyt fish pond), number 13-479 (collected at Mlýnský fish pond) and number 13-502 (collected at Mlýnský fish pond). The minimum prevalence rate of WNV in the examined mosquito pools was therefore 1:8,125 (0.012%). All WNV-2-positive mosquito homogenates were inoculated into SM. While number 13-329 did not kill any mice, the three others did: number 13-104 killed 6 of 11 inoculated SM within 7–8 days post inoculation (DPI) and the average survival time (AST) of SM was 7.7 days; number 13-479 killed 8 of 9 inoculated SM (6–7 DPI; AST 6.1 days); and number 13-502 killed 7 of 10 SM (6–8 DPI; AST 6.4 days). Interestingly, experimentally non-infected mothers of mice inoculated with homogenates from all three infective pools succumbed to infection seven to eight days after cannibalising their dead SM, and WNV was demonstrated by real-time RT-PCR in high concentration (10^7 RNA copies/ml) in the mothers' brains but not in their livers or spleens. This finding supports the hypothesis of oral infection as a (rare) alternative route of WNV transmission, for example, in raptors.

Phylogenetic analysis based on complete WNV-2 genome sequences demonstrated that the four Czech WNV strains identified form two closely related groups: number 13-104 (GenBank: KM203860) with number 13-502 (GenBank: KM203863) and number 13-329 (GenBank: KM203861) with number 13-479 (GenBank: KM203862) and that they cluster together with WNV strains from an Austrian goshawk (isolated in 2008; GenBank: KF179640), Serbian *Cx. pipiens* (in 2012; GenBank: KC407673) and Italian human (in 2011; GenBank: JN858070), while they differ partially from

Figure 2

Phylogenetic positioning of four West Nile virus strains identified in *Culex modestus* mosquitoes, South Moravia, Czech Republic, August 2013



WNV: West Nile virus.

The complete genome nucleotide sequences of the four WNV strains from the Czech Republic (marked in red) were analysed together with representative lineage 2 WNV strains by the neighbor-joining method. GenBank accession numbers, isolation sources, countries of origins and isolation years are indicated at the branches. Supporting (>70%) bootstrap values of 1,000 replicates are displayed at the nodes. The horizontal bar shows genetic distance.

other European WNV-2 strains compared. However, they are all in the same clade (i.e. central and south European WNV-2), while WNV-2 strains from Africa and Russia form distinct clades (Figure 2). Maximum likelihood analysis resulted in a similar tree topology. Although three of the four Czech isolates were found to be neuropathogenic in SM, these virus strains do not carry the putative virulence marker P249 within the NS3 region [17,18].

Conclusions

The discovery of WNV-2 in the Czech Republic has added another country to the list of WNV risk areas in Europe. It also shows that two different lineages of WNV (lineages 2 and 3) co-circulate in the country and that *Cx. modestus* mosquito is a potential vector of WNV in reed belts of South Moravian fish ponds. This ornithophilic mosquito might play an important role in the bird–mosquito cycle of WNV in central Europe.

Our study highlights the need for epidemiological surveillance of (re-)emerging mosquito-borne viruses in central Europe. The seasonal peak activity of the adult *Cx. modestus* population in central Europe is from the beginning of July to late September [19]. Usually, the females do not enter buildings, but readily bite humans outdoors often during the day, at sun- and wind-exposed places, causing a nuisance, especially in late summer when floodwater *Aedes* and *Ochlerotatus* mosquito species have already vanished [19]. The isolation of neuroinvasive WNV strains in South Moravian fish ponds (in a popular recreational and camping area during the summer) raises the question of a possible risk of a local WNF outbreak. Given the mild climate of the 2013–14 winter, we can only speculate on the possible emergence of WNF in this year's WNV season, if favourable conditions for mass breeding of mosquitoes occur. To date, no human WNF cases have been recorded this season, which has just begun (the WNV season in central Europe starts mid-July and the majority of cases are seen in September). While infectious disease specialists in the region are aware of the WNV situation, local general practitioners should also be aware of the circulation of WNV in this area and take it into account during differential diagnosis of late-summer neuroinfections.

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Conflict of interest

None declared.

Authors' contributions

IR, ZH: designed, coordinated and supervised the study, performed laboratory testing, and wrote the manuscript; TB, JM: carried out sequence analysis, processed phylogenetic data, read and revised the manuscript; LB, HB, JP, PS, KV: trapped the mosquitoes, performed molecular analyses, read and revised the manuscript; OS: trapped the mosquitoes and performed their identification, read and revised the manuscript; NN: analysed data, wrote and revised the manuscript.

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PRÁCE 31

Hubálek Z., Šebesta O., Peško J., Betášová L., Blažejová H., Venclíková K., **Rudolf I.** 2014. Isolation of Ťahyna virus (California Encephalitis Group) from *Anopheles hyrcanus* (Diptera, Culicidae), a mosquito species new to, and expanding in, Central Europe. *J. Med. Entomol.* 51: 1264-1267.

Stručná charakteristika: cílem práce bylo vyšetřit patrně nedávno introdukovaný druh *An. hyrcanus* (4568 jedinců) na arboviry pomocí izolačních pokusů na sajících myších a molekulárních metod.

Hlavní přínos práce: podařilo se poprvé izolovat patogenní arbovirus Ťahyňa (2 kmeny) v komárech *An. hyrcanus* v Evropě. Tento druh komára se tak díky své potravní preferenci (mammalofilní) může stát alternativním vektorem pro přenos viru Ťahyňa u nás. *An. hyrcanus* byl patrně teprve nedávno introdukován na naše území a dle našich entomologických výzkumů dokonce rozšiřuje svůj areál. Nedávno introdukovaný komáří druh se tak může stát efektivním vektorem onemocnění, které je dlouhodobě etablováno na daném území.

Příspěvek autora k dané práci: autor se podílel na molekulární analýze komárů (detekce viru) a na přípravě rukopisu.

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Impakt faktor (IF_{2015}): 1,712

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Isolation of Tahyna Virus (California Encephalitis Group) from *Anopheles hyrcanus* (Diptera, Culicidae), a Mosquito Species New to, and Expanding in, Central Europe

Author(s): Z. Hubalek, O. Sebesta, J. Pesko, L. Betasova, H. Blazejova, K. Venclikova and I. Rudolf
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Isolation of Tahyna Virus (California Encephalitis Group) From *Anopheles hyrcanus* (Diptera, Culicidae), a Mosquito Species New to, and Expanding in, Central Europe

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J. Med. Entomol. 51(6): 1264–1267 (2014); DOI: http://dx.doi.org/10.1603/ME14046

ABSTRACT Two strains of Tahyna virus (TAHV; *Orthobunyavirus*, Bunyaviridae) were isolated from 4,568 (92 pools) female *Anopheles hyrcanus* Pallas (Diptera, Culicidae) mosquitoes collected on the fishponds in South Moravia (Czechland, central Europe) during July–August 2013. This is the first isolation of TAHV from *An. hyrcanus* in Europe. *An. hyrcanus* is a species new to Czechland since 2007; its population density was very high in the year 2013 at these ponds. The virus isolation procedure was based on intracerebral inoculation of newborn mice; moreover, the positive pools were also tested by polymerase chain reaction and found to contain TAHV RNA. *An. hyrcanus*, feeding preferentially on mammals including humans, may be a new potential vector for TAHV in Europe.

KEY WORDS Tahyna virus, *Orthobunyavirus*, mosquito, *Anopheles hyrcanus*

Anopheles hyrcanus Pallas (Diptera: Culicidae), a potential vector of malaria, occurs in Asia, southern Russia, Ukraine, throughout the northern Mediterranean (Spain, southern France, Italy, Greece, and Turkey; Ramsdale and Snow 2000, Becker et al. 2010), but it has recently been reported also from central Europe: Hungary (Tóth 2003), Slovakia (Halgoš and Benková 2004), Czechland—the geographic term for the Czech Republic (Votýpka et al. 2008, Šebesta et al. 2009) and Austria (Lebl et al. 2013). At fishponds in south Moravia (Czechland), where *An. hyrcanus* appeared around 2005 (J. Votýpka, personal communication) and occurred sporadically until 2012, its population increased abruptly and 29% of all caught mosquito females belonged to this species in 2013. We were therefore interested whether this insect could transmit pathogenic arboviruses in Moravia, where several mosquito-borne viruses (Tahyna and West Nile) were already found to circulate (Hubálek et al. 2010). This article describes the results of virus isolation attempts aimed at local *An. hyrcanus* mosquitoes carried out in 2013.

Materials and Methods

Study Site. Mosquitoes were caught for virological examination within reed belts of fishponds "Nesyt" (48° 46′35″ N, 16° 42′05″ E; 176 m above sea level) and "Nový" (48° 46′57″ N, 16° 40′13″ E; 177 m above sea level) at Mikulov in the district of Breclav, south Moravia (Czech Republic) during July and August 2013. The local climate is relatively warm and dry: mean annual air temperature is 9.1°C (−1.8°C in January, 19.2°C in July), mean annual precipitation 571 mm (range, 284–919 mm; the mean rain in the vegetation period from April to September is 320 mm). The fishponds are surrounded by fields (corn, maize, and sugar beet), with scattered solitary deciduous broadleaved trees, shrubs or their small clumps, orchards, gardens, and vineyards. A very characteristic plant community on the fishponds is the alliance *Phragmition communis* Koch forming dense and tall (2.8–3.6 m in the littoral zone) reed-beds, covering *c.* 15% of the total pond areas. Mammalian fauna of the study site consists of 33 spp. (*Insectivora* 6, *Chiroptera* 5, *Lagomorpha* 2, *Rodentia* 11, *Carnivora* 8, and *Artiodactyla* 1). Domestic rabbit, pig, and cattle farming occurs in Sedlec village situated directly at the northwest bank of the Nesyt fishpond, while goats and sheep are infrequent. Thirty species of birds have been recorded as breeding in the reed belt; in addition, 51 other, largely terrestrial, avian species breed in close surroundings of the fishpond. Further, additional 54 wild bird species have been recorded as visiting this habitat during seasonal movements—the ponds represent an important resting place especially for waterfowl migrants. The local mosquito fauna involves 30 species of the genera *Anopheles*, *Aedes*, *Ochlerotatus*, *Culex*, *Culiseta*, *Coquillettidia*, and *Uranotaenia* (Šebesta et al. 2012).

Mosquito Collections. Mosquitoes were captured in Centers for Disease Control and Prevention miniature light-CO_2 (dry ice)-baited traps (BioQuip Products Inc., Rancho Dominiquez, CA) exposed at 1 m of height regularly at 2-wk intervals from April to October. The traps were run from 1600–0900 hours on

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two successive nights. The trapped insects were then transported to the laboratory in cooled flasks, and stored at −65°C until examination.

Virus Isolation Procedures. Mosquitoes were sexed and identified (Becker et al. 2010) on a cooled plate under stereomicroscope, and monospecific pools consisting of 50 *An. hyrcanus* females were homogenized in 1.5 ml of cooled phosphate buffered saline (PBS) at pH 7.4 supplemented with 0.4% bovine serum albumin fraction V (Sigma-Aldrich, Saint Louis, MO), penicillin (500 i.u./ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml; PBS). The homogenates were centrifuged at 1,500 *g* for 20 m (at 0°C), and the supernatants inoculated intracerebrally (i.c., 0.02 ml) in SPF suckling ICR mice (SM) 2–3 d old (purchased from the Laboratory Animal Breeding Facility, Medical Faculty, Masaryk University, Brno, Czech Republic). The mice were observed for 20 d postinoculation (DPI); the brains of dead animals were homogenized in PBS, centrifuged, and passaged i.c. in a new batch of suckling mice. Bacterial sterility of the suspensions was checked in meat–peptone broth and thioglycollate broth incubated at 37°C.

From each mosquito suspension, 200-μl aliquots were left aside, frozen and maintained at −65°C for a later molecular analysis of viral RNA by reverse transcriptase–polymerase chain reaction (RT-PCR).

Virus Identification by Neutralization. The viral isolates were identified by the constant serum–serial virus dilution neutralization test on cell cultures (Lennette and Schmidt 1969). Infective mouse brain homogenate was serially 10-fold diluted from 10^{-2} to 10^{-8} in L-15 medium containing 2% heat-inactivated fetal calf serum (FCS, Sigma); 30 μl of the virus dilutions were pipetted in microplates with 96 flat-bottomed wells (Sarstedt), mixed with 30 μl of heat-inactivated (56°C for 30 m) normal or immune (to various arboviruses) murine sera prepared by three intraperitoneal doses at weekly intervals in our laboratory or mouse immune ascitic fluids (IAFs, received from the Ivanovsky Institute of Virology in Moscow), that were diluted 1:4 in L-15 medium. The virus–serum mixtures were incubated at 37°C for 60 m, 60 μl of the Vero E6 trypsinized cell suspension (≈20,000 cells) in L-15 medium with 2% FCS was then added to each well, incubated at 37°C for 4 h, and overlayed with 120 μl of 0.75% carboxymethyl cellulose in L-15 medium. The microplates were sealed in plastic bags, incubated at 37°C for 4 d, and stained with naphthalene black solution (Hubálek et al. 1979). The $\log_{10}$ neutralization indices (NIs: titers with immune serum or IAF vs. normal mouse serum) were estimated for each virus isolate, and log NI ≥2.0 values were regarded as decisive for the virus identification (Lennette and Schmidt 1969). The immune sera used in assays were prepared against the bunyaviruses Tahyna (TAHV, strain T16), Batai (strain Calovo), and Sedlec (strain AV172), and flavivirus West Nile strain Eg-101 (lineage 1), while the used IAFs were prepared against alphavirus Sindbis and flavivirus dengue-1.

RT-PCR Procedure. The virus-positive mosquito pools were tested for TAHV by virus-specific RT-PCR. Viral RNA was extracted from 140 μl of the mosquito homogenate by using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Specific oligonucleotide primers for TAHV in mosquitoes were: forward primer TahS226f (5′-AAGCTGCTCTCGCTCGTAAG-3′) and reverse primer TahS972r (5′-GTGTGCTCCACTGAATACCT-3′; Hubálek et al. 2010). Continuous RT-PCR system encompassed the QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Each 25-μl reaction mixture contained 5 μl of 5× buffer (final $MgCl_2$ concentration 2.5 mmol/liter), 0.4 mmol/liter of each deoxynucleoside triphosphate, 20 pmol of the each primer, 1 μl of enzyme mix (containing Omniscript and Sensiscript Reverse Transcriptases and HotStarTaq DNA polymerase), and 2.5 μl of template RNA. Reverse transcription was carried out at 50°C for 30 m, followed by a denaturation step at 95°C for 15 m. Thereafter, the cDNA was amplified in 40 cycles of heat denaturation at 94°C for 40 s, primer annealing at 57°C for 50 s, and DNA extension at 72°C for 1 m, and the reaction was completed by a final extension for 7 m at 72°C (Bakonyi et al. 2005). The PCR assays were performed in a PTC-200 Gradient Thermal Cycler (MJ Research, Waltham, MA). The PCR products were then separated on 2% agarose gel, stained with nontoxic GelRed (Biotium, Hayward, CA) and visualized under UV light. DNA extraction, PCR handling as well as post-PCR procedures were done in separate rooms to avoid possible cross-contamination of the samples.

Results

Virus Isolation From Mosquitoes. In total, 4,568 female *An. hyrcanus* collected in 2013 were examined in 92 pools by i.c. inoculation of SM, and two viral isolates were obtained: isolate no. 13–42 from mosquitoes collected on the fishpond Nový on 21 August 2013, and no. 13–114 from mosquitoes collected on the fishpond Nesyt on 15 August 2013. The infectious (and bacteriologically sterile) mosquito suspension no. 13–42 killed all the 12 inoculated SM within 4–6 DPI; average survival time (AST) of SM was 4.4 d. A reisolation attempt with the original homogenate stored for an additional 50 d at −65°C resulted again in the death of all 10 inoculated SM (AST was identical, 4.4 d). Moreover, we titrated this original homogenate no. 13–42 in SM, and found that $SMicLD_{50}$ was $10^{-1.51}$ per 0.02 ml, i.e., $10^{-3.39}$ per 1.5 ml (the total volume of the homogenate). We can thus estimate that as many as 2,450 $SMicLD_{50}$ (i.e., ≈1,700 infectious virions, using multiplication of LD_{50} units by the coefficient 0.693; Cunningham 1973) were present in the infected mosquito of the positive pool. The other virus-positive suspension no. 13–114 killed all the 10 inoculated SM within 5–6 DPI; AST of SM was 5.1 d; during the next passage on SM, AST was reduced to 3.5 d.

Identification of Virus Isolates No. 13–42 and No. 13–114. The virus was identified by the constant serum-serial virus dilution neutralization microtest on Vero E6 cell cultures. LogNIs of immune sera or IAFs raised against arboviruses Sindbis, West Nile, dengue,

Batai, and Sedlec tested with both viral isolates were all <0.5, whereas log NI of immune mouse serum prepared against Tahyna virus was 4.5, indicating that the isolates are TAHV.

The virus-positive original mosquito pools no. 13–42 and no. 13–114 were also tested for TAHV RNA by virus-specific RT-PCR. TAHV RNA was detected in both homogenates, confirming the identity of the virus.

Discussion

Tahyna virus has been isolated in South Moravia repeatedly and frequently, mainly from its principal mosquito vector *Aedes vexans* (Meigen) (e.g., Kolman et al. 1964; Danielová et al. 1972, 1976; Rosický and Málková 1980). In 2006, minimum infection rate (expressed as the mean number of virus isolates per 1,000 mosquitoes tested) for TAHV and *Ae. vexans* was 0.58 (1:1,734) in the Breclav district (Hubálek et al. 2010), which is very similar to that found in 1997, when it was 0.60 (1:1,670) in the same area (Hubálek et al. 2000). In the current study, minimum infection rate for TAHV in *An. hyrcanus* has been slightly lower, 0.44 (1:2,284).

Tahyna virus is the causative agent of endemic "Valtice fever," which is a febrile illness lasting about a week, with fever, headache, myalgia, fatigue, pharyngitis, conjunctivitis, nausea, gastrointestinal difficulties, and sometimes also meningitis, and affecting mostly children (Bárdoš et al. 1975) and nonlocal persons in summer season when local mosquito population has the highest abundance. Interestingly, Valtice fever is similar to, but less severe than, the North American LaCrosse encephalitis occurring in the United States, and caused by a related *Orthobunyavirus* LaCrosse.

An. hyrcanus may be considered a potential vector for TAHV in central Europe, but this finding warrants further study. Previous isolation of TAHV from this mosquito species was only reported by Lvov et al. (1972) in Azerbaijan. The mosquito *An. hyrcanus* feeds preferentially on mammals including man (Balenghien et al. 2006, Ponçon et al. 2007, Aldemir et al. 2010, Becker et al. 2010).

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PRÁCE 32

Pachler K., Lebl K., Berer D., **Rudolf I.**, Hubálek Z., Nowotny N. 2014. Putative New West Nile virus lineage in *Uranotaenia unguiculata* mosquitoes, Austria, 2013. *Emerg. Infect. Dis.* 20: 2119–2122.

Stručná charakteristika: dosud bylo popsáno několik linií patogenního arboviru WNV. Cílem práce bylo zařadit do systému virů další WNV, který byl nedávno detegován v komárech *Uranotaenia unguiculata* ve východním Rakousku.

Hlavní přínos práce: na základě fylogenetických analýz se podařilo zařadit novou genomickou linii WNV (linie 9, alternativně 4b), pocházející z komára *Ur. unguiculata*. Patogenita této nové linie pro obratlovce včetně člověka bude předmětem dalších výzkumů.

Příspěvek autora k dané práci: autor se podílel na molekulární analýze komárů *Ur. unguiculata* na přítomnost WNV a na přípravě rukopisu.

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Putative New West Nile Virus Lineage in *Uranotaenia unguiculata* Mosquitoes, Austria, 2013

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West Nile virus (WNV) is becoming more widespread and markedly effecting public health. We sequenced the complete polyprotein gene of a divergent WNV strain newly detected in a pool of *Uranotaenia unguiculata* mosquitoes in Austria. Phylogenetic analyses suggest that the new strain constitutes a ninth WNV lineage or a sublineage of WNV lineage 4.

West Nile virus (WNV), the most widespread flavivirus, is distributed throughout Africa, Asia, Europe, and Australia, and since 1999, WNV has also been present in the Americas (*1*). Within the last 2 decades, WNV infection has caused an increasing number of cases of neuroinvasive disease in humans and become a substantial public health problem (*1*).

Up to 8 lineages of WNV, based on genetic differences, have been proposed (*1*,*2*) (Table 1). Lineage 1 is widely distributed and further divided into lineage 1a, which includes the American strains; lineage 1b, which is also referred to as Kunjin virus and mainly described in Australia; and lineage 1c, which is also referred to as lineage 5 and comprises isolates from India. Lineage 2 has been detected in Africa and several parts of Europe, lineage 3 (Rabensburg virus) has been isolated only in the Czech Republic, and lineage 4 has been reported from Russia (*3*). A putative sixth lineage, based on a small genome fragment, has been described from Spain (*4*), and putative lineages 7 (Koutango virus) and 8 have been reported from Senegal (*2*).

WNV is maintained in an enzootic cycle between mosquitoes and wild birds (*1*). In 2013, ≈100 *Uranotaenia unguiculata* Edwards, 1913, mosquitoes were trapped during mosquito-monitoring projects at Lake Neusiedl-Seewinkel National Park in Austria and near Sedlec in the Czech Republic. In Russia, *Ur. unguiculata* mosquitoes have been described as hosting lineage 4 WNV strains (A. Platonov, unpub. data) (GenBank accession nos. FJ154906–49 and FJ159129–31). To determine whether *Ur. unguiculata* mosquitoes in Austria and the Czech Republic also host WNV, we investigated the mosquitoes collected in 2013 for the presence of WNV, focusing on lineage 4 viruses.

The Study

During May–October 2013, ≈11,300 female mosquitoes belonging to 13 species were trapped at 4 sites in Lake Neusiedl-Seewinkel National Park in Burgenland State, Austria. Mosquito species were determined according to morphologic criteria (*5*). Individual mosquitoes were pooled by species and collection site and date. A total of 47 *Ur. unguiculata* mosquitoes were collected in Austria (12 pools, 1–12 mosquitoes/pool). The relative abundance of *Ur. unguiculata* mosquitoes among the total collected in Austria was 0.42%. During August 2013, ≈39,000 mosquitoes were trapped at 2 fish ponds (Nesyt and Novy) in Sedlec, Czech Republic, near the border with northeastern Austria. A total of 47 female *Ur. unguiculata* mosquitoes were grouped into 4 pools (2 with 1 mosquito each, 1 with 4 mosquitoes, and 1 with 41 mosquitoes). The relative abundance of *Ur. unguiculata* mosquitoes among the total collected in the Czech Republic was 0.12%.

The mosquito pools were homogenized in RNase-free water, and RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). The samples were screened for the presence of flavivirus nucleic acid by reverse transcription PCR, using universal flavivirus primers MAMD (*6*) and CFD2 (*6*,*7*) for amplification of a partial nonstructural protein (NS) 5 sequence. Results were negative for the samples from Czech Republic. One pooled sample from Austria was positive; the pool contained 9 mosquitoes that had been captured in late August in Illmitz, a village east of Lake Neusiedl (47.769997°N, 16.752887°E). We obtained the complete polyprotein coding sequence and partial 5′ and 3′ noncoding ends of this novel WNV strain (GenBank accession no. KJ831223), which was designated West Nile virus-*Uranotaenia unguiculata*-Lake Neusiedl-Austria-2013 (WNV-Uu-LN-AT-2013). Primer sequences and amplification protocols are available upon request.

The complete polyprotein gene sequence of WNV-Uu-LN-AT-2013 shares a maximum identity of ≈83% with lineage 4 WNV strains isolated from *Ur. unguiculata* mosquitoes and *Dermacentor marginatus* ticks in Russia (*3*). At the amino acid level, the entire polyproteins of WNV-

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Table 1. Overview of West Nile virus lineages

Lineage	Representative strain, location	GenBank accession no.
1a	NY 2000-crow3356, New York, USA	AF404756
1b	Kunjin virus, Australia	D00246
1c/5	804994, India	DQ256376
2	Goshawk-Hungary/04, Hungary	AAZ91684
3	Rabensburg virus 97–103, Czech Republic	AY765264
4/4a	LEIV-Krnd88–190, Russia	AY277251
6/4b, putative*	HU2925/06, Spain	GU047875
7 (Koutango virus), putative	Dak-Ar-D-5443, Senegal	EU082200
8, putative*	ArD94343, Senegal	KJ131502
9/4c, putative	WNV-Uu-LN-AT-2013, Austria	KJ831223

*Only partial sequence available.

Uu-LN-AT-2013 and the lineage 4 strains from Russia share ≈96% identity (Table 2). Compared with the Russian lineage 4 strains, a 1,813-nt fragment of the NS5-coding sequence for the putative lineage 6 WNV, isolated from *Culex pipiens* mosquitoes in Spain (*4*), shares slightly higher nucleotide and amino acid identities with WNV-Uu-LN-AT-2013 (Table 2).

Phylogenetic neighbor-joining trees were generated with MEGA5 software, using ClustalW alignments, 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model (*8*). One phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 32 WNV strains representing all previously described lineages for which complete polyprotein-encoding sequences are available. This tree also showed a close relationship between WNV-Uu-LN-AT-2013 and the lineage 4 WNV strains from Russia; however, the newly identified strain forms a distinct branch (Figure, panel A). A second phylogenetic analysis that included the proposed lineage 6 virus from Spain and that was based on 1,813-nt fragments of NS5 showed a close grouping of WNV-Uu-LN-AT-2013 virus from Austria, the virus from Spain, and the lineage 4 viruses from Russia; similarity was slightly higher between the viruses from Austria and Spain (Figure, panel B).

WNV-Uu-LN-AT-2013 encodes a polyprotein of 3,432 aa. The envelope protein carries 1 putative *N*-linked glycosylation site at asparagine residue N-154, which has been associated with increased WNV pathogenicity and neuroinvasiveness (*9*). The 3 highly conserved *N*-linked glycosylation sites at NS1 positions N-130, N-175, and N-207 in WNV strains were also calculated for WNV-Uu-LN-AT-2013 by using NetNGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc/). Glycosylation of NS1 at these 3 positions has been implicated in neuroinvasiveness (*10*), as has proline at NS1 aa position 250 (*11*), which is also present in WNV-Uu-LN-AT-2013. The NS2A-encoding nucleotide region contains a *foo* motif, which can mediate production of NS1′, a variant of NS1 that plays a role in neuroinvasiveness (*12*). A *fifo* motif, which has been described for the nonpathogenic mosquito-specific flaviviruses (*13*), could not be determined for WNV-Uu-LN-AT-2013.

Table 2. Sequence identities between the newly identified WNV strain from Austria, WNV-Uu-LN-AT-2013, and other strains representing different WNV lineages*

	Nucleotide identity or amino acid identity, %, by strain/lineage†‡										
Strain/lineage†	WNV-Uu-LN-AT-2013	1a	1b	1c/5	2	3	4	6 (Spain)§	7 (Koutango virus)	8¶	Usutu virus
WNV-Uu-LN-AT-2013		88.3	87.9	87.0	88.8	86.7	96.2	95.9	85.3	81.2	75.5
1a	76.2		97.6	93.4	94.0	90.4	88.6	91.7	89.2	92.4	76.3
1b	75.4	88.2		92.7	93.5	89.8	88.3	91.2	88.8	92.0	76.1
1c/5	76.3	80.5	79.7		92.1	88.8	87.4	89.1	87.7	91.2	76.1
2	77.0	79.8	79.6	79.1		90.9	89.2	92.6	89.3	92.0	76.0
3	75.9	78.3	77.3	77.3	78.7		87.0	91.4	86.6	89.2	75.5
4	82.8	76.6	76.0	76.2	76.9	76.5		95.0	85.5	81.0	74.7
6 (Spain)§	83.2	78.1	78.1	77.7	78.6	79.5	81.7		88.6	–	80.8
7 (Koutango virus)	75.1	77.7	77.4	77.0	77.8	76.3	75.6	78.0		86.8	75.3
8¶	72.7	78.4	78.0	77.3	78.4	77.7	72.6	–	77.4		76.3
Usutu virus	71.2	72.4	72.6	72.4	71.3	71.0	70.1	73.6	72.4	72.5	

*Alignments were performed by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). WNV, West Nile virus; WNV-Uu-LN-AT-2013, West Nile virus strain *Uranotaenia unguiculata*-Lake Neusiedl-Austria-2013; –, comparison between lineages 6 and 8 was not possible because the available partial sequences do not cover the same nucleotide regions.

†GenBank accession nos. are as follows for the polyprotein genes/polyproteins: WNV-Uu-LN-AT-2013 (KJ831223), lineage 1a (AF404756/AAM81752), lineage 1b (D00246/BAA00176), lineage 1c (DQ256376/ABC40712), lineage 2 (DQ116961/AAZ91684), lineage 3 (AY765264/AAW81711), lineage 4 (FJ159129/ACH99530), lineage 6 (Spain) (GU047875/ADD69956), lineage 7 (Koutango virus) (EU082200/ABW76844), lineage 8 (KJ131502/AHV83443), Usutu virus (AY453411/AAS59402).

‡Amino acid sequences (above the diagonal) and nucleotide sequences (below the diagonal) are based on complete polyprotein genes, with the exception of lineage 6 and 8 strains, for which only partial sequences were available.

§Comparison was based only on partial NS5 gene sequences.

¶Comparison was based only on complete envelope protein gene sequences.

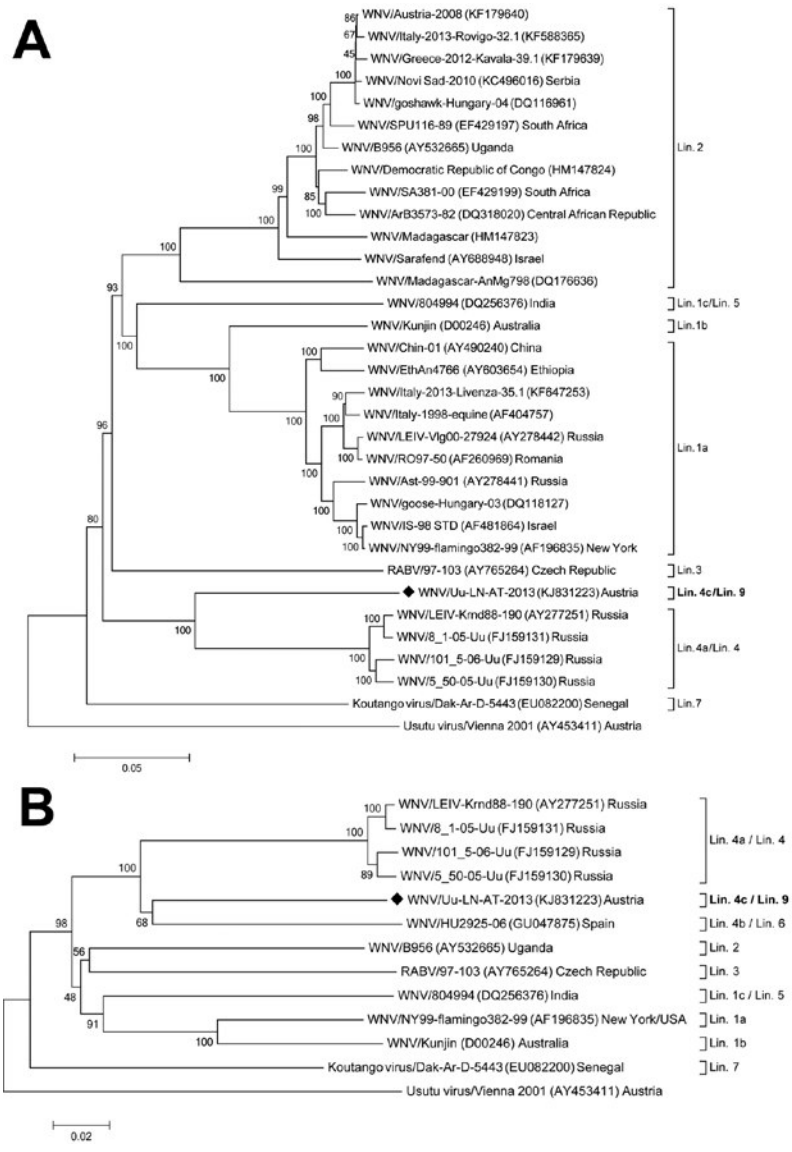

Figure. Phylogenetic positioning of WNV-Uu-LN-AT-2013, a West Nile virus (WNV) strain newly identified in Austria, within the species *West Nile virus*. A) Phylogenetic position as determined on the basis of the full-length polyprotein-coding nucleotide sequences. B) Phylogenetic position as determined on the basis of 1,813-nt fragments of NS5, which enabled inclusion of the proposed lineage 6 virus. The evolutionary history was inferred by using the neighbor-joining method of MEGA5 (*8*) with 1,000-fold bootstrap analysis, rooted against the respective sequence of Usutu flavivirus. Numbers next to the branches indicate the percentage of replicates in the bootstrap analysis. Black diamond indicates the WNV sequence determined in this study. GenBank accession numbers are shown in parentheses with the virus names. Scale bars indicate nucleotide substitutions per site. Lin., lineage; RABV, Rabensburg virus.

Conclusions

WNV lineages 1–4 and putative lineage 6 have been detected in Europe, but only WNV lineage 1a has spread across the American continents. Circulation of such a genetically diverse group of WNV strains in Europe may partly explain the epidemiologic differences observed between WNV disease in Europe and the Americas. In Europe, the presence of less pathogenic WNV strains may inhibit the spread of more pathogenic strains.

We propose that the WNV-Uu-LN-AT-2013 strain from Austria either constitutes a new lineage (lineage 9) or can be grouped into lineage 4 as sublineage 4c, with the strains from Russia and Spain as sublineages 4a and 4b, respectively. However, the short sequence available for the strain from Spain does not allow a clear-cut conclusion to be drawn with regard to lineage 4. We suggest that future designation of new WNV lineages should be restricted to viruses for which at least the complete polyprotein gene sequences have been determined. In addition, rules for defining virus lineages should be established by the International Committee on Taxonomy of Viruses.

Strain WNV-Uu-LN-AT-2013 has been detected only in *Ur. unguiculata* mosquitoes. These mosquitoes are mainly distributed in the southern half of Europe (*5*); in eastern Europe, they have spread from southern Ukraine and the Volga Delta through middle and southwestern Asia to Iran and Pakistan (*5*). In the Lake Neusiedl area of Austria, *Ur. unguiculata* mosquitoes seem to be an indigenous species, which was first reported in 1970 (*14*). In the Czech Republic, *Ur. unguiculata* mosquitoes have been detected only in Moravia, in the southern part of the country (*15*). Although there are anecdotal reports of *Ur. unguiculata* mosquitoes feeding on mammals, including humans, they feed mainly on amphibians and reptiles (*5*).

The pathogenicity of strain WNV-Uu-LN-AT-2013 in humans and animals has not been elucidated. Genetic data show that the strain carries typical WNV pathogenicity markers and suggest that WNV-Uu-LN-AT-2013 is not restricted to mosquitoes. Additional monitoring studies involving cell culture and animal isolation experiments are necessary to evaluate the pathogenic potential of this virus for humans and animals.

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PRÁCE 33

Kolodziejek J., Seidel B., Jungbauer C., Dimmel K., Kolodziejek M., **Rudolf I.**, Hubálek Z., Allerberger F., Nowotny N. 2015. West Nile virus positive blood donation and subsequent entomological investigation, Austria, 2014. *PLoS One.* 10: e0126381.

Stručná charakteristika: při přenosu WNV se kromě klasické cesty přenosu (sáním komára) uplatňují i další alternativní cesty, jako je např. přenos krevní cestou (transplantace, dárcovství krve). Některé země včetně Rakouska mají zavedenou účinnou kontrolu krevních derivátů na WNV, což bylo startovacím momentem pro danou studii.

Hlavní přínos práce: podařilo se izolovat a molekulárně charakterizovat kmen WNV linie 2 z plazmy asymptomatické dárkyně krve v Rakousku. Při následném entomologickém průzkumu v okolí jejího bydliště se podařilo odchytit komáry, u kterých byl zjištěn WNV (dokonce v nedospělých stádiích) a tak potvrzen autochtonní výskyt WNV. Práce má dva hlavní aspekty, které je nutné zdůraznit: v zemích s endemickým výskytem západonilské horečky je nezbytné monitorovat krevní deriváty na možný výskyt WNV. Druhým aspektem je záchyt viru v kukle a vajíčku komára *Cx. pipiens* naznačující možný transstadiální a transovariállní přenos WNV.

Příspěvek autora k dané práci: autor se podílel na izolaci WNV z plazmy pacienta (donora krve) a na přípravě rukopisu.

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RESEARCH ARTICLE

West Nile Virus Positive Blood Donation and Subsequent Entomological Investigation, Austria, 2014

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Abstract

The detection of West Nile virus (WNV) nucleic acid in a blood donation from Vienna, Austria, as well as in *Culex pipiens* pupae and egg rafts, sampled close to the donor's residence, is reported. Complete genomic sequences of the human- and mosquito-derived viruses were established, genetically compared and phylogenetically analyzed. The viruses were not identical, but closely related to each other and to recent Czech and Italian isolates, indicating co-circulation of related WNV strains within a confined geographic area. The detection of WNV in a blood donation originating from an area with low WNV prevalence in humans (only three serologically diagnosed cases between 2008 and 2014) is surprising and emphasizes the importance of WNV nucleic acid testing of blood donations even in such areas, along with active mosquito surveillance programs.

Introduction

West Nile virus (WNV) is the most widespread flavivirus present in all continents except Antarctica. Up to 9 different genetic lineages have been described so far [1]; medically most important are lineages 1 and 2. WNV is maintained in a mosquito-bird transmission cycle, humans and horses are considered dead-end hosts. Most human infections are asymptomatic, however approximately 20% of cases develop a febrile illness with flu-like symptoms (West Nile fever, WNF) and less than 1% West Nile neuroinvasive disease (WNND), the latter associated with a mortality rate of about 10% [2]. The vast majority of patients acquire WNV infection through the bite of an infected mosquito, mainly of the genus *Culex* [3]. Other routes of transmission include blood transfusion, solid organ transplantation, congenital infection, and laboratory

accidents [2]. Ticks may play an alternative role as vectors, especially in the introduction of WNV to new areas when attached to birds [4].

The first virologically confirmed WNV infections in Austria were reported for 2008 [5]. Goshawks proved to be especially vulnerable [5], [6]. In 2008, WNV nucleic acid was also detected in seven pools of adult female *Culex (Cx.) pipiens* mosquitoes [5]. Three autochthonous human WNV infections (two in 2009 and one in 2010; two cases of WNND and one case of WNF) were retrospectively diagnosed in the Greater Vienna area by specific serological testing [7].

In this paper we report on an acute WNV infection in a Viennese blood donor and the results of subsequent entomological investigations.

Material and Methods

Case record

As of June 2014, all blood donations originating from the province of Vienna are screened for WNV-RNA (PCR performed by the German Red Cross, Blood Service for Baden-Württemberg-Hessen, Frankfurt, Germany). A blood donation from 12 August 2014 tested positive for WNV-RNA. Serologically, the sample was WNV-IgM-positive and WNV-IgG-negative. Re-examination of the sample was performed at the Department of Virology of the Medical University of Vienna, the Austrian National Reference Laboratory for Flavivirus Infections. Following confirmation of the positive result, the Austrian Federal Office for Safety in Health Care (BASG), and the Austrian Federal Ministry of Health (BMG) were informed. Appropriate actions were taken according to the guidelines of the BMG from August 2014 (http://www.bmg.gv.at/cms/site2/attachments/2/7/5/CH1082/CMS1408527163324/westnilvirus_leitfaden_20140820.pdf), which correspond to the respective European legislation. On 19 August 2014 this case was also reported to ECDC (http://www.ecdc.europa.eu/en/healthtopics/west_nile_fever/West-Nile-fever-maps/pages/index.aspx).

Ethics statement

Written informed consent to conduct this study was obtained from the affected blood donor. This investigation was part of a non-research public health emergency response and therefore exempt from the Institutional Review Board process of the City of Vienna. The Ethics Committee of Vienna as the competent Institutional Review Board has previously stated in writing (decision number EK 13-151-VK_NZ of July 1, 2013) that public health surveillance and public health emergency responses performed by the Austrian Agency for Health and Food Safety (AGES) are exempted from the Institutional Review Board process of the City of Vienna.

Human WNV positive plasma sample

WNV-RNA positive plasma of the blood donor was provided by the Blood Service for Vienna, Lower Austria and Burgenland of the Austrian Red Cross. For PCR, pathogens in the sample were inactivated by immediately adding DNA/RNA Shield solution (Zymo Research, Irvine, USA) in the proportion 1:4 as described by [8], and stored at -20°C until further processing. The original plasma sample was independently investigated in the Czech laboratory for the presence of neutralizing antibodies against WNV strain Eg-101 by plaque-reduction neutralization test (PRNT) as described previously [9] and it was also used for virus isolation attempts (see below section "Virus isolation").

Mosquito collection

Following confirmation of the WNV-positive result of the human sample at the Medical University of Vienna, the BMG designated AGES to survey mosquitoes in close vicinity to the WNF patient's residence. The sampling started on 28 August 2014. The mosquitoes investigated in this study were collected on public land as part of the Austrian mosquito surveillance program. No additional permits to collect mosquitoes were required.

Trapping was performed using carbon-oxygen and attractants for pregnant female mosquitoes by BG-Sentinel traps (Biogents, Regensburg, Germany) and by manual exhauster. Because the field survey was carried out during a long-term rainfall period, when mosquitoes show low activity, the manual trapping was extended to aquatic mosquito stages. Collected mosquitoes were examined morphologically according to the identification key of Mohrig [10]. The specimens were sorted by species, their developmental stages, collection sites and dates, and pooled to a maximum number of 25 individuals.

Each mosquito pool was subsequently homogenized in an appropriate amount (300–700 μl, depending on the number of individuals) of cooled minimal essential medium (MEM, Gibco by Life Technologies, Grand Island, USA) supplemented with Earle's salts, non-essential amino acids (NEAA, 1%, Gibco by Life Technologies), and antibiotics. Homogenization was performed using an automatic TissueLyser II (Qiagen, Redwood City, USA) and Tungsten Carbide Beads 3 mm (Qiagen) followed by centrifugation at 10,000 x g for 5 min. All mosquito suspensions were stored at -80° C until processed.

Sample screening

For nucleic acid extraction 140 μl of each sample was used. All extractions were performed with the QIAamp Viral RNA Mini Kit (Qiagen, USA), following the manufacturer's instructions. For detection of both WNV lineages 1 and 2 in the samples, a RT-qPCR targeting the highly conserved 5´ non-coding region (NCR) was performed as described recently [4]. For confirmation and lineage determination a second RT-qPCR specific for WNV lineage 2 within the NS3 protein coding region was conducted as previously described [5].

Virus isolation

The WNV positive samples were subjected to virus isolation attempts by intracerebral inoculation into suckling mouse brain (SMB), as described earlier [11]. The bacteriologically sterile SMB suspension (designated SMB_1) was subsequently used for differential inoculation of adult (5–6 week old) ICR female specific-pathogen-free (SPF) mice. They were injected with 1% infectious SMB_1 suspension intracerebrally (i.c., 0.04 ml, under anesthesia), 0.2 ml intraperitoneally (i.p.) and 0.2 ml subcutaneously (s.c.), respectively. Each of the three groups consisted of 4 mice.

All experiments with laboratory mice were conducted in Valtice, Czech Republic according to the Czech Animal Protection Act no. 246/1992. The protocols were approved by the Institutional and Central Care and Use Committees at the Academy of Sciences of the Czech Republic in Prague and by the Veterinary Service in Brno. The facility in Valtice is accredited by the Czech National Committee on Care and Use of Laboratory Animals (6630/2008-10001).

RT-PCR, sequencing and sequence alignment

All positive samples and isolates were identified by various RT-PCRs targeting the complete WNV genome by employing published primer pairs specific for WNV lineage 2 [12], as well as

self-designed primers specific for the Austrian WNV strains (primer sequences available upon request).

The RT-PCR assays were carried out using One Step RT-PCR Kit (Qiagen, USA) following the manufacturer's instructions. Prior to sequencing, all specific amplification products were purified using PCR Kleen Spin Columns (BIO-RAD, Hercules, USA) following the manufacturer's protocol. The purified PCR fragments were then premixed with the corresponding individual PCR primers (concentration of 2 μM each) in a volume of 15 μl. Sequencing in both directions was performed by Microsynth (http://www.microsynth.ch/). The obtained WNV sequences were manually verified and compiled to continuous sequences. Thereafter nucleotide sequences of the new WNVs were submitted to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) for further comparison with other WNV sequences deposited in GenBank databases. All complete genomic WNV sequences were downloaded individually in FASTA format. Sequences determined in this study were then compared to each other and to sequences from GenBank by using the Align Plus 4 program (Scientific & Education Software). Their nucleotide identities were determined.

Polyprotein sequences and determination of pathogenicity and neuroinvasiveness markers

Translation of the new WNV sequences was carried out using the EMBOSS translation tools program (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Deduced entire polyprotein sequences were then compared to each other and to other polyprotein sequences deposited in GenBank databases employing the Align Plus 4 program (Scientific & Education Software) as described for the nucleotide sequences above.

To explore the pathogenicity and neuroinvasiveness markers of the newly determined WNV strains, predicted N-glycosylation sites of the relevant viral proteins E [13], [14] and NS1 [15] were analyzed using the program NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Certain amino acids (P249 in NS3 and P250 in NS1) assumed to be associated with increased virulence were explored according to [16] and [17], respectively, summarized in [18]. Ability for neuroinvasiveness was furthermore investigated by laboratory mice experiments as described above (see section Virus isolation).

Phylogenetic analysis

For phylogenetic analysis, nucleic acid sequences which encode the entire polyproteins but represent unique genomes were selected. For better resolution only WNV lineage 2 sequences were chosen, altogether 36 sequences (including sequences determined in this study). Synthetic constructs, incomplete (with gaps) and wrong (with Ns) sequences were excluded from the analysis.

Prior to phylogenetic analysis ClustalW multiple sequence alignments were conducted using BioEdit Sequence Alignment Editor Version 7.0.9.0. Several phylogenetic trees on both nucleotide and amino acid basis were constructed with the MEGA6 program [19] using the Maximum Composition Likelihood (MCL) and Kimura 2-parameter models of the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods. In each case bootstrap resampling analysis with 1,000 replicates was employed. The most likely tree was chosen.

Intra- and inter-sequence groups distances

The MCL and p-distance algorithms of the MEGA6 were conducted for the determination of genetic distances within and between nucleotide and amino acid sequence groups, respectively. For this purpose sequence groups according to the clustering of the phylogenetic tree were

defined. Furthermore, within the Central/Southern European cluster all new Austrian viruses and isolates and the nine most closely related viruses were subjected to detailed genetic distance analysis.

Molecular determination of mosquito species

For confirmation of the morphologic typing, WNV-positive mosquito samples were investigated by a PCR assay within the mitochondrial 12S rDNA gene recommended for molecular determination of widely divergent arthropods [20]. For the genomic DNA-PCRs a Fast Cycling PCR Kit (Qiagen, USA) was applied. The specificity of PCR products was verified by sequencing, as mentioned above (see section RT-PCR, sequencing and sequence alignment).

GenBank accession numbers

The newly described Austrian complete WNV sequences are available from GenBank under accession numbers KP109691 (WNV lin. 2, blood donor/Vienna/2014) and KP109692 (WNV lin. 2, *Cx. pipiens*/Vienna/2014). The 388 bp long 12S rDNA gene sequence of the WNV-positive *Cx. pipiens* is available in GenBank under accession number KP109693.

Results

Blood donor

The blood donor was a 44-year-old Viennese female. Three days after her blood donation on 12 August 2014 she developed myalgia, and later a generalized maculopapular rash. Her travel history outside Austria only revealed a trip to Barcelona, Spain, in February 2014. She remembered numerous mosquito bites in the weeks before blood donation when gardening at her home in the city of Vienna.

Human plasma sample

The neutralizing antibody titer of the human plasma sample against WNV by PRNT was 1:40.

The positive WNV-RNA results of this sample were confirmed by the two RT-qPCR assays described above, revealing WNV lineage 2. In addition WNV from the original plasma sample of the blood donor was successfully isolated in suckling mice: one of five mice died on day 6 post inoculation (p.i.). The brain suspension of the dead mouse SMB_1 was further passaged. In the next passage all of the 11 inoculated mice died on days 3 and 4 p.i. with neurological symptoms.

Further experimental inoculation of 1% centrifuged mouse brain homogenate of the SMB_1 plasma isolate into 12 adult SPF mice by 3 different routes caused death of all mice inoculated. I.c. inoculated mice died after 6–7 days (average survival time AST = 6.7 days), i.p. inoculated mice died after 6–11 days (AST = 8.5 days), and s.c. inoculated mice died after 10 and more days. The obtained isolates are subsequently named SPF i.c. and SPF i.p.

The presence of WNV-RNA in the plasma isolates SMB_1, SPF i.c. and SPF i.p. was confirmed by RT-qPCR.

By a series of specific RT-PCRs and subsequent sequencing the entire WNV genomes of the original plasma sample as well as of its isolates SMB_1, SPF i.c. and SPF i.p. were determined and compared to each other. Mutations were analyzed.

Mosquitoes

A total of 603 mosquitoes (when one egg raft was counted as one individual mosquito) were trapped in Vienna between 28 August and 10 September 2014, and assigned to 45 pools (Table 1).

Mosquito trapping was performed in five locations in Vienna, near to the patient's home: Baumgarten cemetery (48°12'07.7"N / 16°16'44.8"E; 8 pools, n = 122), Lainz zoo (48°12'09.3"N / 16°13'57.0"E; 6 pools, n = 102), Schönbrunn (48°10'55.5"N / 16°19'20.5"E; 11 pools, n = 109), Lobau Polzer (48°11'40.6"N / 16°28' 06.7"E; 1 pool, n = 2), and Ottakring cemetery (48° 12'52.6"N / 16°17'58.0"E; 19 pools, n = 268).

A total of 4 different mosquito species were morphologically identified: *Cx. pipiens* (41 pools, n = 595), *Anopheles (An.) maculipennis* group (2 pools, n = 6), *Culiseta (Cs.) annulata* (1 pool, n = 1), and *An. plumbeus* (1 pool, n = 1); thus *Cx. pipiens* represented 98.67% of our mosquito collection. All developmental stages were collected: egg rafts (4 pools, n = 11), larvae (25 pools, n = 421), pupae (8 pools, n = 130) and adult mosquitoes (8 pools, n = 41); therefore non-adult mosquitoes represented 93.20% of the mosquito collection.

Out of the 45 mosquito pools investigated, two pools proved positive for WNV lineage 2: one pool of 15 *Cx. pipiens* pupae, and one pool of two *Cx. pipiens* egg rafts, both collected on 08 September 2014 near Ottakring cemetery, 500m distance to the patient's home. Molecular determination confirmed the mosquito species.

The relative abundance of WNV in the investigated mosquito pools was 4.44%. The minimal infection rate (MIR) for all mosquitoes collected was 0.332 (converted to MIR per 1,000 mosquitoes, 0.551).

Virus isolation attempt on the *Cx. pipiens* pupae suspension failed; no suckling mouse of 10 inoculated died. The *Cx. pipiens* egg rafts suspension was not subjected to virus isolation due to unsufficient quantity and quality of the sample.

Out of the mosquito pool containing 15 *Cx. pipiens* pupae a complete WNV genomic sequence was determined, while only a few partial sequences (approx. 20% of the genome) could be obtained from the sample which contained two *Cx. pipiens* egg rafts due to above mentioned reason. However, their corresponding sequences were 100% identical to each other.

Comparison of human- and mosquito-derived WNV strains

While the viral loads in the human plasma sample and in the mosquito pool consisting of 15 pupae were identical (quantification cycle [Cq] for both = 32), the quantity of viral RNA was less in the sample containing the two egg rafts (Cq = 37). In the SMB_1, SPF i.p. and SPF i.c. plasma isolates 10^6-, 10^7- and 10^8-fold more viral RNA was detected than in the original plasma sample.

WNV genomes determined in this study were 10,988 nucleotides in length. The deduced complete polyproteins of all strains consisted of 3,434 amino acids, along which the three known flaviviral structural and eight non-structural proteins could be defined. The lengths of the corresponding individual proteins were: 123 (C), 167 (prM/M), 501 (E), 352 (NS1), 231 (NS2A), 131 (NS2B), 619 (NS3), 122 (NS4A), 27 (2K), 256 (NS4B), and 905 (NS5), respectively.

The Austrian human plasma-derived WNV showed the least nucleotide and amino acid divergences (0.2% and 0.1%, respectively) to WNV strains Cz 13–329 and Cz 13–479, both isolated in 2013 from *Cx. modestus* mosquitoes in the Czech Republic, belonging to the Central/Southern European WNV lineage 2 group (Table 2A, Fig 1). The Austrian mosquito-derived WNV nucleotide sequence exhibited the least nucleotide (0.3%) and amino acid (0.1%) genetic distance to the Czech strain Cz-104 and the Austrian goshawk-derived WNV (Table 2A, Fig 1). Detailed nucleotide and amino acid distances over the Austrian strains (including SMB_1

Table 1. Mosquitoes collected in the city of Vienna between 28 August and 10 September 2014.

No.	Location	Date	Species	Stage	Quantity	MEM [µl]
1	BFH	28.08.14	*Cx. pipiens*	L	20	600
2	BFH	28.08.14	*Cx. pipiens*	L	2	300
3	BFH	28.08.14	*Cx. pipiens*	L	20	600
4	BFH	29.08.14	*Cx. pipiens*	L	16	500
5	BFH	29.08.14	*Cx. pipiens*	L	20	600
6	BFH	29.08.14	*Cx. pipiens*	L	21	600
7	BFH	30.08.14	*Cx. pipiens*	E	5	300
8	BFH	30.08.14	*Cx. pipiens*	L	18	600
9	FH Ottakring	08.09.14	*Cx. pipiens* (+)	E	2	300
10	FH Ottakring	08.09.14	*Cx. pipiens*	E	2	300
11	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
12	FH Ottakring	08.09.14	*Cx. pipiens*	L	14	500
13	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
14	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
15	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
16	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
17	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
18	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
19	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
20	FH Ottakring	08.09.14	*Cx. pipiens* (+)	P	15	500
21	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
22	FH Ottakring	08.09.14	*Cx. pipiens*	P	15	500
23	FH Ottakring	08.09.14	*Cx. pipiens*	P	25	700
24	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
25	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
26	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
27	FH Ottakring	08.09.14	*Cx. pipiens*	L	15	500
28	Lainz Zoo	29.08.14	*Cx. pipiens*	E	2	300
29	Lainz Zoo	30.08.14	*Cx. pipiens*	L	20	600
30	Lainz Zoo	30.08.14	*Cx. pipiens*	L	20	600
31	Lainz Zoo	30.08.14	*Cx. pipiens*	L	20	600
32	Lainz Zoo	30.08.14	*Cx. pipiens*	L	20	600
33	Lainz Zoo	30.08.14	*Cx. pipiens*	L	20	600
34	Lobau Polzer	06.09.14	*An. maculipennis*	A	2	300
35	Schönbrunn	02.09.14	*Cx. pipiens*	L	15	500
36	Schönbrunn	02.09.14	*Cx. pipiens*	L	15	500
37	Schönbrunn	02.09.14	*Cx. pipiens*	L	20	600
38	Schönbrunn	02.09.14	*Cx. pipiens*	L	20	600
39	Schönbrunn	02.09.14	*An. maculipennis*	A	4	300
40	Schönbrunn	02.09.14	*Cs. annulata*	A	1	300
41	Schönbrunn	02.09.14	*Cx. pipiens*	A	1	300
42	Schönbrunn	02.09.14	*An. plumbeus*	A	1	300
43	Schönbrunn	03.09.14	*Cx. pipiens*	A	2	300
44	Schönbrunn	10.09.14	*Cx. pipiens*	A	15	500
45	Schönbrunn	10.09.14	*Cx. pipiens*	A	15	500

The two WNV-positive samples are marked with (+). Abbreviations used: BFH, Baumgarten Cemetery; FH Ottakring, Ottakring Cemetery; *An.*, *Anopheles*; *Cs.*, *Culiseta*; *Cx.*, *Culex*; A, adults; E, egg rafts; L, larvae; P, pupae; MEM, minimal essential medium.

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Table 2. Estimates of evolutionary pairwise distances A. over the Austrian strains (including the SMB_1 plasma isolate) and their nine closest relatives, B. five major groups (clades), and C. six minor groups (clusters) among clade 2d, all defined according to the clustering in the phylogenetic tree (Fig 1).

A

	At-bd	SMB_1	Cz 329	Cz 479	At-Cx	It AN2	It 32.1	It 33.2	It 34.1	Cz 104	Cz 502	At-gh
At-bd	-	0.0003	0.0009	0.0015	0.0015	0.0026	0.0023	0.0026	0.0035	0.0012	0.0018	0.0012
SMB_1	0.0001	-	0.0012	0.0018	0.0018	0.0029	0.0026	0.0029	0.0038	0.0015	0.0020	0.0015
Cz 329	0.0020	0.0021	-	0.0012	0.0012	0.0023	0.0026	0.0023	0.0032	0.0009	0.0015	0.0009
Cz.479	0.0018	0.0019	0.0021	-	0.0018	0.0029	0.0032	0.0029	0.0038	0.0015	0.0020	0.0015
At-Cx	0.0032	0.0033	0.0035	0.0031	-	0.0023	0.0026	0.0023	0.0032	0.0009	0.0015	0.0012
It AN2	0.0036	0.0037	0.0037	0.0035	0.0034	-	0.0038	0.0035	0.0044	0.0020	0.0026	0.0023
It 32.1	0.0042	0.0043	0.0047	0.0043	0.0043	0.0047	-	0.0003	0.0012	0.0023	0.0029	0.0026
It 33.2	0.0041	0.0042	0.0044	0.0040	0.0040	0.0044	0.0005	-	0.0009	0.0020	0.0026	0.0023
It 34.1	0.0046	0.0047	0.0049	0.0045	0.0045	0.0049	0.0010	0.0007	-	0.0029	0.0035	0.0032
Cz 104	0.0031	0.0032	0.0032	0.0030	0.0030	0.0034	0.0040	0.0037	0.0042	-	0.0006	0.0009
Cz 502	0.0037	0.0038	0.0038	0.0036	0.0036	0.0040	0.0046	0.0043	0.0048	0.0014	-	0.0015
At-gh	0.0028	0.0029	0.0029	0.0027	0.0027	0.0029	0.0037	0.0034	0.0039	0.0020	0.0026	-

B

	2d	2c	2bc	2b	2a
2d	-	0.0114	0.0121	0.0227	0.0335
2c	0.0689	-	0.0125	0.0226	0.0332
2bc	0.0744	0.0813	-	0.0239	0.0344
2b	0.1204	0.1255	0.0194	-	0.0401
2a	0.1988	0.2045	0.1999	0.2060	-

C

	2d-1	2d-2	2d-3	2d-4	2d-5	2d-6
2d-1	-	0.0063	0.0057	0.0054	0.0061	0.0067
2d-2	0.0257	-	0.0053	0.0050	0.0058	0.0063
2d-3	0.0238	0.0202	-	0.0042	0.0052	0.0058
2d-4	0.0353	0.0317	0.0194	-	0.0050	0.0053
2d-5	0.0410	0.0389	0.0264	0.0313	-	0.0055
2d-6	0.0394	0.0356	0.0240	0.0284	0.0311	-

The average numbers of substitutions per site between nucleotide and amino acid sequences are indicated below and above the diagonal, respectively. Estimation of evolutionary distances was conducted in MEGA6 [19] using the MCL and p-distance algorithms for nucleotide and amino acid sequences, respectively. Group 2d-1 contains the Central/Southern European viruses including Austrian strains, and group 2d-5 consists of the Eastern European WNVs.

Abbreviations used: At-bd = strain Blood donor/Vienna/2014Austria (KP109691), SMB_1 = first passage of the suckling mouse brain isolate from the Austrian blood donor's plasma, Cz 329 = strain Cz 13–329 (KM203861), Cz 479 = strain Cz 13–479 (KM203862), At-Cx = strain Cx pipiens/Vienna/2014Austria (KP109692), It AN2 = isolate Italy/2011/AN-2 (JN858070), It 32.1 = strain Italy/2013/Rovigo/32.1 (KF588365), It 33.2 = strain Italy/2013/Rovigo/33.2 (KF647249), It 34.1 = strain Italy/2013/Padova/34.1 (KF647251), Cz 104 = s train CZ 13–104 (KM203860), Cz 502 = strain Cz 13–502 (KM203863), At-gh = WNV strain Austria/2008_goshawk (KF179640).

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plasma isolate) and their nine relatives defined according to the clustering in the phylogenetic tree (Fig 1) are indicated in Table 2A.

The comparison of the entire polyprotein sequences of the Viennese human- and mosquito-derived WNV strains revealed 36 nucleotide (genetic distance 0.003) and five amino acid substitutions (genetic distance 0.001) which were found among the E (A 159 T, T 424 A) and

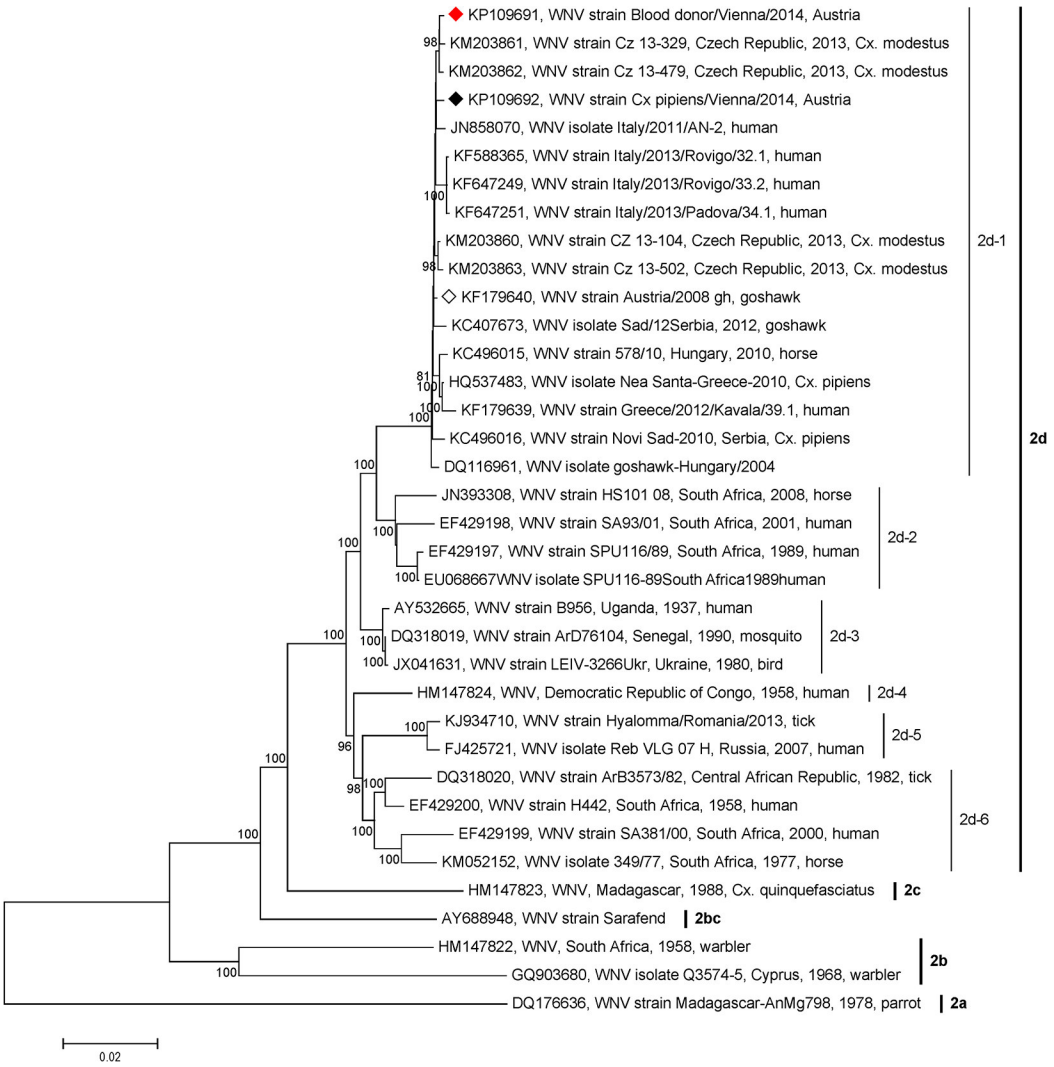

Fig 1. Phylogenetic tree of 36 West Nile virus lineage 2 full length polyprotein-coding nucleotide sequences. The sequences determined in this study are marked with a red diamond (Viennese blood donor-derived WNV) and a black diamond (Viennese *Culex pipiens*-derived WNV), respectively. The Austrian goshawk-derived sequence from 2008 is marked with a contoured diamond. Five major clades and six clusters among clade 2d are indicated. Group 2d-1 contains the Central/Southern European viruses including Austrian strains, and group 2d-5 consists of the Eastern European WNVs. The phylogenetic tree was constructed using the NJ method with MCL algorithm of MEGA6 [19] with 1,000-fold bootstrap analysis. GenBank accession numbers, strain names, and (if known) species, countries of origins and years of isolations are indicated at the branches. Supporting bootstrap values >80% (the percentage of replicates in the bootstrap analysis) are displayed next to the nodes. The horizontal scale bar indicates genetic distances (here 2% nucleotide sequence divergence).

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NS5 (R 314 K, R 576 Q, K 638 E) genes. All remaining genes exhibited identical amino acid sequences.

The comparison of the entire polyprotein sequences of the Viennese human WNV strain and its isolate SMB_1 revealed only one nucleotide substitution (T to A at position 1332 of the entire polyprotein coding nucleotide sequence), resulting in amino acid change asparagine (N) to lysine (K) at position 154 within the E gene. The subsequent WNV plasma passages SPF i.c. and SPF i.p exhibited 100% identity to isolate SMB_1.

Markers associated with increased pathogenicity and neuroinvasiveness could be identified in all Austrian WNV strains and isolates: three potential N-glycosylation sites at positions N-130, N-175, and N-207 [15] and proline at position 250 of the NS1 gene [17]. Proline at

position 249 of the NS3 gene responsible for higher strain virulence [16] was, however, not identified in any of the Austrian strains. At this position histidine was determined. Furthermore, while in both the original human plasma- and mosquito-derived sequences N-glycosylation motif NYS at positions 154–156 of the E protein were identified [13], [14], it was no longer found in the sequences obtained from SMB_1, SPF i.c. and SPF i.p. passages of the plasma isolate. As mentioned above, at position 154, lysine instead of asparagine was identified. Experimental infection of adult SPF mice with mouse brain homogenate SMB_1 resulted in 100% mortality.

Phylogenetic analysis and genetic distances

The phylogenetic tree based on nucleotide sequences coding for entire polyproteins constructed by the NJ method and MCL model of the MEGA6 program support a clear division of the WNV lineage 2 strains into the recently described four clades (2a-2d) [21]. While clades 2a and 2c consist of only one strain each (both from Madagascar), clade 2b is composed of two WNV strains, one from South Africa 1958 and the other from Cyprus 1968 (Fig 1). Central/Southern European strains (cluster 2d-1) cluster within the largest clade 2d together with Eastern European strains (cluster 2d-5) and several viruses mostly from Africa, isolated between 1937 and 2008 (clusters 2d-2, 2d-3, 2d-4 and 2d-6; Fig 1). As strain Sarafend was not included in the study by Mac Mullen [21], we denoted it clade 2bc due to its position between clades 2b and 2c. The division into the above clades and clusters was confirmed using the ML method of MEGA6 (data not shown). Similar clustering was also obtained by analyzing the corresponding entire polyprotein sequences (data not shown).

In the phylogenetic analysis both newly determined Austrian WNV strains cluster next to recently isolated Czech and Italian strains as well as to the 2008 goshawk-derived Austrian strain and other Central/Southern European lineage 2 strains, sorted by temporal sequence of WNV detections from 2004 to 2014 (Fig 1, cluster 2d-1).

The nucleotide and amino acid genetic distances over all sequence pairs between major sequence groups (clades) 2a-2d and 2bc as well as between clusters 2d-1 and 2d-6 are shown in Table 2B and 2C, respectively. The least distances on both nucleotide and amino acid levels were calculated between clusters 2d-3 and 2d-4 (0.0194, 0.0042), and the maximum distances between clades 2a and 2b (0.2060, 0.0401). The genetic distance between the Central/Sothern (2d-1) and Eastern (2d-5) European lineage 2 sequence groups was calculated with 0.0410 (nucleotides) and 0.0061 (amino acids). The numbers of differences per site over all sequence pairs within clades 2b and 2d were 0.0983 and 0.0228 for nucleotides and 0.0242 and 0.0050 for amino acids, respectively. The numbers of differences per site over all sequence pairs within clusters 2d-1, 2d-2, 2d-3, 2d-5 and 2d-6 were 0.0045, 0.0124, 0.0016, 0.0054 and 0.0215 for nucleotides, and 0.0027, 0.0046, 0.0021, 0.0017 and 0.0055 for amino acids, respectively.

Discussion

The potential for WNV transmission by blood transfusion during the acute phase of infection, when infected individuals are asymptomatic but viremic, was first recognized in the United States [22]. Soon thereafter WNV transmission by organ transplantation was reported [23]. Twenty-three confirmed cases of WNV transmission by blood or blood components were documented in 2002 [23], resulting in the implementation of a stringent blood safety monitoring system in the U.S. ([24], http://www.cdc.gov/westnile/resources/pdfs/wnvguidelines.pdf).

Independent introductions of two different WNV lineage 2 strains from Africa to Europe occurred recently: the first strain was introduced to Central Europe (South-Eastern Hungary) in or before 2004 [12], dispersed all over Hungary and the eastern part of Austria in 2008 [5],

[6], has spread in the following years via the Balkan Peninsula [25] to Southern European countries [26], [27], and arrived to the Czech Republic in 2013 [11], while the other strain emerged in Eastern Europe (Russia and Romania) since 2007 and 2010, respectively [28], [29], [4]. Both strains have been responsible for several outbreaks in the EU with 128 autochthonous cases reported in 2011, 242 in 2012, and 228 in 2013 (http://www.ecdc.europa.eu/en/healthtopics/west_nile_fever/West-Nile-fever-maps/Pages/historical-data.aspx), with case fatality rates of 8% in Romania in 2010 [29], 15% in Greece between 2010 and 2011 [30] and about 10% in Italy between 2008 and 2012 [31]. In 2014, apart from the Austrian case, 73 further human cases of WNV infection have been reported in the EU and 136 in neighboring countries (http://www.ecdc.europa.eu/en/healthtopics/west_nile_fever/West-Nile-fever-maps/pages/index.aspx).

Nucleic acid testing (NAT) of blood supplies was initiated in Italy following the first human cases of WNND during the 2008 outbreak [31]. Since then, a total of 71 human cases of WNND have been reported in Italy until 2012 and 26 WNV positive blood donations could be detected by NAT [31].

In order to ensure safety and quality of the blood transfusion chain in Europe, a guidance was introduced at the European Union level (http://ec.europa.eu/health/blood_tissues_organs/docs/wnv_preparedness_plan_2012.pdf), which has been continuously updated.

As the incubation period of WNF is typically between 2 and 15 days, the use of NAT techniques has provided an opportunity to diagnose WNV in patients prior to the production of specific IgM antibodies, as the circulation of detectable levels of WNV RNA in blood occurs, on average, 4 days prior to the first detection of IgM antibodies [32]. WNV RNA generally became undetectable after 13.2 days [33], thus, the detection of both IgM antibodies and viral RNA of the Viennese patient indicates very recent infection.

As the Viennese blood donor was not abroad in the last 6 months before infection, it was considered an autochthonous case. This is supported by the identification of WNV-positive *Cx. pipiens* mosquitoes collected in the residential area of the blood donor. The genetic differences between the two virus strains are not surprising, as the co-circulation of similar, but not identical WNV strains in restricted areas, has been reported previously [11].

The Austrian WNV strains investigated here carry only a few suspected neuroinvasiveness and pathogenicity markers. Interestingly, the highly conserved N-glycosylation site N-154 of the E gene, which has been associated with significant human outbreaks including the North American epidemic [13] and which was initially identified in both the Austrian human plasma and mosquito pool, mutated to lysine (K-154) during the virus isolation process of the plasma sample in suckling and adult mice. Despite (or because) of lack of glycosylation of this site, the WNV plasma isolate turned out to be highly neuroinvasive for adult mice. Such a mutation was also observed among the Russian WNV strains isolated in Volgograd 1999 from human brains indicating their high neuroinvasivness and pathogenicity (GenBank acc. nos. AY277252 and AF317203) [21]. Studies in mice revealed that—while both non- and E-glycosylated WNV strains were equally neurovirulent—the latter were more neuroinvasive [13]. The WNV strain investigated in our study had fortunately caused only mild febrile illness in the Viennese patient, possibly related to the comparatively young age of the patient. In addition, we do not expect that the neuroinvasive properties of the plasma isolate were due to the presence of lysine at position E-154 or due to the lack of the N-154 glycan, however this exceptional point mutation observed in the present study requires further analysis.

Despite the limited number of mosquitoes collected for this study (45 pools), WNV was detected at least in two individuals. Compared to recent Czech and Hungarian studies, in which WNV-positive mosquitoes were found in four of 650 pools [11] and in three of 645 pools [34], respectively, the MIR in our mosquito collection seems to be relatively high. A possible

explanation for this phenomenon could be the fact that more than 90% of mosquitoes collected in Vienna represented non-adult individuals, including egg rafts, pupae and larvae, which may be progenies of a few infected adult mosquitoes. Most published WNV detections in mosquitoes were in adult individuals only, and reports of detections in different developmental stages of mosquitoes are scarce [35].

While over 65 mosquito species have been implicated in the transmission of WNV, the principal mosquito vector species are those belonging to the genus *Culex* [3], [33], Fig 1. Although *Cx. pipiens* are essentially ornithophilic mosquitoes, their blood meal may be taken from mammals, including humans [36]. Hence, infected females may contribute to avian and human infections by horizontal transmission of the virus during their blood feedings, but also to vertical virus transmission [37]. Vertically infected *Cx. pipiens* that entered diapause in late autumn are able to initiate infection in the following spring [37]. Thus, detection of WNV-RNA in egg rafts and pupae deposited by infected females during late summer or fall may provide evidence for the vertical passage of WNV to overwintering cohorts.

The detection of WNV in a blood donation originating from an area with rather low WNV prevalence in humans is surprising and emphasizes the importance of NAT screening of blood donations even in areas of low WNV prevalence, along with active mosquito surveillance programs.

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Author Contributions

Conceived and designed the experiments: JK NN FA ZH. Performed the experiments: JK BS ZH IR KD MK. Analyzed the data: JK. Contributed reagents/materials/analysis tools: CJ BS. Wrote the paper: FA NN CJ BS ZH IR KD MK.

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PRÁCE 34

Straková P., Šikutová S., Jedličková P., Sitko J., **Rudolf I.**, Hubálek Z. 2015. The Common Coot as sentinel species for the presence of West Nile and Usutu flaviviruses in Central Europe. *Res. Vet. Sci.* 102: 159–161.

Stručná charakteristika: vodní ptactvo hraje klíčovou roli v přenosu některých arbovirů v přírodním ekosystému. V práci jsme testovali séra 146 lysek černých (*Fulica atra*) na přítomnost specifických protilátek k WNV a USUV. Prevalence protilátek činila 1,4% k WNV, zatímco pro USUV byla překvapivě zjištěna prevalence 6,2%.

Hlavní přínos práce: podařilo se prokázat specifické protilátky k WNV a USUV u lysek černých naznačující jejich roli jako obratlovčích rezervoáru virů v endemických oblastech. Lyska černá by se mohla jevit i jako vhodný sentinel odrážející recentní aktivitu viru v dané oblasti.

Příspěvek autora k dané práci: autor se podílel na sběru sér lysek, jejich následném zpracování, vyhodnocení testů včetně přípravy rukopisu.

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Short Communication

The common coot as sentinel species for the presence of West Nile and Usutu flaviviruses in Central Europe

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ABSTRACT

We examined 146 common coots (*Fulica atra*) on fishponds in central Moravia, Czech Republic, for antibodies to West Nile (WNV) and Usutu (USUV) flaviviruses. Eighteen birds reacted in the plaque-reduction neutralization test against WNV; these WNV seropositive samples were then titrated in parallel against USUV and tick-borne encephalitis virus (TBEV) to exclude flavivirus cross-reactivity. Two birds (1.4% overall) had the highest titers against WNV while 9 birds (6.2% overall) were seropositive for USUV, and in 7 birds the infecting flavivirus could not be differentiated with certainty. Our results indicate that both WNV and USUV infections occur in common coots; these birds might serve as a 'sentinel' species indicating the presence of these viruses at fishpond and wetland habitats in Central Europe.

West Nile virus (WNV) and Usutu virus (USUV) belong to the genus *Flavivirus* (family *Flaviviridae*) (Hubálek, 2008). Both viruses circulate in nature between birds and bird-feeding mosquitoes. Migratory birds may be infected with WNV or USUV in their African wintering grounds and could carry the virus during spring migrations northward to European sites (Hannoun et al., 1972; Watson et al., 1972; Calistri et al., 2010).

Usutu virus is an African virus but in 2001 it emerged surprisingly in Austria, causing fatal outbreaks in blackbirds (*Turdus merula*) and some other avian species (Weissenböck et al., 2002). In the following years, it spread to Hungary, Italy, Switzerland, Germany, Spain and Czechland (Bakonyi et al., 2007; Calzolari et al., 2010, 2012; Manarolla et al., 2010; Steinmetz et al., 2011; Jost et al., 2011; Becker et al., 2012; Vazquez et al., 2011; Hubálek et al., 2014). WNV and USUV can circulate together in certain ecosystems (Calzolari et al., 2010). In contrast to WNV, USUV has rarely caused human disease — only in immunocompromised persons (Vazquez et al., 2011). However, neutralizing antibodies against Usutu virus were documented recently in sera of 3 patients with neuroinvasive disease (one patient presented with meningitis and two with meningoencephalitis) in Croatia (Vilibic-Cavlek et al., 2014).

In a previous study, we found that among 391 wild birds in Moravia (Czechland, i.e. territory of the Czech Republic), 13 had specific antibodies to WNV — including several common coots (*Fulica atra*), and one coot had specific antibodies also against USUV (Hubálek et al., 2008a). We decided to assess prevalence of antibodies against WNV and USUV in this particular bird species in Moravia by examining a greater number of individuals.

The birds were legally shot by fishermen and gamekeepers (they received a permit from Přerov and Kojetín municipalities) at fishponds in Záhlinice (49°17′ N, 17°29′ E) near Přerov in central Moravia, Czech Republic, during September to October 2011. The serum samples were maintained at −20 °C.

All serum samples were inactivated at 56 °C for 30 min and diluted 1:5 in Leibowitz L-15 medium. In a plaque-reduction neutralization microtest (PRNT: Hubálek et al., 2008a), the diluted serum samples (30 μl) were mixed in the microtiter plate wells with test dose of virus (30 μl, containing about 20 to 40 PFU) and incubated at 37 °C for 60 min. Three viruses were used for neutralization tests – WNV Eg-101, TBEV Hypr, and USUV 939 – all prepared as infected suckling mouse brain suspension in L-15 medium with 2% of fetal calf serum. During an initial screening (all sera diluted 1:5 and 1:10, i.e. final dilutions were 1:10 and 1:20), only WNV was used. Vero E6 cells grown at 37 °C for 3 days in L-15 medium with 10% fetal calf serum and antibiotics were added to each well and incubated at 37 °C for 4 h. After incubation, 120 μl of carboxymethylcellulose overlay was poured into each well, and after 3 to 5 days at 37 °C, the cells were stained with 0.1%

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solution of naphthalene black. The controls were titrations of test doses of the Eg-101 strain of WNV, immune mouse WNV reference serum, control negative bird serum and the cells without viruses (to reveal potential cytotoxic effect of individual avian sera). A 90% reduction of plaque-forming units (PFU) was used in this study as a measure of neutralization ($PRNT_{90}$), and reciprocal serum titers 20 or higher were considered positive. All WNV positive sera were then titrated in parallel with two other flaviviruses present in Czechland, i.e. USUV and TBEV, to exclude serological cross reactions.

Serum samples from 146 common coots (*F. atra*) were first examined for the presence of WNV antibodies. During this initial screening, 18 coots were positive for WNV. However, when these sera were titrated against all three viruses in parallel, 9 tested birds were found to have specific antibodies against USUV, two birds had specific antibodies against WNV, while the prevalence of antibodies in 7 birds could not be differentiated by PRNT with certainty (Table 1). Local circulation of WNV in Czechland was first proved indirectly in 1985 and then in 1990 by hemagglutination-inhibition test in free-living wetland birds and sentinel ducks on South Moravian fishponds (Hubálek et al., 1989; Juřicová and Halouzka, 1993; Juřicová et al., 1993). After a big flood in Moravia in 1997, a higher prevalence rate of arboviruses in local mosquitoes was observed, and WNV-3 (Rabensburg) was isolated in that area repeatedly (Hubálek et al., 2000; Bakonyi et al., 2005). Moreover, WNV-2 was detected in south Moravia recently (Rudolf et al., 2014). In another study, 13 WNV specifically seroreactive birds were found, including 5 common coots (Hubálek et al., 2008a). These common coots came from fishponds at Zahlinice near Přerov. Interestingly, WNV antibodies were detected in coots also in other countries — Spain (Figuerola et al., 2007), southern Russia (Lvov et al., 2008), Iran (Fereidouni et al., 2011) and India (Mishra et al., 2012).

There are not enough data on the prevalence of USUV and antibodies against it because USUV is relatively new to Europe. Weissenböck et al. (2013) did a retrospective analysis of archived bird tissue samples and found USUV to be present in northern Italy as early as 1996. In Austria, USUV is endemic since its first occurrence in 2001 (Chvala et al., 2007; Meister et al., 2008). Bakonyi et al. (2007) tested dead birds in Hungary between years 2003 and 2006: they found one positive blackbird in 2005 and six positive blackbirds in 2006. Llorente et al. (2013) tested in parallel antibodies against WNV, Bagaza virus and USUV in partridges and pheasants in South Spain and recorded overall prevalence 10% against USUV. Steinmetz et al. (2011) noticed a mass mortality due to USUV in wild and captive songbirds and owls around the Zurich Zoo in Switzerland. In 2010, a strain of USUV was isolated from mosquitoes *Culex pipiens pipiens* in Germany where the first dead bird (mostly blackbirds) cases appeared in 2011 (Jost et al., 2011; Becker et al., 2012). In the same year, several blackbirds killed by USUV were reported in Czechland (Hubálek et al., 2014). Recent evidence of USUV RNA in *Culex modestus* in South Moravian fishponds indicates possible establishment of this virus in that country (Rudolf et al., in preparation). It is interesting that USUV strains from Germany, Switzerland, Austria, Hungary, Italy and Czechland are nearly identical in nucleotide sequence. Serological surveys sporadically detected antibodies to USUV in wild and game birds in additional European countries — Great Britain (Buckley et al., 2003, 2006), Spain (Llorente et al., 2013) and Poland (Hubálek et al., 2008b).

Reports on mosquito-borne viruses in the target bird of this study – the common coot – are sporadic. In India, Mishra et al. (2012) did a serosurvey of 1058 wild birds for WNV: 26 samples (2.5%) were positive (including common coots). In southern Spain, a total of 1213 birds belonging to 72 species were examined during preliminary screening for antibodies against WNV and 43 common coots reached positive WNV titres ranging from 1:20 to 1:640 (Figuerola et al., 2008). On the basis of this finding they focused on coots in Doñana NP, Spain, and detected WNV seroconversion in nine birds during the 2004–2005 season (Figuerola et al., 2007). They also did parallel neutralization against USUV but all titers of 47 serum samples from the coots were higher to WNV than to USUV. According to an experimental study, American coots (*Fulica americana*) have very low competence to WNV (but only one bird was tested) and therefore another transmission mechanism should be taken into account, such as fecal–oral transmission of WNV (Komar et al., 2003). A very interesting finding is that of Alkhovskij et al. (2003) who detected RNA of WNV in 15% of coots examined in the Volga delta which might indicate significant role of common coots in circulation and spread of WNV in that region.

Detection of antibodies in migratory birds such as the common coot need not mean that the bird was infected at the place of sampling. For instance, the coots occurring in central Moravia during autumn migration (this study) breed in Czechland, but also in Poland and Baltic countries, while the coots breeding in Czechland usually migrate southwest to Austria, Switzerland, Italy, France and Spain (Cepák et al., 2008), where USUV might occur. This fact must be taken into account at interpretation of findings. Herein we examined serum samples obtained from 146 common coots in central Moravia for the specific WNV and USUV antibodies by $PNRT_{90}$. Two birds (1.4%) had specific antibodies against WNV and nine birds (6.2%) had specific antibodies against USUV.

In conclusion, common coots might serve as a 'sentinel' species indicating the presence of WNV and USUV at fishpond and wetland habitats in Central Europe and serological examinations of this species could be a potentially useful tool for surveillance of mosquito-borne viruses in Europe.

Table 1
Antibody reciprocal titers ($PRNT_{90}$) of 18 bird sera tested against three flaviviruses (West Nile, tick-borne encephalitis, Usutu). Specific reactions for particular viruses are printed in bold.

Bird no.	WNV	TBEV	USUV
42	20	<20	**80**
43	40	40	**80**
45	40	<20	40
46	40	20	**80**
47	40	20	40
50	40	80	80
56	40	40	**80**
57	20	<20	**80**
60	20	<20	**40**
155	20	20	**40**
175	40	<20	40
176	40	40	**80**
178	40	<20	**80**
179	20	<20	20
182	40	20	40
184	**160**	20	20
186	20	<20	20
187	**80**	20	20

Conflicts of interest

The authors declare that they have no competing interests.

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PRÁCE 35

Rudolf I., Bakonyi T., Šebesta O., Mendel J., Peško J., Betášová L., Blažejová H., Venclíková K., Straková P., Nowotny N., Hubálek Z. 2015. Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem. *Parasites&Vectors.* 8: 520.

Stručná charakteristika: v práci byla sledována prevalence patogenních flavivirů v komárech *Cx. modestus* v několika rákosinných biotopech Jižní Moravy, kde byl současně zjištěn WNV.

Hlavní přínos práce: jde o první detekci viru Usutu v rákosinném druhu komára *Cx. modestus* v celosvětovém měřítku. Na základě naší práce je možné zvážit sylvatický cyklus viru Usutu, který zahrnuje cirkulaci viru mezi komáry *Cx. modestus* a lyskami černými, zatímco v již popsaném urbánním cyklu mezi komáry *Cx. pipiens* a synantropními pěvci, zejména kosy.

Příspěvek autora k dané práci: autor se podílel na designu studie, extenzivním sběru komárů v průběhu několika sezón, molekulárních analýzách včetně jejich vyhodnocení a na přípravě rukopisu.

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RESEARCH Open Access

Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem

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Abstract

Background: Mosquito-borne flaviviruses are a major public health threat in many countries worldwide. In Central Europe, West Nile virus (WNV) and Usutu virus (USUV), both belonging to the Japanese encephalitis virus group (*Flaviviridae*) have emerged in the last decennium. Surveillance of mosquito vectors for arboviruses is a sensitive tool to evaluate virus circulation and consequently to estimate the public health risk.

Methods: Mosquitoes (*Culicidae*) were collected at South-Moravian (Czech Republic) fishponds between 2010 and 2014. A total of 61,770 female *Culex modestus* Ficalbi mosquitoes, pooled to 1,243 samples, were examined for flaviviruses by RT-PCR.

Results: One pool proved positive for USUV RNA. Phylogenetic analysis demonstrated that this Czech USUV strain is closely related to Austrian and other Central European strains of the virus. In addition, nine strains of WNV lineage 2 were detected in *Cx. modestus* collected in the same reed bed ecosystem.

Conclusions: This is the first detection of USUV in *Cx. modestus*. The results indicate that USUV and WNV may co-circulate in a sylvatic cycle in the same habitat, characterised by the presence of water birds and *Cx. modestus* mosquitoes, serving as hosts and vectors, respectively, for both viruses.

Keywords: *Culex modestus*, Usutu virus, West Nile virus, Flavivirus, Arbovirus, Surveillance, Mosquitoes

Background

Usutu virus (USUV) is a mosquito-borne virus (Japanese encephalitis group, genus *Flavivirus*; family *Flaviviridae*) that was originally isolated in Africa. In or before 1996, the virus was introduced to Europe [1]. It circulates in nature between birds (as amplifying hosts) and bird-feeding mosquitoes, principally *Culex* spp., as vectors. USUV is taxonomically and ecologically very similar to West Nile virus (WNV) [2, 3]. Contrary to WNV, USUV has rarely caused human disease – only in immunocompromised persons [4]. However, USUV antibodies were recently reported in three patients with neuroinvasive disease in Croatia [5].

In the Czech Republic, two strains of USUV were isolated from dead blackbirds (*Turdus merula*) in Brno, 2011 and 2012 [6]. In addition, specific neutralizing antibodies against USUV were found in common coots (*Fulica atra*) in Moravia [7, 8].

Neutralizing antibodies against WNV were rarely found in the local human population, but five cases of West Nile fever in humans were reported after heavy floods in 1997 [9]. More frequently WNV antibodies occur in apparently healthy wild birds in this region [7]. Three identical strains of WNV (proposed genomic lineage 3: Rabensburg) were isolated from *Culex pipiens* and *Aedes rossicus* mosquitoes in 1997, 1999 and 2006 [10]. In a previous study, we reported four strains of lineage 2 WNV from *Culex modestus* mosquitoes collected in reed beds at South-Moravian fishponds (Czech Republic) during August 2013 [11].

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Within the scope of the joint European EDENext project we extended our virological surveillance of local *Cx. modestus* mosquitoes for pathogenic flaviviruses, including USUV.

Methods

Study sites

Female mosquitoes were collected using CDC minilight CO_2-baited traps (EVS CO_2 Mosquito Trap, BioQuip Products Inc., United States) at three study sites (fishponds "Nesyt" and "Nový" near Sedlec, and "Mlýnský" near Lednice: 48°47′ N and 16°42′ – 16°49′ E; 175–177 m a.s.l.) in the district of Břeclav, South Moravia, Czech Republic, as described in a preceding paper [11], during July and August from 2010 to 2014. All study sites are characterised by reed bed ecosystem (*Phragmites communis* alliance) situated at the littoral zone of the fishponds (Fig. 1). Thirty species of birds have been recorded breeding in the reed bed, and an additional 54 wild wetland and terrestrial bird species visit this ecosystem during seasonal movements [12]. A characteristic mosquito species for this ecosystem in this part of Moravia is *Cx. modestus*. From an epidemiological point of view it is noteworthy that all study sites represent favourite recreational areas during the summer season.

Mosquito processing, RNA extraction, PCR and sequencing

Caught insects were transported to the laboratory in cooled flasks, and stored at −65 °C until examination. Mosquitoes were determined on a chilled table under a stereomicroscope according to an entomological key [13], and monospecific pools consisting of up to 50 female *Cx. modestus* (other species were not tested in this study) were homogenized in 1.5 ml of cooled phosphate-buffered saline pH 7.4 supplemented with 0.4 % bovine serum albumin (Sigma) and antibiotics (PBS-BSA), and centrifuged.

RNA was extracted from 140 µl mosquito homogenates using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Generic oligonucleotide primers targeting the NS5 region of flaviviruses were used for screening purposes [14]. Samples positive by the above pan-flavivirus PCR were subjected to USUV [15, 16] as well as WNV [11] -specific RT-PCR assays for amplification of overlapping genome regions. Amplification

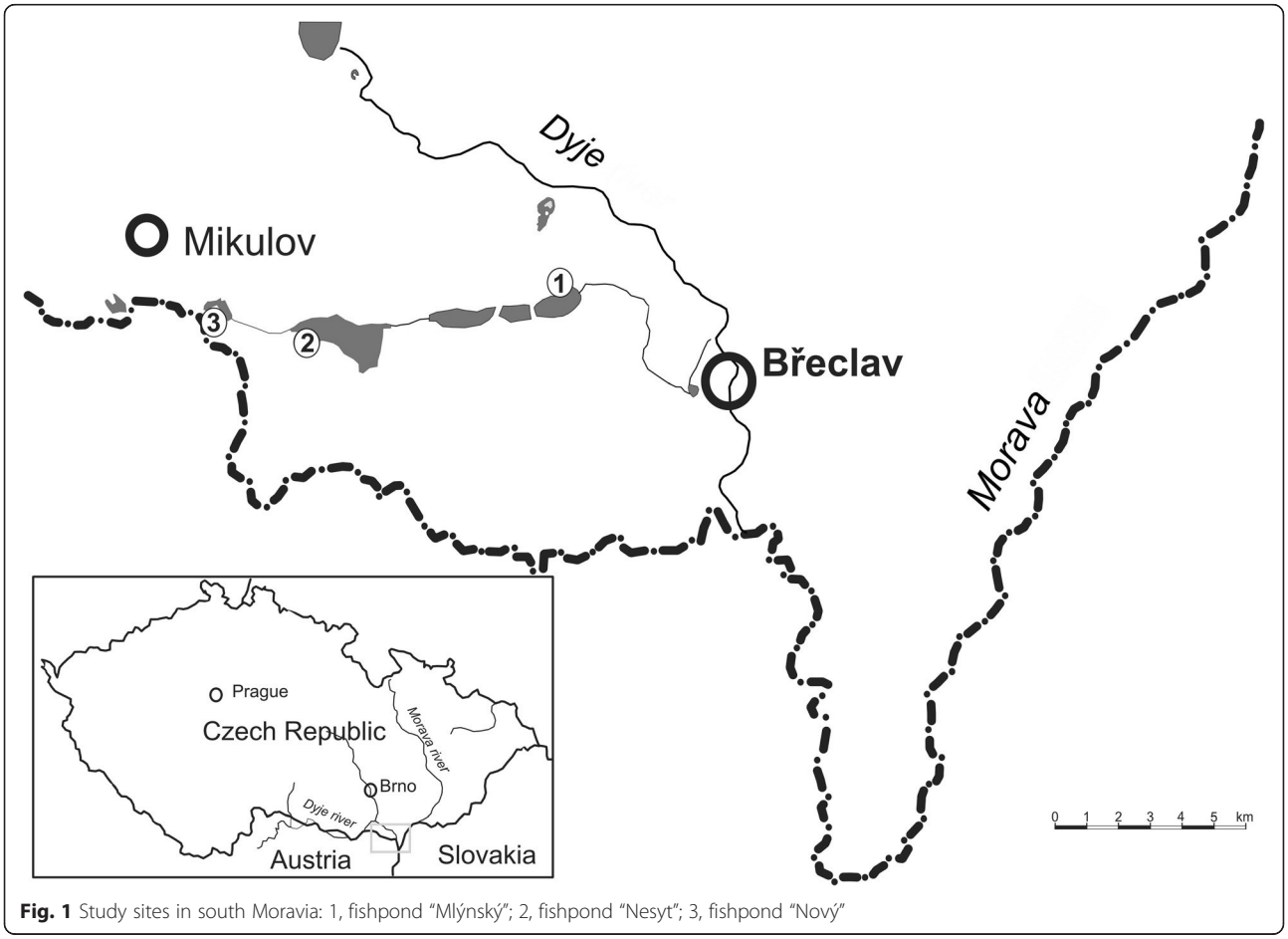

Fig. 1 Study sites in south Moravia: 1, fishpond "Mlýnský"; 2, fishpond "Nesyt"; 3, fishpond "Nový"

products were directly sequenced (Microsynth, Balgach, Switzerland); the sequences were aligned and compiled, and identified by BLAST search against GenBank database.

Phylogenetic analyses

Phylogenetic and molecular evolutionary analyses of concatenated sequences were conducted using neighbor-joining, maximum likelihood, minimum evolution, UPGMA and maximum parsimony algorithms (MEGA version 6, with 1000 replicates for bootstrap testing).

Virus isolation

The original mosquito homogenates of PCR-positive samples were inoculated intracerebrally (i.c., 20 μl) into specific pathogen free suckling ICR mice (SM). Brains of dead animals were homogenized in PBS-BSA, centrifuged, and passaged (i.c.) in a new batch of SM. Bacterial sterility of the suspensions was checked in meat-peptone and thioglycollate broths incubated at 37 °C [10]. All experiments with laboratory mice were conducted in accordance with the Czech Animal Protection Act no. 246/1992, and the protocols were approved by the Institutional and Central Care and Use Committees at the Academy of Sciences of the Czech Republic in Prague and by the Veterinary Service in Brno. The facility is accredited by the Czech National Committee on Care and Use of Laboratory Animals (6630/2008-10001).

Results and discussion

A total of 61,770 female *Cx. modestus* mosquitoes in 1,243 pools (including 32,500 individuals in 650 pools, collected in the same place and evaluated in 2014 [11]) were examined for flaviviruses by RT-PCR (Table 1). USUV RNA was detected in one pool (#13-662) of *Cx. modestus* collected at Mlýnský fishpond on 7 August 2013; the overall minimum prevalence rate of USUV in *Cx. modestus* was therefore 0.016 per 1,000 mosquitoes. When the mosquito homogenate #13-662 was inoculated into 13 SM, two of them were missing on day 4 p.i. (most probably cannibalized by the mother after they became ill or died). Repeated inoculation of another litter of 12 SM with the same homogenate was negative, all SM survived.

A total of 4218 nucleotides in five genome regions (corresponding to 38 % of the genome) of the USUV-positive pool #13-662 was determined. It revealed 12 substitutions when compared to the complete genome sequence of the USUV Vienna strain from 2001 ([2, 15]; GenBank: AY453411), thus indicating a high (99.7 %) nucleotide identity rate: genome region nt 1610–2980 (E–NS1; 1371 nt): 1 substitution; genome region 3021–3685 (NS1–NS2a; 665 nt): 2 substitutions; genome region 4444–5615 (NS2b–NS3; 1172 nt): 6 substitutions; genome region 6582–6907 (NS4a–2 K; 326 nt): no substitution; genome region 7351–8034 (NS4b–NS5; 684 nt): 3 substitutions. Nucleotide sequences were deposited in GenBank database under accession number KT445930. The phylogenetic relationship of the Czech *Cx. modestus*-derived USUV strain with other USUV strains is displayed in Fig. 2. Neighbor-joining, maximum likelihood, minimum evolution, UPGMA and maximum parsimony algorithms revealed almost identical trees; the phylogeny shown in Fig. 2 is based on the neighbor-joining algorithm. The Czech USUV clustered together with Austrian and Hungarian viruses detected between 2001 and 2005 [16]. However, recently identified USUV strains from Germany and Italy formed separate branches.

Also in South Moravia (Czech Republic), USUV strains had been previously isolated, namely from blackbirds, which were found dead in 2011 and 2012, respectively [6]. The 2011 isolate was sequenced in two regions: partial E + NS1 (GenBank: JX236666) and partial NS5-5′UTR (GenBank: JX236667). In the E + NS1 region there is a 1371 nt overlap (nts 1610–2980 referring to the USUV sequence AY453411) between the 2011 blackbird-derived USUV sequence [6] and the 2013 *Cx. modestus*-derived sequences (this paper), and in the E protein coding region of this overlap the two genomes differ in (only) three nucleotides at positions 1872 (T *vs.* C), 2322 (C *vs.* T), and 2419 (C *vs.* T), resulting in a 99.78 % identity between the two Czech USUV strains in this genomic region; none of the substitutions lead to putative amino acid changes.

Furthermore, WNV genomic lineage 2 (WNV-2) was detected in nine pools of *Cx. modestus* mosquitoes collected in August 2013: #13-104 (collected at Nový fishpond); #13-329 (coll. at Nesyt fishpond); #13-479 (coll. at Mlýnský fishpond); #13-502 (coll. at Mlýnský fishpond) (these four records were reported in a

Table 1 Numbers of female *Culex modestus* mosquitoes examined for flaviviruses in individual years and at three study sites

Fishpond	2010	2011	2012	2013	2014	*Total*
Nesyt	11 (1)	1,304 (27)	2,649 (54)	8,400 (168)	100 (2)	*12,464 (252)*
Nový	0	0	0	10,835 (217)	206 (4)	*11,041 (221)*
Mlýnský	5,450 (109)	533 (11)	4,079 (82)	22,050 (441)	6,153 (127)	*38,265 (770)*
Total	*5,461 (110)*	*1,837 (38)*	*6,728 (136)*	*41,285 (826)*	*6,459 (133)*	*61,770 (1243)*

Number of pools is shown in parentheses

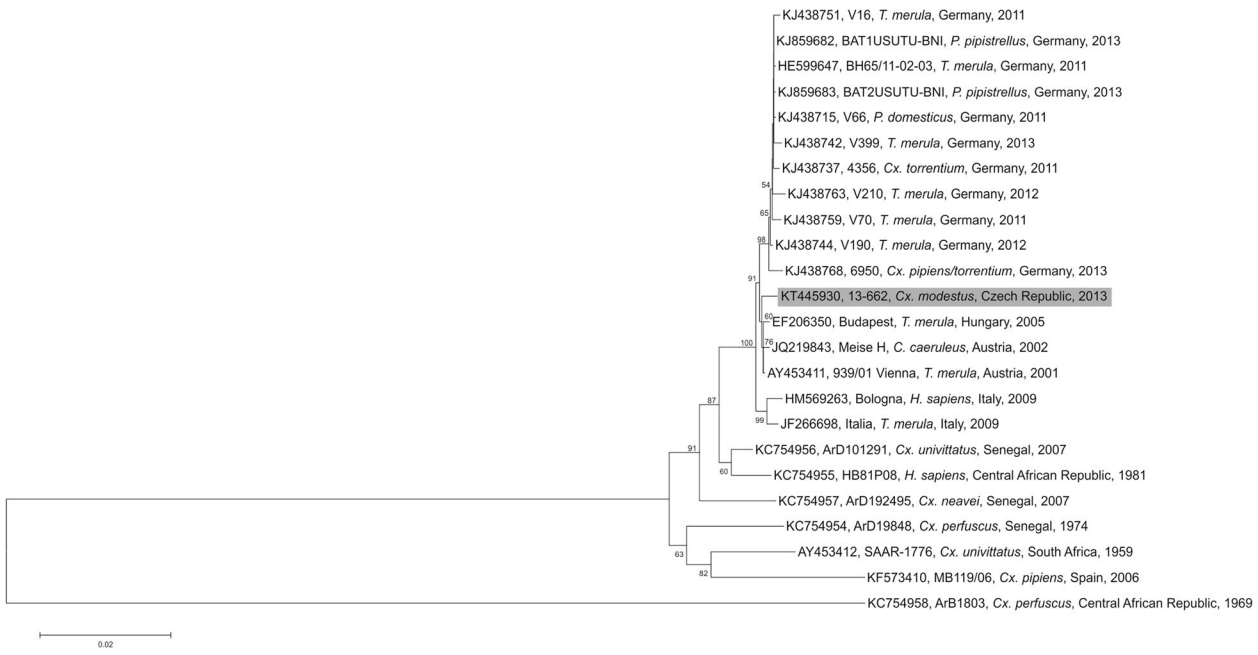

Fig. 2 Phylogram demonstrating the genomic relationships among Usutu viruses based on concatenated nucleotide sequences. Partial coding regions were E, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 proteins. Sequences are labelled by codes containing the GenBank accession number, the name of the virus strain, host (*T. merula*: *Turdus merula* [Blackbird], *P. pipistrellus*: *Pipistrellus pipistrellus* [Common pipistrelle], *P. domesticus*: *Passer domesticus* [House sparrow], *Cx.*: *Culex*, *C. caeruleus*: *Cyanistes caeruleus* [Blue tit], *H. sapiens*: *Homo sapiens* [Human]), country of origin, and year of sample collection. The Czech sequence described in this paper is highlighted with grey background. Bootstrap values ≥ 500 (50 %) are displayed. Bar on the left represents the genetic distance

previous study: [11]); #13-670 (coll. at Mlýnský fishpond); #13-743 (coll. at Nesyt fishpond); #13-853 (coll. at Mlýnský fishpond); #13-859 (coll. at Nesyt fishpond); #13-862 (coll. at Nesyt fishpond); the overall minimum prevalence rate of WNV in *Cx. modestus* was therefore 0.146 per 1,000 mosquitoes, about ten times higher than that for USUV. All WNV RNA positive original mosquito homogenates were then inoculated into SM. While the homogenates #13-329, #13-670, #13-743, and #13-853 did not kill any mice, the five others did: #13-104 killed 6 of 11 inoculated SM within 7–8 days post inoculation (DPI), average survival time (AST) of SM was 7.7 days; #13-479 killed 8 of 9 inoculated SM (6–7 DPI; AST 6.1 d); #13-502 killed specifically 7 of 10 SM (6–8 DPI; AST 6.4 d); #13-859 killed 5 of 11 SM (6–7 DPI; AST 6.4 d); and #13-862 killed all 11 inoculated SM (6–7 DPI; AST 6.7 d).

To the best of the authors' knowledge, this is the first detection of USUV in *Cx. modestus.* It indicates that USUV may co-circulate with WNV in certain habitats – this phenomenon was demonstrated previously in northern Italy, where the principal mosquito vector of USUV (and WNV as well) is *Cx. pipiens* [3, 17–21]. A comprehensive review on the co-circulation of the two arboviruses in Europe has recently been written [22]. Contrary to northern Italy, where USUV occurs in *Culex* mosquitoes much more frequently than WNV, reverse proportion was found in South Moravia in this study.

Interestingly, both viruses (USUV, WNV) were detected in South Moravia in 2013, but not in the years 2010, 2011, 2012 and 2014. This result could be affected by the number of *Cx. modestus* mosquitoes examined in individual years, which was much higher in 2013 than in the other years (Table 1). Moreover, mosquitoes were not collected in August 2014 (only in July).

Our previous finding that the common coot (*Fulica atra*) relatively often reveals specific antibodies to USUV [7, 8] might indicate a specific role of this avian species in the circulation of USUV in wetlands.

Conclusions

This is the first detection of USUV in *Cx. modestus.* The results indicate that USUV and WNV may co-circulate in a sylvatic cycle in the same habitat, characterised by the presence of water birds and *Cx. modestus* mosquitoes, serving as hosts and vectors, respectively, for both viruses. The present finding suggests that USUV (similar to WNV) may circulate in two types of ecosystems: (i) sylvatic cycle between *Cx. pipiens/ Cx. modestus* and water birds - such as coots, based on a previous serosurvey study [7]; (ii) urban cycle involving *Cx. pipiens* and blackbirds or occasionally some other synanthropic avian species.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
IR and ZH designed, coordinated and supervised the study, performed laboratory testing, and wrote the manuscript; TB and JM carried out sequence analysis, processed phylogenetic data, read and revised the manuscript; LB, HB, JP, PS and KV trapped the mosquitoes, performed molecular analyses, read and revised the manuscript; OS trapped the mosquitoes and performed their identification, read and revised the manuscript; NN analysed data, wrote and revised the manuscript. All authors read and approved the final manuscript.

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PRÁCE 36

Rudolf I., Šebesta O., Straková P., Betášová L., Blažejová H., Venclíková K., Seidel B., Tóth S., Hubálek Z., Schaffner F. 2015. Overwintering of *Uranotaenia unguiculata* adult females in Central Europe: a possible way of persistence of the putative new lineage of West Nile virus? *J. Am. Mosquito Contr. Assoc.* 31: 364–365.

Stručná charakteristika: při sběru a determinaci přezimujících komárů jsme nalezli jeden exemplář komára *Uranotaenia unguiculata* a tím prokázali jeho přezimování v podmínkách střední Evropy.

Hlavní přínos práce: podařilo se potvrdit data o přezimování druhu *Ur. unguiculata* ve Střední Evropě, které má další epidemiologické konsekvence pro možné přezimování viru WNV (linie 9) ve střední Evropě. Nová WNV linie byla teprve nedávno detegována právě v komárech *Ur. unguiculata*. Data byla doplněna o podobná pozorování ze sousedního Rakouska a Maďarska.

Příspěvek autora k dané práci: autor se podílel na designu a hodnocení studie a přípravě rukopisu.

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Overwintering of *Uranotaenia Unguiculata* Adult Females in Central Europe: A Possible Way of Persistence of the Putative New Lineage of West Nile Virus?

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SCIENTIFIC NOTE

OVERWINTERING OF *URANOTAENIA UNGUICULATA* ADULT FEMALES IN CENTRAL EUROPE: A POSSIBLE WAY OF PERSISTENCE OF THE PUTATIVE NEW LINEAGE OF WEST NILE VIRUS?

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ABSTRACT. We report the overwintering of *Uranotaenia unguiculata* adult females in Central Europe (Czech Republic, Hungary, Austria). This finding suggests a potential mode of winter persistence of putative novel lineage of West Nile virus in the temperate regions of Europe.

KEY WORDS Mosquito, mosquito-borne viruses, overwintering, *Uranotaenia unguiculata*, West Nile virus

Uranotaenia unguiculata Edwards is a common mosquito species throughout the Mediterranean region (Becker et al. 2010). In Europe, its northernmost distribution range reaches southern part of the Czech Republic (Ryba et al. 1974, Šebesta et al. 2013) and Germany (Becker and Kaiser 1995). In eastern Europe the species can be found in southern Ukraine and the Volga delta to the Caucasus (Gutsevich et al. 1974). Typical larval habitats for *Ur. unguiculata* are marshes, pools, ditches, or canals with stagnant or softly running water, all with rich aquatic vegetation (Schaffner et al. 2001, Becker et al. 2010). In the Czech Republic, the species is reported from southern Moravia province only. It has been observed as both larval and adult stages from July to September, typically in reed beds (Vaňhara 1991, Šebesta et al. 2013), where it often co-occurs with *Anopheles hyrcanus* (Pallas), *Culex pipiens* Linnaeus, and *Cx. modestus* Ficalbi. From an epidemiological point of view, *Ur. unguiculata* could act as a vector of a putative novel lineage of West Nile virus (WNV) in Central Europe (Kemenesi et al. 2014, Pachler et al. 2014).

To the best of our knowledge, there is little information about overwintering of *Ur. unguiculata* in Europe. In southern Europe, development is supposed to be continuous throughout the year (Schaffner et al. 2001). However, in southern France (Camargue), numerous females have been observed overwintering in sheltered places, often with standing water on the ground, and without evidence of continuous larval development (Mouchet and Rageau 1965). Scarce data are available for more temperate regions, but because of late-summer activity, it has been hypothesized that the species hibernates in the adult stage (Mihalyi and Gulyás 1963, Schaffner et al. 2001, Becker et al. 2010).

During long-term investigation and within the scope of European EDENext project, we collected overwintering putative WNV mosquito vectors in the Czech Republic, in order to elucidate possible WNV persistence in Central Europe. However, on basis of our unexpected finding, we decided to supplement data on overwintering *Ur. unguiculata* from 2 additional countries.

In the Czech Republic, overwintering female mosquitoes were collected by battery-operated aspirators from walls and ceilings of cellars in wine cellars, basements, and a castle underground. The collections were carried out at localities Sedlec, Lednice, Hlohovec, and Břeclav in southern Moravia province, from February to March during 2011 through 2014 (shortly before mosquitoes left their winter hibernacula). The insects were transported to the laboratory and maintained at −65°C until identification and virological examination. In Hungary, mosquitoes were collected irregularly in caves situated in the Bakony Mountains between 1966 and 2014. In Austria, female mosquitoes were collected irregularly during autumn inspection of wine cellars and shelters. Mosquito specimens were determined morphologically by standard identification keys (Schaffner et al. 2001, Becker et al. 2010).

A total of 14,776 overwintering mosquito females of *Cx. pipiens*, 282 *Culiseta annulata* (Schrank), 39 *An. maculipennis* Meigen sensu lato, and 1 *Ur. unguiculata* specimen were collected from 2011 to 2014 in southern Moravia, Czech Republic. The finding of the *Ur. unguiculata* female originated from a ceiling in a basement located near Sedlec village, very close to an endemic

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Table 1. Summary of overwintering females of *Uranotaenia unguiculata* collection in Central Europe.

Country	Study site	Geographical coordinates	No. of specimens	Date of collection
Czech Republic	Sedlec	48.767400°N, 016.683967°E	1	February 26, 2014
Austria	Jedenspeigen	48.484217°N, 016.866983°E	1	September 23, 2013
	Hundsheim	48.116800°N, 016.933717°E	1	November 25, 2013
Hungary	Ajka: Bújó-lik	47.100217°N, 017.550100°E	1	November 23, 1997
	Dudar: Csapóné-konyhája	47.300417°N, 017.933883°E	2	January 16, 2014
	Pénzesgyőr: Tilos-erdei-barlang	47.217233°N, 017.783767°E	1	December 1, 2013

circulation site of WNV lineage 2 (Rudolf et al. 2014). Similar findings of overwintering *Ur. unguiculata* were documented in nearby Hungary during winter collection in caves Ajka: Bújó-lik, Dudar: Csapóné-konyhája, and Pénzesgyőr: Tilos-erdei-barlang (Table 1). Additional supportive finding was observed recently in neighboring Austria, where 2 females of *Ur. unguiculata* were collected from walls in a large baroque storage cellar in the village of Jedenspeigen and in a small wine cellar in the village of Hundsheim (Table 1).

These are the first solid data describing *Ur. unguiculata* hibernation in the adult stage in Central Europe. Despite that only a few specimens could be caught, and considering the absence of reports of overwintering larvae, we assume that the species overwinters in the adult stage in Europe, and that females may hide in the vegetation of natural shelters (dense vegetation, ground and rock holes) as does, e.g., *Cx. modestus*.

Scientific relevance of this finding is underlined by two very recent detections of putative novel lineage of WNV in field-caught *Ur. unguiculata* mosquitoes from Lake Neusiedler-Seewinkel in Austria and from southwestern Hungary, respectively (Kemenesi et al. 2014, Pachler et al. 2014). It is worth mentioning that pathogenic potential of this new putative WNV lineage is not yet characterized. *Uranotaenia unguiculata* females were rarely reported to bite humans (Baghirov et al. 1994), and other *Uranotaenia* spp. are commonly considered to feed on amphibians and reptiles (Becker et al. 2010). Because of limited information on its biting behavior, no assumption can be made for its potential vector role. However, overwintering of females suggests that this new WNV lineage could overwinter in Central Europe within overwintering *Ur. unguiculata* females and then be transmitted to vertebrate hosts during spring. Further studies are needed to elucidate bloodfeeding host preferences of *Ur. unguiculata* females as well as public health and animal health relevance as potential WNV vector in Europe.

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PRÁCE 37

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Stručná charakteristika: cílem review bylo podat ucelený přehled méně známých arbovirů vyskytujících se na našem území s důrazem na jejich taxonomické zařazení, určení vektorů a hostitelů a patogenitu pro člověka.

Hlavní přínos práce: shrnutí nejnovějších poznatků o patogenních arbovirech vyskytujících se na našem území a také zdroj informací pro infekcionisty a epidemiology o onemocněních, jež způsobují. Jde totiž mnohdy o opomíjené nákazy, které unikají zejména v letních měsících pod diagnózou *status febrilis* neznámého původu.

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Opomíjené virové infekce přenášené hematofágními členovci v České republice

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Souhrn

Nejčastějším onemocněním způsobeným arboviry v České republice je středoevropská klíšťová encefalitida (s průměrnou roční incidencí 6 případů/100 000 obyvatel). Na našem území se však občas vyskytuje dalších sedm méně známých arbovirů (*Flavivirus* West Nile, *Bunyavirus* Ťahyňa, *Bunyavirus* Batai, *Bunyavirus* Sedlec, *Bunyavirus* Lednice, *Orbivirus* Tribeč, *Uukuvirus* Uukuniemi), z nichž onemocnění člověka prokazatelně způsobují viry West Nile, Ťahyňa, Tribeč, a pravděpodobně také Batai. Navíc byly u nás prokázány protilátky k dalším dvěma patogenním arbovirům vyskytujícím se na evropském kontinentu (*Alphavirus* Sindbis, *Coltivirus* Eyach). Cílem předkládané práce je podat ucelený, stručný přehled méně známých arbovirů vyskytujících se na území ČR s důrazem na jejich taxonomické zařazení, určení vektorů a hostitelů, a patogenitu pro člověka. Zmíněné arboviry mohou vyvolávat horečnaté stavy až aseptické (meningo)encefalitidy s tzv. nejasnou etiologií. Přehled zdůrazňuje problém možné emergence těchto opomíjených arbovirů v blízké budoucnosti, a uvádí diagnostické postupy.

Klíčová slova: arboviry – klíšťata – komáři – emergentní nákazy – surveillance.

Summary

Rudolf I., Hubálek Z., Šikutová S., Švec P.: Neglected Arthropod-Borne Viral Infections in the Czech Republic

Central European encephalitis is the most common arthropod-borne virus disease in the Czech Republic, with the mean annual incidence of 6 cases per 100 000 population. However, seven less known arboviruses (*Flavivirus* West Nile, *Bunyavirus* Ťahyňa, *Bunyavirus* Batai, *Bunyavirus* Sedlec, *Bunyavirus* Lednice, *Orbivirus* Tribeč, *Uukuvirus* Uukuniemi) also circulate in this country, of which West Nile, Ťahyňa, Tribeč and possibly Batai have been reported to cause human disease. Moreover, antibodies against two other pathogenic viruses found in Europe, i.e. *Alphavirus* Sindbis and *Coltivirus* Eyach, have been detected in the Czech Republic. The aim of this study is to review briefly the less known arboviruses found in the Czech Republic with emphasis on the taxonomic status, identification of their hosts and vectors, and pathogenicity to humans. These arboviruses can cause febrile illness to aseptic (meningo)encephalitis of unclear etiology. The review points out the possible emergence of these neglected arboviruses in the foreseeable future and provides diagnostic guidance.

Key words: arboviruses – ticks – mosquitoes – emerging diseases – surveillance.

Motto: V tomto roce si připomínáme 50. výročí události, kdy dva naši badatelé, Vojtech Bárdoš a Vlasta Danielová, izolovali na východním Slovensku první evropský arbovirus přenášený komáry a nazvali jej podle místa objevu Ťahyňa. Tento přehled je věnován nejen jim, ale i mnoha dalším vědcům, kteří se zasloužili o rozkvět české a slovenské arbovirologie v evropském a snad i ve světovém měřítku.

Úvod

Virové nákazy přenášené hematofágními členovci (arbovirózy) patří mezi nejdůležitější emergentní infekční nemoci, kterým čelí lidstvo na začátku třetího tisíciletí, a stávají se (vedle malárie, malnutrice a helmintóz) největším zdravotnickým problémem zejména v zemích třetího svě-

Tab. 1. Přehled významných arbovirů včetně onemocnění, jež způsobují ve světě

Table 1. Review of important arboviruses and diseases caused by these agents in the world

Zařazení	Přenašeč	Onemocnění	Rozšíření	BSL
Togaviridae				
EEE	komáři *Culicinae*	encefalomyelitida EEE	Severní Amerika	2
WEE	komáři *Culicinae*	encefalomyelitida WEE	Severní Amerika	2
VEE	komáři *Culicinae*	encefalomyelitida VEE	Jižní Amerika	3
CHIK	komáři *Culicinae*	horečka chikungunya	tropická Afrika, Asie	3
ONN	komáři *Anophelinae*	horečka o'nyong nyong	tropická Afrika	2
SIN	komáři *Culicinae*	horečka Sindbis (Ockelbo,	kosmopolitní (mimo Ameriku)	
		Karelská, Pogosta)		2
MAY	komáři *Culicinae*	horečka Mayaro	Jižní Amerika	3
RR	komáři *Culicinae*	epidemická polyartritida	Austrálie	2
BF	komáři *Culicinae*	epidemická polyartritida	Austrálie	2
Flaviviridae				
JE	komáři *Culicinae*	japonská encefalitida	východní a jižní Asie	3
CEE, LI, RSSE, POW	klíšťata *Ixodes*	klíšťová encefalitida	Eurasie, Severní Amerika	3, 4
WN	komáři *Culicinae*	západonilská horečka	kosmopolitní	3
SLE	komáři *Culicinae*	saintlouiská encefalitida	Severní Amerika	3
MVE	komáři *Culicinae*	encefalitida údolí Murray	Austrálie	3
YF	komáři *Culicinae*	žlutá zimnice	tropická Afrika a Jižní Amerika	3
DEN	komáři *Culicinae*	horečka dengue	tropy	2
ROC	komáři *Psorophora*	encefalitida Rocio	Jižní Amerika	3
OHF	klíšťata *Dermacentor*	omská hemoragická horečka	Jižní Sibiř	4
KFD	klíšťata *Haemaphysalis*	kjasanurská hemoragická horečka	Indie	4
Bunyaviridae				
TAH, CE, LAC, SSH, INK, JC	komáři *Culicinae*	valtická horečka, kalifornská encefalitida	Eurasie, Afrika, Sev. Amerika	2
ORO	pakomárci, komáři	horečka Oropouche	Jižní Amerika	3
KET	klíšťáci *Argasidae*, komáři	horečka Keterah	Střední Asie	2
BHA	klíšťata Metastriata	horečka, meningoencefalitida	Afrika, Eurasie	3
CCHF	klíšťata Metastriata	krymsko-konžská hemor. horečka	Afrika, Eurasie	4
RVF	komáři, pakomárci, flebotomové	horečka údolí Rift	Afrika	3
SFN, SFS, TOS	flebotomové	horečka papatači	Středomoří, Asie	2
Reoviridae				
CTF	klíšťata Metastriata	koloradská klíšťová horečka	Severní Amerika	2
KEM (TRB)	klíšťata *Ixodes*	horečka Kemerovo, Tribeč	Eurasie	2
Rhabdoviridae				
VSV	hematofágní dvoukřídlí	vezikulární stomatitida	Amerika	2
Orthomyxoviridae				
THO	klíšťata Metastriata	horečka Thogoto	Eurasie, Afrika	3
DHO	klíšťata Metastriata	horečka Dhori	Eurasie, Afrika	3

Vysvětlivky: BSL–úroveň zabezpečení laboratoře pro práci s patogenními mikroorganismy, EEE–východoamerická encefalomyelitida koní, WEE–západoamerická encefalomyelitida koní, VEE–venezuelská encefalomyelitida koní, CHIK–chikungunya, ONN–o'nyong nyong, SIN–Sindbis, MAY–Mayoro, RR–Ross River, BF–Barmah Forest, JE– japonská encefalitida, CEE–středoevropská klíšťová encefalitida, LI–louping ill, RSSE–ruská jaro-letní encefalitida, POW–Powassan, WN–West Nile, SLE– saintlouiská encefalitida, MVE–encefalitida údolí Murray, YF–žlutá zimnice, DEN–dengue, ROC–Roccio, OHF–omská hemoragická horečka, KFD–nemoc kjasanurského pralesa, TAH–Ťahyňa, CE–kalifornská encefalitida, LAC–LaCrosse, SSH – Snowshoe hare, INK–Inkoo, JC–Jamestown Canyon, ORO – Oropouche, KET–Keterah, BHA–Bhanja, CCHF–krymsko-konžská hemoragická horečka, RVF–horečka údolí Rift, SFN–Sandfly-Naples, SFS–Sandfly-Sicily, TOS–Toscana, CTF–koloradská klíšťová horečka, KEM (TRB)–Kemerovo, Tribeč, VSV–virus vezikulární stomatitidy, THO–Thogoto, DHO–Dhori

Explanations: BSL-biosafety level for handling with pathogenic microorganisms, EEE–Eastern equine encephalomyelitis, WEE–Western equine encephalomyelitis, VEE–Venezuelan equine encephalomyelitis, CHIK–Chikungunya, ONN–O'nyong nyong, SIN–Sindbis, MAY–Mayoro, RR–Ross River, BF–Barmah Forest, JE– Japanese encephalitis, CEE–Central european encephalitis, LI–Louping ill, RSSE–Russian spring-summer encephalitis, POW–Powassan, WN–West Nile, SLE–St. Louis encephalitis, MVE–Murray Valley encephalitis, YF–yellow fever, DEN–dengue, ROC–Roccio, OHF–Omsk hemorrhagic fever, KFD–Kyasanur Forest disease, TAH–Ťahyňa, CE–California encephalitis, LAC–LaCrosse, SSH – Snowshoe hare, INK–Inkoo, JC–Jamestown Canyon, ORO – Oropouche, KET–Keterah, BHA–Bhanja, CCHF–Crimean-Congo hemorrhagic fever, RVF–Rift Valley fever, SFN–Sandfly-Naples, SFS–Sandfly-Sicily, TOS–Toscana, CTF–Colorado tick fever, KEM (TRB)–Kemerovo, Tribeč, VSV–Vesicular stomatitis virus, THO–Thogoto, DHO–Dhori

ta [23]. Celosvětově je podle *International Catalogue of Arboviruses* registrováno téměř 500 arbovirů [48]: pomineme-li duplikátní viry a nearboviry typu hantavirů, v katalogu rovněž zařazené. Arboviry nejsou taxonomickou, ale ekologickou skupinou a její příslušníci náležejí do 7 čeledí: *Bunyaviridae* (51% arbovirů), *Reoviridae* (14%), *Flaviviridae* (12%), *Rhabdoviridae* (10%), *Togaviridae* (8%), *Orthomyxoviridae* (<1%) a *Poxviridae* (<1%) [37]. Asi u 134 arbovirů byla prokázána souvislost s lidským onemocněním [22], mnohé z nich jsou původci lokálních epidemií na všech kontinentech s výjimkou Antarktidy (Tabulka 1). V Evropě se v současnosti vyskytuje asi 50 arbovirů náležejících do 6 čeledí RNA virů, některé z nich však s efemérním výskytem (např. *Flavivirus* dengue, nebo veterinárně významné viry africké nemoci koní a africké horečky prasat), mnohé pak cirkulují v pobřežních ekosystémech mezi mořským ptactvem (např. *Flavivirus* Tyuleniy, *Uukuvirus* Zaliv Terpenija a další). Mezi arboviry významné pro humánní medicínu vyskytující se v Evropě řadíme především tyto zástupce: *Alphavirus* Sindbis, flaviviry West Nile, dengue, louping ill, středoevropské klíšťové encefalitidy a ruské jaro-letní encefalitidy, bunyaviry Ťahyňa, Inkoo, Batai a Bhanja, fleboviry horeček papatači (Sandfly – Naples, Sandfly – Sicily) a Toscana, nairovirus krymsko-konžské hemoragické horečky, orbivirus Tribeč a také orthomyxoviry Dhori a Thogoto [29]. Pro úplnost je třeba zmínit, že veterinárně významnými arboviry jsou: West Nile, způsobující encefalomyelitidu u koní [75], louping ill, původce encefalitidy u ovcí [18], Bhanja, původce encefalitidy u ovcí [36], Thogoto, dále původce africké nemoci koní a africké horečky prasat, a v neposlední řadě *Orbivirus* bluetongue, způsobující vážné onemocnění ovcí i skotu – nyní v centru pozornosti epizootologů v důsledku lavinovitého šíření napříč evropským kontinentem; první onemocnění horečkou bluetongue bylo nedávno hlášeno i v západních Čechách [74].

Arboviry jsou přenášeny hematofágními členovci řádů roztoči (*Acarina*) a dvoukřídlí (*Diptera*) - nejčastěji však zástupci čeledí klíšťatovití (*Ixodidae*) a komárovití (*Culicidae*). V České republice se kromě viru středoevropské klíšťové encefalitidy vyskytuje dalších 7 arbovirů (*Flavivirus* West Nile, *Bunyavirus* Ťahyňa, *Bunyavirus* Sedlec, *Bunyavirus* Lednice, *Bunyavirus* Batai, *Orbivirus* Tribeč, *Uukuvirus* Uukuniemi), z nichž však onemocnění člověka prokazatelně způsobují pouze viry West Nile, Ťahyňa, Tribeč a pravděpodobně i Batai. Navíc byly u nás detegovány protilátky k dalším dvěma pro člověka patogenním arbovirům vyskytujícím se na evropském kontinentu (*Alphavirus* Sindbis a *Coltivirus* Eyach), aniž však tyto viry byly izolovány [37].

Jako základ pro tento přehled sloužily 3 anglicky psané práce [29, 64, 73], které jsme doplnili o nejnovější poznatky. V nich nalezne zvídavý čtenář mnohé další podrobnosti o pojednávaných „českých" arbovirech. Pro přehlednost budou v textu arboviry rozděleny na agens přenosná klíšťaty a komáry. Virus středoevropské klíšťové encefalitidy není součástí tohoto přehledu.

Arboviry přenášené klíšťaty

Virus Eyach

Poprvé byl izolován z nenasátého klíštěte *Ixodes ricinus* poblíž obce Eyach u města Tübingen v Německu v roce 1972 (prototypový kmen Eyach-38) [70]. Jde o segmentovaný dsRNA virus rodu *Coltivirus* čeledi *Reoviridae* patřící do antigenní skupiny viru koloradské klíšťové horečky [1]. Jeho přenašečem jsou klíštata *Ixodes ricinus*

a *I. ventalloi* [43]. Hostiteli viru jsou pravděpodobně některé druhy hlodavců a zajíci [29].

Charakteristickým ekosystémem cirkulace viru jsou smíšené lesy. Virus je rozšířen v západní a střední Evropě (Francie, Německo); byl dokonce reizolován z klíšťat po 25 letech v Německu [25]. Protilátky k viru byly nalezeny i u pacientů s encefalitidou a polyradikulineuritidou v Čechách. Arbovirus Eyach je pravděpodobně původcem některých neuropatií a meningoencefalitid s tzv. nejasnou etiologií [65]. Průkaz možné příčinné souvislosti s lidským onemocněním v ČR by však vyžadoval další výzkum. Dosud se nepodařilo kultivovat virus na savčích buněčných liniích (izolace viru se daří jen při intracerebrální inokulaci sajících myší) a to ztěžuje jeho diagnostiku pomocí neutralizačního nebo komplement fixačního testu [42]. Taxonomicky velmi blízký až identický virus koloradské klíšťové horečky způsobuje v USA horečnaté onemocnění s bolestmi hlavy, svalů, fotofobií, nauzeou, celkovou slabostí a někdy i vyrážkou a je přenášen klíštětem *Dermacentor andersoni*. Podle domněnky jednoho z autorů (Z.H.) mohl být tento virus importován ze Severní Ameriky s armádními psy a klíšťaty je parazitujícími na vojenskou základnu USA v Německu.

Virus Tribeč

Virus byl poprvé izolován z klíštěte *I. ricinus* současně ve třech regionech Slovenska (Malé Karpaty, Tribeč, Slovenský kras) v roce 1963 (prototypový kmen: Tribeč, západní Slovensko, 1963; topotypový kmen: LIP-91 Lipovník, východní Slovensko, 1963) [20, 59]. Jde o dsRNA segmentovaný virus náležející do rodu *Orbivirus* čeledi *Reoviridae* antigenní skupiny Kemerovo [1]. V Česku byl virus izolován na severní Moravě pod odlišným názvem Cvilín [57]. Principiálním přenašečem je klíště *I. ricinus*, méně často *Haemaphysalis punctata*. Hostiteli jsou hlodavci (*Myodes glareolus*, *Microtus subterraneus*), zajíci [15], koza a ptáci (*Sturnus vulgaris*, *Fringilla coelebs*). Protilátky k viru byly v endemických oblastech nalezeny u pasených přežvýkavců. Typickým biotopem jsou boskematické (pastorální) a teriodické (lesní se zvěří) ekosystémy. Geografické rozšíření viru pokrývá střední, východní, jižní i severní Evropu včetně Ruska, mimo Evropu se virus vyskytuje také v Ázerbajdžánu. Stěhovaví ptáci mohou hrát významnou roli v šíření virů skupiny Kemerovo na velké vzdálenosti [29]. Virus Tribeč způsobuje horečnaté onemocnění [19], někdy s aseptickou meningitidou, sérokonverze byla prokázána u pacientů v Čechách [17, 65] i na Moravě [32], kde byla navíc zjištěna akutní nákaza virem Tribeč u 14 osob na Znojemsku: převažující klinickou manifestací byla serózní meningitida. Na východní Moravě byly zjištěny protilátky k viru Tribeč u 16 % pacientů s diagnózou meningoencefalitidy [58]. Navíc při experimentální inokulaci opic *Macaca mulatta* virem Tribeč se u nich po 11 dnech vyvinula lymfocytární meningitida [60]. U arboviru Tribeč platí podobný závěr jako u viru Eyach: žádoucí by byl další výzkum, např. studium sérokonverze k viru Tribeč u pacientů s výskytem aseptické meningitidy s nejasnou etiologií.

Virus Uukuniemi

Poprvé byl tento virus izolován z klíštěte *I. ricinus* sbíraného z paseného dobytka u obce Uukuniemi (jihovýchodní Finsko) v roce 1959 (prototypový kmen: S-23; topotypový kmen: Poteplí PO-63, *I. ricinus*, Čechy 1963) [48, 51]. Jde o RNA virus rodu *Uukuvirus* čeledi *Bunyaviridae* náležející do antigenní skupiny Uukuniemi [47]. Hlavním přenašečem jsou klíšťata *I. ricinus*, méně pak *I. persulcatus*. Možným mechanickým vektorem jsou také komáři čeledi *Culicidae* (*Aedes vexans*, *Ochlerotatus. punctor*, *Oc. communis* a další). Hostiteli jsou lesní hlodavci (*Myodes glareolus*, *Apodemus flavicolis*), ještěrky a ptáci (*Turdus merula*, *Erithacus rubecula*, *Sylvia communis*), kteří mohou hrát významnou roli v zánosu viru na velké vzdálenosti [29, 64].

Typickým ekosystémem viru Uukuniemi jsou lesní biotopy, nelze ovšem vyloučit ani urbánní ohniska [63]. Virus je rozšířen především v severní, střední a východní Evropě, mimo Evropu v Ázerbajdžánu a asijské části Ruska [29]. Infekce zapříčiněné virem Uukuniemi manifestující se klinicky nejsou známé [19, 64], s výjimkou ojedinělých případů hlášených z jižního Ruska, kde virus údajně způsobil horečnaté onemocnění s náhlým nástupem horečky, bolestmi hlavy, svalů, kloubů, hyperémií v obličeji a někdy s vyrážkou po celém těle [10]. Toto sdělení vyžaduje ovšem verifikaci, neboť klinické příznaky ukazují spíše na infekci virem Sindbis nebo West Nile. V České republice byl virus Uukuniemi opakovaně izolován z klíšťat *I. ricinus*, avšak protilátky k viru byly u lidí detegovány jen výjimečně nebo vůbec ne [32, 38, 50].

Arboviry přenášené komáry

Virus Sindbis

Poprvé byl izolován ze směsi komárů *Culex pipiens* a *Cx. univittatus* ve vesnici Sindbis v deltě řeky Nilu v roce 1952 (prototypový kmen: EgAr-399) [82], v Evropě poprvé z mozku rákosníka obecného (*Acrocephalus scirpaceus*) u Malacek v roce 1971 (topotypový kmen: R-33) [16]. Nej-

více studií týkajících se viru Sindbis bylo uskutečněno ve Skandinávii, kde se vyskytuje epidemicky a periodicky od roku 1974. Virus Sindbis patří do antigenní skupiny západní koňské encefalomyelitidy rodu *Alphavirus* čeledi *Togaviridae* [79]. Je přenášen převážně ornitofilními komáry *Cx. univittatus*, *Cx. pipiens*, *Cx. modestus* [29]; vzácně byly zaznamenány izolace z klíšťat rodu *Hyalomma* [21]. Typickým stanovištěm jsou mokřady, kde se uskutečňuje enzootický cyklus mezi komáry a ptáky. Přirozeným hostitelem viru jsou převážně ptáci (*Corvus corone*, *Motacilla alba*, *A. scirpaceus* a další), méně často hlodavci, netopýři, žáby [29]. Stěhovaví ptáci mohou hrát významnou roli v přenosu viru Sindbis na velké vzdálenosti (fylogenetické studie naznačují zános viru z místa původního výskytu v Africe do Skandinávie). Virus byl izolován v Africe i Evropě (Itálie, Slovensko, Maďarsko, nejvíce však Finsko, Švédsko a ruská Karélie), dále v Asii i Austrálii. Protilátky k viru byly detegovány i v Česku u ptáků [46] a také ojediněle u lidí, aniž byl virus zachycen [41]. Je původcem horečky Sindbis – s bolestmi hlavy, myalgií, artralgií, polyartritidou, únavou, konjunktivitidou, faryngitidou, svěděním a vyrážkou [53]. Jsou zaznamenány i trvalé následky spojené s postižením kloubů po infekci tímto virem [54]. Od roku 1974 se opakují epidemie ve Finsku s periodicitou 7 let (1974, 1981, 1988, 1995, 2002) [55]. To pravděpodobně souvisí s 6-7letým populačním cyklem tetřívků, kteří jsou společně s některými pěvci považováni za hostitele amplifikátory [9]. Podle místa výskytu se onemocnění liší svým názvem – 'Ockelbo' ve Švédsku, 'Pogosta' ve Finsku a karelská horečka v severozápadním Rusku [47]. Rozšíření viru v České republice je možné při zánosu agens infikovanými stěhovavými ptáky a etablováním viru v místní komáří populaci, pravděpodobně podobným způsobem jako u virového kmene Rabensburg (třetí genomické linie viru West Nile), který byl izolován opakovaně z komárů *Cx. pipiens* v letech 1997 a 1999 na jižní Moravě [30, 39].

Virus West Nile

Poprvé byl tento virus izolován z krve nemocné ženy v ugandské provincii West Nile v roce 1937 (prototypový kmen: B-956), později také v Egyptě z dítěte (neotypový kmen: Eg-101) [67, 78]. V Evropě byl poprvé izolován z klíštěte *Hyalomma marginatum* v jižním Rusku, ale také z komárů a pacientů při epidemii západonilské horečky v jihofrancouzském Camargue v roce 1964 [29]. Virus West Nile patří do antigenní skupiny japonské encefalitidy čeledi *Flaviviridae* a dělí se do několika genomických linií: linie 1 zahrnuje kmeny ze Severní Ameriky, Evropy, Afriky, Asie a Austrálie (subtyp Kunjin), zatímco linii 2 tvoří kmeny původem ze subsaharské Afriky a Madagaskaru [26], linii 3 tvoří Rabensburg [2]. V současnosti se však uvažuje ještě o dalších dvou liniích (Indie, Jižní Rusko) [8]. Jeho přenašečem jsou zejména ornitofilní komáři rodu *Culex* (např. *Cx. pipiens*, *Cx. univittatus*, *Cx. modestus*), méně často *Oc. cantans* nebo *Anopheles maculipennis*. Výjimečně mohou virus přenášet také některá klíšťata rodu *Hyalomma* nebo klíšťáci rodů *Ornithodoros* a *Argas*, kteří slouží jako alternativní vektor viru především v suchých a teplých oblastech [29]. Hostitelem viru jsou vodní i terestričtí ptáci, hlodavci, člověk, netopýři, velbloudi, koně, ovce, vlci, obojživelníci, aligátoři a hadi [29, 83]. Cirkulace viru v Evropě je charakterizována dvěma cykly a ekosystémy: exoantropním (sylvatickým) zahrnujícím převážně vodní a mokřadní ptáky jako hostitele a amplifikátory viru a ornitofilní komáry (*Cx. pipiens pipiens*, *Cx. modestus*, *Coquillettidia richiardii*) jako vektory, a synantropní (urbánní) cyklus zahrnující synantropní ptáky jako hostitele a komáry sající na ptácích i savcích jako vektory (*Cx. pipens molestus*) [28, 34]. Virus způsobuje západonilskou horečku, mezi jejíž hlavní příznaky patří dále bolesti hlavy, zad a kloubů, laryngitida, myalgie, konjunktivitida, nitrooční tlak, nechutenství, nevolnost, zvracení, nespavost, makulopapulární vyrážka, lymfadenopatie, někdy však s výskytem hepatitidy, pankreatitidy, myokarditidy, meningitidy nebo encefalitidy [34]. Až 80 % lidských infekcí probíhá asymptomaticky. Letalita onemocnění se pohybuje kolem 5-10 % a ohrožuje zejména pacienty starší 60 let [27]. West Nile virus je typickou reemergentní nákazou, na konci 20. století byly zaznamenány lokální epidemie nebo případy západonilské horečky v Alžírsku (1994), Maroku (1996), Tunisku (1997 a 2003), Rumunsku (1996-2000), České republice (1997), Izraeli (1999-2000), Rusku (1999-2001), Francii (2003) a rozsáhlá epidemie ve Spojených státech (1999-2004) [27]. Na jižní Moravě bylo po povodních v roce 1997 dokumentováno pět případů klinického onemocnění tímto virem [39].

Virus Ťahyňa

Virus byl poprvé izolován ze směsi komárů *Ae. vexans* a *Oc. caspius* z obcí Ťahyňa a Križany na východním Slovensku v roce 1958. Šlo o první arbovirus teplokrevných obratlovců izolovaný v Evropě (prototypový kmen: Ť-92) [5]. Jedná se o *Orthobunyavirus* kalifornské antigenní skupiny z čeledi *Bunyaviridae*. Přenašečem viru jsou komáři *Ae. vexans*: prokázán dokonce transovariální přenos [13], dále *Oc. caspius*, *Ae. cinereus*, *Oc. cantans*, *Oc. communis* a další [29, 73]. Hostitelem viru je především zajíc (*Lepus europaeus*), králík, hlodavci, netopýři, ježek, sysel, *Oc.*, *ondat-*

ra, veverka, kuna, tchoř, liška, jezevec, netopýři, protilátky k viru byly detegovány u šelem, koní, skotu, prasat, a mokřadních druhů ptáků.

Preferovaným místem výskytu je záplavový ekosystém v inundačních oblastech řek včetně urbánních ekosystémů [19, 73].

Nemoc způsobená virem Ťahyňa se nazývá valtická horečka. Jde o chřipkovité onemocnění vyskytující se v letních a časně podzimních měsících převážně u dětí. Mezi příznaky patří náhlý nástup horečky, bolesti hlavy a končetin, únava, konjunktivitida, faryngitida, myalgie, nauzea, střevní potíže, anorexie, artralgie, meningitida, vzácněji bronchopneumonie [29]. Podobné onemocnění vyskytující se v Severní Americe a pojmenované kalifornská encefalitida nebo encefalitida LaCrosse má za následek i letalitu a s vysokou prevalencí se objevuje hlavně u dětí [24]. V České republice byla cirkulace viru Ťahyňa v přírodním ohnisku intenzívně zkoumána především v minulých desetiletích [64, 73]. Protilátky k viru byly prokázány u většiny dospělé populace v endemických oblastech, především na jižní Moravě [31], ale i jinde – např. ve středních Čechách [41, 49] v záplavových oblastech velkých řek, kde dochází k pravidelnému přemnožení lokální komáří populace. Virus byl opakovaně izolován z krve febrilních dětí [6] i dospělých [80]. Desítky kmenů viru byly izolovány také z komárů při monitorování aktivity ohniska v posledních desetiletích [39]. Při přemnožení komáří populace, převážně po povodních nebo při umělén jarním povodňování lužních lesů, stoupá i riziko nákazy valtickou horečkou. Určité procento febrilních stavů dětí v letních měsících, stejně tak dospělých, kteří se s infekcí dosud nesetkali, může být způsobeno valtickou horečkou, avšak onemocnění běžně uniká pozornosti infektologů i epidemiologů.

Virus Batai (Čalovo)

Poprvé byl izolován z komára *Cx. gelidus* na pastvinách v oblasti Kuala Lumpur v Malajsii v roce 1955 (prototypový kmen: AMM-2222) [48], v Evropě byl antigenně identický kmen 'Čalovo' izolován z komára *An. maculipennis* sensu lato u obce Trstená blízko Čalova na jižním Slovensku v roce 1960 (evropský topotypový kmen: Čalovo-184) [4]. Virus je přenášen převážně zoofilními komáry *An. maculipennis* [77], dále *An. claviger*, *Oc. punctor*, *Oc. communis*, *Ae. vexans*. Cirkuluje v agroekosystémech převážně v enzootickém cyklu mezi zoofilními komáry a domácími přežvýkavci. Hostitelem viru jsou prase domácí a ptáci (*Corvus corone*, *Fulica atra*, *Perdix perdix*) [29].

Virus Batai způsobuje u člověka horečnaté onemocnění provázené únavou, myalgií a anorexií [7, 29, 76]. Horečnaté onemocnění způsobené virem Batai bylo pozorováno u pacientů v Thajsku, virus byl izolován z febrilní krve pacientů v Súdánu [29]. Protilátky k viru Batai u lidí byly detegovány jak v Čechách [41], tak i na Moravě u pacientů s horečnatým onemocněním [32]. Pro studium epidemiologie viru Batai v České republice by byl žádoucí také veterinární monitoring domácích přežvýkavců společně se sérologickými přehledy lidské populace na specifické protilátky včetně monitoringu lokální komáří populace (především anofelů).

Další arboviry s možným výskytem na našem území

Existuje několik dalších arbovirů, které by se mohly potenciálně vyskytovat nebo v budoucnu rozšířit na naše území. Jedná se o *Bunyavirus* Inkoo z čeledi *Bunyaviridae*, který je přenášen komáry *Ae. communis* a způsobuje chřipkovité onemocnění, někdy až aseptickou meningitidu, faryngitidu, konjunktivitidu, závrať, a někdy i vyrážku [69]. Virus byl dosud izolován převážně na severu Evropy [47]. Samostatnou kapitolou je emergence *Flaviviru* Usutu (původně arbovirus endemický na africkém kontinentu) v Rakousku v letech 2001-2002, který zapříčinil masivní hynutí některých pěvců, zejména kosů (*Turdus merula*) v dolním Rakousku a protilátky k viru jsou v populaci přítomny dosud, i když ptáci jsou na virus pravděpodobně již adaptováni [66, 85]. Virus byl také později prokázán u kosů v okolí Budapešti v Maďarsku [3] a protilátky byly nalezeny dokonce u racka chechtavého (*Larus ridibundus*) v Polsku [40]. Patogenita viru pro člověka je sporná, i když 52 pacientů s horečnatým onemocněním v endemické oblasti výskytu viru vykazovalo protilátky v hemaglutinačně inhibičních titrech 1:20 až >1:160 a v jednom případě byla dokonce detegována nukleová kyselina viru pomocí RT-PCR [84].

Diagnostické postupy při průkazu arbovirů

Diagnostika arboviróz spočívá převážně v sérologii, optimálně ve vyšetření párových vzorků krevního séra, odebraných s odstupem 2-3 týdnů; za průkaz recentní infekce se považuje sérokonverze nebo minimálně čtyřnásobný vzestup titru protilátek mezi prvním a druhým vzorkem v enzymové imunoanalýze, hemaglutinačně inhibičním testu, komplement fixační reakci, virus neutralizačním testu, nepřímé imunofluorescenci či jiných testech [11, 33, 34, 44, 45, 56, 62]. Je-li k dispozici jediný vzorek rekonvalescentního séra pacienta, pomůže mnohdy k odlišení paralelní vyšetření na protilátky IgG a IgM – u recentních

infekcí převažují IgM nad IgG. U flavivirů je nutná obezřetnost při interpretaci výsledků testů v důsledku možné zkřížené reaktivity sér. Velmi průkazná, avšak obtížná, je izolace viru z krve, séra, likvoru nebo bioptických vzorků pacienta metodou inokulace sajících myší, buněčných kultur (nejčastěji VERO, XTC-2, SPEV, BHK-21, CV-1, GMK) nebo kuřecích embryí, a také molekulární detekce viru v krvi, CNS a tkáních pacienta [29, 37, 45, 72]. Možnost záchytu (detekce) viru v krvi je totiž nadějná jen v prvních dnech akutní fáze. V poslední době jsou do virologické diagnostiky intenzivně zaváděny molekulárně biologické techniky (PCR, RT-PCR, nested RT-PCR, reverseline blotting, real-time PCR, sekvencování, a další, které jsou využitelné pro detekci a typizaci většiny známých patogenů [37, 52]. Především detekce virové RNA v reálném čase spolu s kvantifikací nukleové kyseliny nahrazuje dnes již klasické molekulární metody díky vyšší specificitě i citlivosti, která je při diagnostice arbovirů rozhodující [61]. Pro detekci viru ve tkáních obratlovců se také využívá vysoce specifických imunohistochemických metod [44].

Terapie a prevence arboviróz

Specifická terapie arboviróz neexistuje, doporučuje se symptomatická léčba, klid na lůžku, příjem tekutin, podávání antipyretik; v kritických případech někdy pomáhá antisérum (specifický imunoglobulin), pokud je podáno bezprostředně po infekci. U některých virových nákaz však mohou být relativně účinná analoga nukleotidů, např. ribavirin u RNA virů. Jediným efektivním specifickým opatřením proti virózám je však očkování, u zoonotických nákaz přenášených hematofágními členovci bohužel omezené jen na nevelký počet virových infekcí (tj. pokud existuje vakcína: klíšťová encefalitida, žlutá zimnice, japonská encefalitida, západní koňská encefalomyelitida, východní koňská encefalomyelitida, venezuelská koňská encefalomyelitida, horečka údolí Rift). U virů pokrytých tímto přehledem bohužel žádná vakcína neexistuje.

Při pobytu v přírodním ohnisku nákazy je vhodným preventivním opatřením použití repelentů (na oděv i pokožku) proti vektorům, a účinnou prevencí je samozřejmě také vyhýbání se kontaktu s vektory (např. v případě komárů sítě v oknech, moskytiéry nad lůžkem atp.) [37].

Surveillance arbovirálních infekcí přenášených hematofágními členovci v České republice

Povodně v roce 1997 na Moravě [31] a v roce 2002 v Čechách [41] nám nastavily zrcadlo v problematice monitorování přírodních ohnisek nákaz. V roce 1997 byly zjištěny protilátky proti viru Ťahyňa u 53,8 % sér z počtu 619 vyšetřovaných osob z oblasti Břeclavska a byla zaznamenána jedna subklinická infekce valtickou horečkou. Dále bylo dokumentováno pět případů onemocnění kompatibilních se západonilskou horečkou včetně dvou dětí (byla zaznamenána sérokonverze mezi časným a rekonvalescentním sérem) s horečkou, vyrážkou a encefalitidou a to poprvé ve střední Evropě [31]. Při pozdějších povodních v Čechách roku 2002 bylo v Polabí v širším okolí Mělníka vyšetřeno 497 obyvatel na komáry přenosné nákazy: séroprevalence k viru Ťahyňa u místní vyšetřované populace dosahovala až 14 % (a byla prokázána i 1 sérokonverze), dále byly nalezeny v omezené míře protilátky k viru Sindbis (1%) a Batai (0,2%) [41].

Nesmí se ovšem opomenout ani monitorování importovaných případů arboviróz především u turistů vracejících se z endemických oblastí výskytu exotických virů. Za zmínku stojí např. první import západonilské horečky z USA do ČR v období její epidemie na severoamerickém kontinentu [35] nebo importovaná infekce horečky chikungunya u turistky vracející se z dovolené na ostrově Mauritius v Indickém oceánu, kde právě probíhala rozsáhlá epidemie tohoto horečnatého onemocnění [86].

Mezi metody surveillance řadíme především periodické vyšetřování vektorů v endemické oblasti výskytu viru (s jejich následným hubením v případě přemnožení), sérologické přehledy hostitelů (hlodavci, volně žijící zvěř, stálí i stěhovaví ptáci), monitoring domácích sentinelů (slepic a kachen) na specifickou sérokonverzi, vyšetřování lokální lidské populace na protilátky k virům přenosným hematofágními členovci (zvláštní pozornost by měla být soustředěna při zjišťování etiologie letních chřipkovitých stavů, spalničkového exantému, aseptických meningitid nebo meningoencefalitid nejasného původu) a důsledný monitoring importovaných nákaz [14]. Při šetření v přírodním ohnisku nákazy v epidemickém období je pak více než žádoucí spolupráce širokého týmu odborníků z řad epidemiologů, medicínských akaroentomologů, zoologů, veterinářů, terénních i klinických mikrobiologů a infektologů [34, 37].

Perspektivy výzkumu arbovirů u nás

Nové studie přinášejí nejen zprávy o rozšíření některých arbovirů a jejich příbuzných do míst, o nichž se dosud nevědělo, ale ukazují, že je nutno počítat i se vznikem kombinací virů s novými vlastnostmi, které mohou kdykoli přinést velká překvapení. Viry, které jsou dnes málo významné, se mohou stát velkými patogeny, mohou měnit svá působiště, hostitele i přenašeče. Je na místě skromnost a smíření se s tím, že všechny vědecké

poznatky mohou platit jen dočasně, protože příroda a přírodní ohniska se vyvíjejí a mění dál, i když velmi pomalu [12]. Tato slova významného českého virologa nelze jistě brát na lehkou váhu a lze si jen přát, aby se komplexní výzkum arbovirů a onemocnění, jež přenášejí, nadále rozvíjel. Výzkum ekologie arbovirů u nás má jistě na co navazovat. Studium jejich biologie a ekologie, které intenzivně probíhalo v 50. až 80. letech 20. století v bývalém Československu, posunulo tuto vědní disciplínu významně vpřed. Mnozí čeští a slovenští virologové (abecedně: Vojtech Bárdoš, Rudolf Benda, Dionýz Blaškovič, Luděk Daneš, Vlasta Danielová, Elo Ernek, Milota Grešíková, Jaroslava Holubová, Jiří Januška, Jan Mária Kolman, Otto Kožuch, Milan Labuda, Helena Libíková, Doubravka Málková, Josef Nosek, a mnozí další) patří k průkopníkům arbovirologie jak v evropském, tak i světovém měřítku, a některé jejich práce jsou stále pro svou platnost hojně citovány.

V budoucnu nás jistě čekají nové hrozby, kterým budeme muset čelit a na které bychom měli být připraveni (import exotických arbovirů z tropických oblastí v důsledku migrace obyvatel a zvířat [81], posun vektorů do vyšších zeměpisných šířek v důsledku změn klimatu). Evropská unie si uvědomuje tato reálná rizika, a proto posílila svoji podporu financováním projektů 6. a 7. rámcového programu se zaměřením na reemergentní nákazy včetně přenášených hematofágními členovci. Historie nedávných epidemií západonilské horečky v Americe [68] a horečky chikungunya na ostrovech Indického oceánu (Mauritius, Seychelly, Mayotte a Reunion) včetně první evropské autochtonní epidemie horečky chikungunya v Itálii v okolí Ravenny (asi 200 laboratorně potvrzených případů) [71] budiž nám varováním, že boj s arboviry zdaleka nekončí.

Poděkování.

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PRÁCE 38

Hubálek Z., **Rudolf I.** 2011. Microbial Zoonoses and Sapronoses, Springer, 1st Edition., 457 p., ISBN: 978-90-481-9656-2.

Stručná charakteristika: komplexní monografie o zoonózách v anglickém jazyce, která shrnuje současné poznatky o nákazách, vektorech, geografickém rozšíření vektorů a patogenů, klinických aspektech onemocnění včetně diagnostiky a léčby. Úvodní kapitoly se věnují historii výzkumu zoonotických onemocnění a také nastiňují některé pojmy eko-epidemiologické. Následuje výčet vektorů zoonotických agens, obratlovčích hostitelů zoonóz a komplexní charakteristika jednotlivých patogenů. Práce je doplněna bohatou obrazovou přílohou.

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Zdenek Hubálek · Ivo Rudolf

Microbial Zoonoses and Sapronoses

This book presents the state of art in the field of microbial zoonoses and sapronoses. It could be used as a textbook or manual in microbiology and medical zoology for students of human and veterinary medicine, including Ph.D. students, and for biomedicine scientists, medical practitioners and specialists as well.

Surprisingly, serious zoonoses and sapronoses still appear that are either entirely new (e.g., SARS), newly recognized (Lyme borreliosis), resurging (West Nile fever in Europe), increasing in incidence (campylobacterosis), spatially expanding (West Nile fever in the Americas), with a changing range of hosts and/or vectors, with modified clinical manifestations or caused by agents acquiring antibiotic resistance. The collective term for those diseases is (re)emerging infections, and most of them represent zoonoses and sapronoses (the rest are anthroponoses). The number of known zoonotic and sapronotic pathogens of humans is continually growing – over 800 today.

In the introductory part, short characteristics are given of infectious and epidemic processes, including the role of environmental factors, possibilities of their epidemiological surveillance, and control. Much emphasis is laid on ecological aspects of these diseases (haematophagous vectors and their life history; vertebrate hosts of zoonoses; habitats of the agents and their geographic distribution; natural focality of diseases). Particular zoonoses and sapronoses are then characterized in the following brief paragraphs: source of human infection; animal disease; transmission mode; human disease; epidemiology; diagnostics; therapy; geographic distribution.

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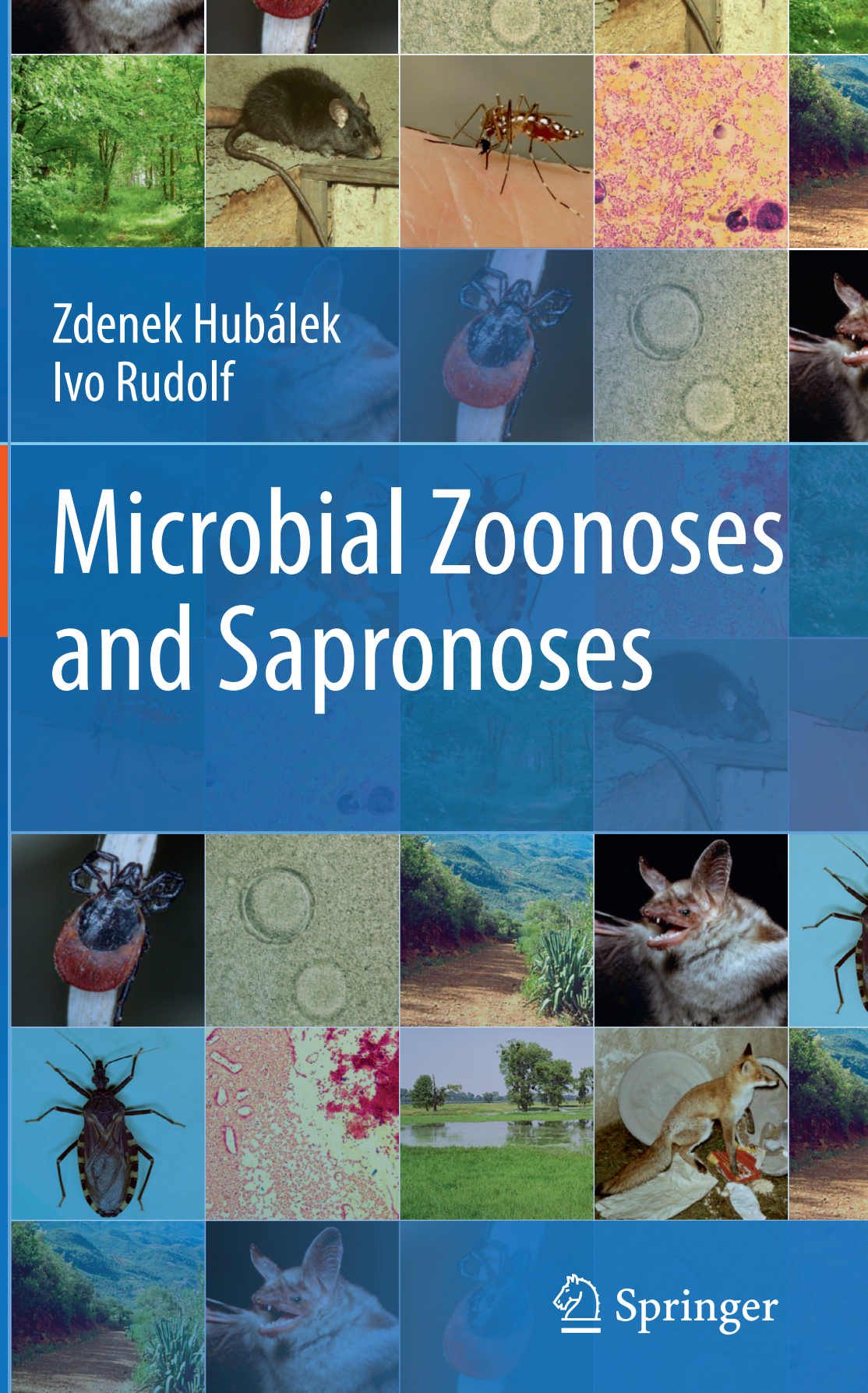

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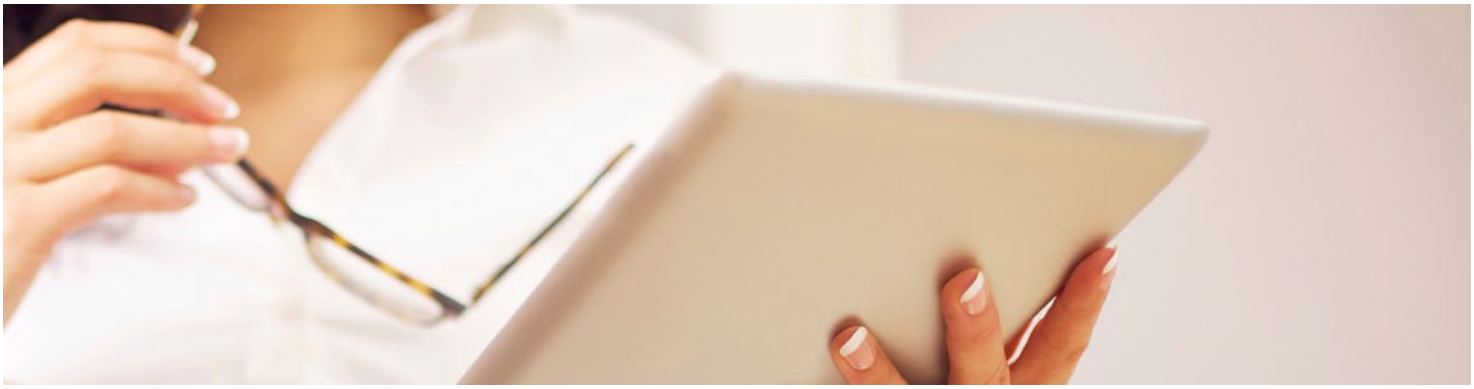

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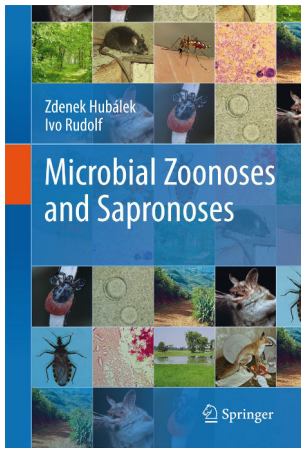

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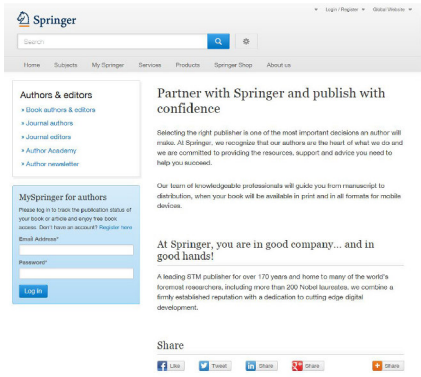

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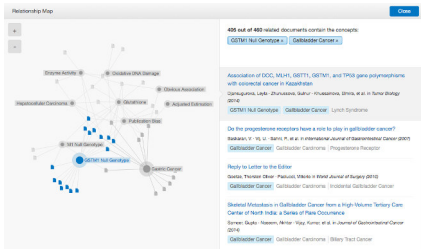

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PRÁCE 39

Hubálek Z., **Rudolf I.** 2012. Tick-borne viruses in Europe. *Parasitol. Res.* 111: 9–36.

Stručná charakteristika: práce je komplexním review, které shromažďuje nejnovější data o ekologii, taxonomii, geografickém rozšíření, obratlovčích hostitelích a zdravotnickém významu celkem 27 tzv. tibovirů (akronym z angl. termínu 'tick-borne viruses') patogenních pro člověka i virů s dosud neprokázanou patogenitou.

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REVIEW

Tick-borne viruses in Europe

Zdenek Hubálek · Ivo Rudolf

Received: 23 January 2012 / Accepted: 20 March 2012 / Published online: 18 April 2012

Abstract The aim of this review is to present briefly background information on 27 tick-borne viruses ("tiboviruses") that have been detected in Europe, viz flaviviruses tick-borne encephalitis (TBEV), louping-ill (LIV), Tyuleniy (TYUV), and Meaban (MEAV); orthobunyaviruses Bahig (BAHV) and Matruh (MTRV); phleboviruses Grand Arbaud (GAV), Ponteves (PTVV), Uukuniemi (UUKV), Zaliv Terpeniya (ZTV), and St. Abb's Head (SAHV); nairoviruses Soldado (SOLV), Puffin Island (PIV), Avalon (AVAV), Clo Mor (CMV), Crimean-Congo hemorrhagic fever (CCHFV); bunyavirus Bhanja (BHAV); coltivirus Eyach (EYAV); orbiviruses Tribec (TRBV), Okhotskiy (OKHV), Cape Wrath (CWV), Mykines (MYKV), Tindholmur (TDMV), and Bauline (BAUV); two thogotoviruses (Thogoto THOV, Dhori DHOV); and one asfivirus (African swine fever virus ASFV). Emphasis is laid on the taxonomic status of these viruses, range of their ixodid or argasid vectors and vertebrate hosts, pathogenicity for vertebrates including humans, and relevance to public health. In general, three groups of tibovirus diseases can be recognized according to main clinical symptoms produced: (i) febrile illness—usually with a rapid onset, fever, sweating, headache, nausea, weakness, myalgia, arthralgia, sometimes polyarthritis and rash; (ii) the CNS affection—meningitis, meningoencephalitis or encephalomyelitis with pareses, paralysis and other sequelae; (iii) hemorrhagic disease. Several "European" tiboviruses cause very serious human (TBEV, CCHFV) or animal (LIV, ASFV) diseases. Other arboviruses play definite role in human or animal pathology though the disease is usually either less serious or infrequently reported (TYUV, BHAV, AVAV, EYAV, TRBV, DHOV, THOV). The other European arboviruses are "orphans" without a proven medical or veterinary significance (BAHV, MTRV, MEAV, GAV, PTVV, ZTV, SAHV, UUKV, SOLV, PIV, AVAV, CMV, OKHV, CWV, MYKV, TDMV, BAUV). However, certain arbovirus diseases of free-living vertebrates (but also those of domestic animals and even man) may often pass unnoticed or misdiagnosed and eventually, they might potentially appear as emerging diseases. Active search for new tiboviruses or for new, pathogenic variants of the known tiboviruses in Europe should therefore continue.

Abbreviations

CF(T)	Complement fixation (test)
CPE	Cytopathic effect
HA	Hemagglutinin
HI(T)	Hemagglutination-inhibition (test)
i.c.	Intracerebral
i.m.	Intramuscular
i.n.	Intranasal
IFA	Immunofluorescent antibody assay
i.p.	Intraperitoneal
i.v.	Intravenous
p.o.	Peroral
PRNT	Plaque-reduction neutralization test
s.c.	Subcutaneous
TOT	Transovarial transmission (in arthropods)
TST	Transstadial transmission (in arthropods)
VN(T)	Virus neutralization (test)

Introduction

Tick-borne viruses (acronym "tiboviruses" might be used, for short) belong to an ecological group of viruses characterized by their specific biological transmission via competent hematophagous hard (ixodid) or soft (argasid) ticks

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(*Ixodidae* and *Argasidae*, respectively) to endotherm (homeotherm, warm-blooded) vertebrates. Competent vectors are those arthropods that are able to imbibe the virus in the course of blood-feeding on an infected donor vertebrate host, to support the multiplication of the virus in their organism and to deliver a sufficiently large inoculum to the recipient, uninfected vertebrate host. Usually certain minimum level of viremia ("infection threshold") in a donor vertebrate host is necessary for an efficient infection of particular arthropod vectors. Therefore, only those vertebrate species that produce at least moderate viremia have been regarded as competent, "true" or "amplifying" hosts of particular arboviruses (Bárdoš 1979). However, co-feeding ixodid ticks on a viremia-free host can sometimes also contribute to infection of noninfected ticks (Jones et al. 1987; Alekseev and Chunikhin 1990; Labuda et al. 1993). Some tiboviruses are transmitted from larvae to nymphs and imagoes during metamorphosis (transstadial transmission, TST), from infected female to the offspring (transovarial transmission, TOT), and from male to female tick during copulation (venereal or horizontal transmission). These modes are extremely important ecologically: e.g., under conditions of TOT, the tick vector also plays the role of a long-term reservoir of the virus.

In addition to two "major" severe, occasionally re-emerging virus diseases transmitted by ixodid ticks in Europe, viz tick-borne encephalitis and Crimean-Congo hemorrhagic fever, there is a number of other, neglected tick-borne virus infections of vertebrates. They are usually infrequent, although some of them are probably underdiagnosed, and other of these tiboviruses are nonpathogenic, or of low pathogenicity, for vertebrates (Tables 1 and 2).

This review briefly summarizes present knowledge especially on the taxonomy, ecology, epidemiology and distribution of European tiboviruses; for related reviews and additional, more detailed data, see, e.g. Theiler and Downs (1973), Karabatsos (1985), Málková et al. (1986), Lvov et al. (1989), Hubálek and Halouzka (1996), Charrel et al. (2004), Labuda and Nuttall (2004), Gratz (2006), and Dobler (2010). The virus taxonomy and nomenclature has been adopted from King et al. (2012).

Family *Flaviviridae*

Flavivirus of tick-borne encephalitis (TBEV)

There are three recognized TBEV subtypes: (1) Western or European subtype (TBEV-W), also called Central European (CEEV—topotype strains are Hypr and Neudoerfl) or sometimes "ricinus" subtype (Clarke 1964; Votyakov et al. 1978; Rubin and Chumakov 1980; Calisher 1988; Calisher et al. 1989; Gritsun et al. 2003; Lindquist and Vapalahti 2008)—varieties of this subtype are Spanish sheep encephalitis (SSE), Turkish sheep encephalitis (TSE) and Greek goat encephalitis ("Vergina") viruses; these three varieties are antigenically more closely related to TBEV-W (CEEV) than to louping ill virus (Hubálek et al. 1995); (2) (Ural-)Siberian subtype (TBEV-S: the prototype strains are Aina and Vasilchenko), sometimes called "persulcatus" subtype, causing Russian spring–summer encephalitis (RSSEV:); (3) Far Eastern subtype (TBEV-FE with prototype strain Sofyin, isolated from human brain in Khabarovsk, 1937). However, all three subtypes occur in Europe—the TBEV-S and TBEV-FE subtypes were recently detected in the Baltic republics and eastern Finland (Golovljova et al. 2004; Jääskeläinen et al. 2010). A taxonomic and nomenclatural confusion around TBEV has repeatedly been emphasized (Clarke 1964; Calisher 1988; Holzmann et al. 1992). In addition, TBEV is very closely related to louping-ill virus which should be regarded in fact as the fourth (or, historically, the first) subtype of TBEV (see below).

History: in Europe, RSSEV subtype of TBEV was first isolated in the Russian Ural Mts. in 1938 (Chumakov and Zeitlenok 1939), and CEEV (strain "256") from *Ixodes ricinus* ticks collected near Minsk, Belarus in 1940 (Levkovich and Karpovich 1962; Votyakov et al. 1978). Further isolations of CEEV were reported in Czechland from human patients and *I. ricinus* ticks in 1948–1949 (Gallia et al. 1949; Krejčí 1949; Rampas and Gallia 1949).

Principal arthropod vectors are ticks of the genus *Ixodes*: for CEEV *I. ricinus* (TST, TOT: Benda 1958b; Řeháček 1962; the infection rate may attain 0.5 % to 3 % in valent natural foci: Grešíková 1972), and *Ixodes gibbosus* (a vicariant, marginal vector in the Mediterranean). Occasional vectors are other tick species such as *Ixodes hexagonus* (Křivanec et al. 1988), possibly *Ixodes arboricola* (successful experimental transmission: Lichard and Kožuch 1967), while only sporadically metastriate tick species *Haemaphysalis inermis*, *Haemaphysalis concinna* (Riedl et al. 1971; TOT), *Haemaphysalis punctata*, *Dermacentor marginatus*, *Dermacentor reticulatus* (Georgiev et al. 1971; Kožuch and Nosek 1971; Naumov et al. 1980; Nosek and Kožuch 1985), and *Hyalomma marginatum* (Crimea). Main vector for RSSEV is *Ixodes persulcatus* (infection prevalence rates can reach frequently >2 %; TST, TOT: Chunikhin 1990), less often *Ixodes ovatus*, but also *Dermacentor silvarum, D. reticulatus, D. marginatus, H. concinna* (TOT), *Haemaphysalis longicornis*, and *Haemaphysalis japonica* (Naumov et al. 1980).

Competent vertebrate hosts of TBEV are small forest mammals, especially rodents and insectivores (*Apodemus flavicollis*, *Apodemus sylvaticus*, *Myodes glareolus*, *Myodes rufocanus*, *Microtus agrestis*, *Sciurus vulgaris*, *Talpa europaea*, *Sorex araneus, Erinaceus concolor*); additional hosts

Table 1 Experimental pathogenicity of tiboviruses occurring in Europe (Karabatsos 1985; Hubálek and Halouzka 1996)

	SM i.c.	SM i.p.	M i.c.	M i.p.	H i.c.	H i.p.	GP i.c.	GP i.p.	C s.c.	CE y.s.	Other
TBEV	3–5	3–6	4–7	5–9	4–6	4–12	7–8	(−)	−	3–7	RM, lamb ic + sc−
LIV	3–4	3–5	7	10	+	(+)	9–12	−	−	(+)	Lamb and goat ic+ M(+), grouse sc+
TYUV	3–6	4–8	3–7	(−)	nd	nd	(+)	−	−	nd	R ip−, RM in(−)
MEAV	5	+	+	−	nd	nd	nd	nd	−	nd	
BAHV	3–4	+	(+)	−	nd	nd	nd	nd	nd	nd	
MTRV	3	10	6	−	nd	nd	nd	nd	nd	nd	
GAV	7	nd	−	−	nd	nd	nd	nd	nd	nd	
PTVV	6	nd	7–8	−	nd	nd	nd	nd	nd	nd	
UUKV	4–6	+	−	−	−	−	−	−	−	3–7	RM ic(−) ip−
ZTV	+	−	−	−	nd	nd	nd	nd	(+)	nd	
SAHV	(+)	−	−	−	nd	nd	nd	nd	nd	nd	
SOLV	4–7	(−)	5–9	−	−	nd	5–8	nd	+	4–5	R, pigeon ic−
PIV	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	
AVAV	7–12	(+)	(+)	−	nd	−	nd	−	−	nd	Rat ic−
CMV	4–11	6–15	−	−	nd	−	nd	−	−	nd	
CCHFV	4–7	5–9	(+)	−	−	−	−	−	nd	nd	RM, sheep ic (+)
BHAV	3–5	5–6	5–8	−	−	−	5–6	−	nd	4–6	RM, lamb ic(+)
EYAV	6–8	(+)	−	−	nd	−	nd	nd	nd	nd	
TRBV	3	4–6	(−)	−	(−)	−	−	−	−	2–4	ic: SH + RM(+)
OKHV	3	−	−	−	nd	nd	nd	nd	nd	nd	
CWV	2–4	3–8	−	−	nd	nd	nd	−	−	nd	Chick ic−
MYKV	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	
TDMV	4	nd	nd	nd	nd	nd	nd	nd	nd	nd	
BAUV	3–4	4–5	−	−	nd	nd	nd	nd	3–5	nd	Chick ic +
THOV	3	3–4	4–8	(+)	+	3	nd	−	nd	nd	Sheep iv fever
DHOV	2–5	3–7	3–6	5–8	+	+	nd	−	nd	−	Ad rat ip,sc−
ASFV	nd	nd	nd	nd	nd	nd	nd	nd	nd	6–7	Pig sc+

The figures show the average survival time (days) of laboratory animals inoculated with particular viruses established after several mouse passages; +, death; (+), irregular death; (−), irregular encephalitis or pareses, but survival; −, no death; nd, not done. Animals: *SM* suckling mouse, *M* adult mouse, *H* adult Syrian hamster, *GP* guinea pig, *C* chick (newly hatched), *CE* chick embryo (inoculated into yolk sac), *RM* rhesus monkey, *R* rabbit. Inoculation mode: *i.c.* intracerebrally, *i.p.* intraperitoneally, *s.c.* subcutaneously, *i.n.* intranasally

may be (due to viremia) goat, sheep, rarely cattle (Brummer-Korvenkontio et al. 1973; Kožuch et al. 1966, 1967a, b; Kiffner et al. 2011). The role of some forest passerines and other birds as hosts of TBEV has not yet been fully elucidated; the virus was isolated occasionally from *Turdus pilaris, Turdus iliacus*, other *Turdus* spp., *Corvus monedula*, *Corvus corone, Pica pica, Sturnus vulgaris*, *Lanius collurio*, *Fringilla montifringilla, Fringilla coelebs*, *Loxia curvirostra, Carduelis flammea*, *Anthus trivialis, Motacilla alba, Motacilla flava*, *Emberiza* spp., *Jynx torquilla, Bonasa bonasia, Crex crex, Scolopax rusticola, Clangula hyemalis, Melanitta fusca*, *Anas querquedula*, *Fulica atra* (Brummer-Korvenkontio et al. 1973; Ernek 1959; Ernek et al. 1977; Grešíková 1972; Grešíková et al. 1975; Hubálek 1994; Lvov and Ilyichev 1979; Saikku 1973; Soběslavský et al. 1960; van Tongeren 1962). A potential for TOT was demonstrated in some avian species (*T. iliacus, T. pilaris*, *Turdus ruficollis*, *Turdus pallidus*, *Lanius cristatus, Emberiza fucata, Troglodytes troglodytes, Accipiter gentilis*) in Asian Russia by isolation of TBEV from their eggs (Kraminskiy et al. 1972). Experimental viremia has been demonstrated in many mammalian, avian, amphibian, and reptilian species (Naumov and Gutova 1979; Naumov et al. 1983, 1984a, b; Gutova et al. 1985; Chunikhin 1990): *Micromys minutus, Microtus arvalis*, *Microtus subterraneus* (Radda et al. 1968), *Myodes rufocanus, Myodes rutilus*, *Glis glis* (Kožuch et al. 1963), *Myotis myotis, Plecotus auritus, Barbastella barbastellus* (Nosek et al. 1961), cat, *Mustela nivalis*, *Mustela erminea* (Radda et al. 1969), *Coturnix coturnix*, *Anas platyrhynchos* (van Tongeren 1983), *Lacerta viridis*,

Table 2 Susceptibility of cell cultures to tiboviruses occurring in Europe (David-West 1971, 1972; Karabatsos 1985; Hubálek and Halouzka 1996)

	CEC, DEC	BHK	VERO	CV-1	GMK	LLC-MK2	PS, SPEV	HeLa	XTC-2	Other
TBEV	p	(+)	(p)	+	+	p	+	(+)	m	
LIV	p	(+)	(p)	+	+	p	+	(+)	m	
TYUV	p	p	p	(+)	(+)	(+)	+	−	nd	
MEAV	−	nm	nm	nd	nd	p	−	nd	nd	
BAHV	+	nd	(+)	nd	nd	nd	nd	nd	nd	
MTRV	+	nd	p	nd	nd	p	nd	nd	nd	
GAV	nd	nd	nd	nd	nd	nd	nd	nd	nd	
PTVV	nd	p	nd	nd	nd	nd	nd	nd	nd	
UUKV	+	+	(+)	+	m	p	+	m	p	BSC-1(+)
ZTV	m	nd	nd	nd	nd	nd	nd	nd	nd	
SAHV	+	+	(+)	nd	nd	nd	nd	nd	+	
SOLV	nd	−	(+)	nd	nd	−	nd	nd	+	
PIV	−	−	−	nd	nd	nd	nd	nd	+	
AVAV	m	m	(+)	nd	nd	nd	m	+	−	
CMV	nd	nd	+	nd	nd	nd	nd	nd	+	
CCHFV	nm	m	nm	p	(p)	(+)	(p)	−	nd	Lamb kidney (p)
BHAV	m	+	+	+	+	m	+	(+)	−	BSC-1 (+)
EYAV	−	m	(p)	(p)	−	−	m	−	nd	
TRBV	(+)	+	+	nd	nd	+	+	+	nd	L, Hep-2, RU-1 +
OKHV	+	(+)	(+)	nd	nd	nd	+	−	nd	
CWV	+	+	p	nd	nd	nd	nd	nd	(+)	
MYKV	nd	nd	p	nd	nd	nd	nd	nd	nm	
TDMV	nd	nd	nd	nd	nd	nd	nd	nd	nd	
BAUV	nd	nd	+	nd	nd	nd	nd	nd	nd	
DHOV	p	+	+	−	nd	+	+	nd	nd	BSC-1−
THOV	p	+	+	nd	nd	p	nd	nd	nd	BSC-1m
ASFV	+	+	+	nd	nd	nd	+	nd	nd	Lamb testis +

Explanations: +, CPE and plaques produced; (+), faint CPE formed; p, plaques produced (under overlay) but no CPE; (p), indistinctive plaques produced, usually no CPE; −, neither CPE nor plaques produced (data on multiplication missing); *m*, multiplication without CPE/plaques production; *nm*, no multiplication; *nd*, not done

Lacerta agilis (Grešíková-Kohútová and Albrecht 1959) and some other vertebrate species (Chunikhin 1990; Gutova et al. 1985; Hubálek 1994; Naumov et al. 1983, 1984a, b; Naumov and Gutova 1979).

TBEV causes fatal disease in suckling and adult laboratory mouse at any route including i.n. and p.o., suckling rat (i.c.) but not adult rat (i.c., i.p.), newborn guinea pig (i.c.), suckling hamster (i.c., i.p.), rhesus monkey (i.c., but not all strains, and not at i.n., i.p., s.c. or i.v. routes: Ilyenko et al. 1974; Zlotnik et al. 1976; Pogodina et al. 1981, 1986), lamb and kid (i.c., i.n. but not s.c.). The diffuse meningoencephalitis is characterized by perivascular infiltration, neuronal degeneration and necrosis, and focal glial proliferation. On the other hand, no mortality is produced by TBEV in adult forest rodents *Apodemus* and *Myodes* spp. (i.p., s.c.), adult rabbit (i.c., i.p.). Encephalitis with ataxia, jumping, tremor, and convulsions can affect lambs, kids or, exceptionally, dogs (Tipold et al. 1993; Pfeffer and Dobler 2011). CEEV infection is usually subclinical in adult ruminants and pig; goats, sheep, and cows excrete virus in the milk (Smorodintsev et al. 1953; van Tongeren 1955; Benda 1958a; Grešíková 1958a, b). TBEV (especially TBE-S and TBE-FE virus subtypes) occasionally kills birds of some species, e.g., *C. flammea* (long-term viremia and the virus excretion in droppings up to 11 months was confirmed experimentally), *Passer domesticus*, and *F. atra* (van Tongeren 1962; Hubálek 1994), amphibians *Rana temporaria* and *Bufo bufo* (s.c.).

Natural foci of TBE have been classified (Rosický 1959) as "theriodic" (situated in deciduous and mixed forest ecosystems, often game preserves), "boskematic" (pastoral), mixed "theriodic-boskematic" or "mountain" (Rosický and Bárdoš 1966; Nosek et al. 1982). Urban foci of CEE have also been described in Europe (Málková et al. 1983).

There are two basic modes of human infection with TBEV—by the bite of an infective tick or by consumption of infected raw (unpasteurized) goat (less often sheep or cow) milk or dairy products (Smorodintsev et al. 1953; Grešíková 1972; Grešíková et al. 1975). Whereas the tick-transmitted cases are sporadic, the milk-borne infections usually affect whole families or population groups in outbreaks. For instance, a large milk-borne TBE epidemic occurred in Rožňava, East Slovakia in 1951, when 660 persons were infected and 274 of them hospitalized (Blaškovič 1954). As much as 76 % of human infections have been alimentary in Belarus (Ivanova 1984). The virus may resist in milk at 60°C for more than 10 min and partially even the pasteurization at 62°C for 20 min, and it is not inactivated at pH 2.8 within 24 h/4°C. In addition, many laboratory infections (usually by infectious aerosol) have been reported in unvaccinated personnel.

Human disease caused by TBEV is meningoencephalitis, usually with typical biphasic course: the first phase starts with sudden fever and flu-like symptoms (pronounced headache, general weakness, nausea, myalgia, arthralgia), sometimes conjunctivitis; the second phase appears after an interval of usually 4–7 days of an apparent recovery, with affection of the CNS (meningoencephalitis) accompanied with fever, retrobulbar pain, photophobia, stiff neck, sleep disorders, excessive sweating, drowsiness, tremors, nystagmus, meningeal signs, ataxia, pareses of cranial nerves and extremities, dizziness, confusion, psychic instability, excitability, anxiety, disorientation, memory loss, and sometimes personality changes. In the CNS, the virus produces diffuse degenerative changes of neurons, perivascular lymphocytic infiltration ("cuffing") and damage to Purkinje cells. Case fatality rate in humans ranges from *c*. 1 % (in TBEV-W), 7–8 % (in TBEV-S), up to 20–40 % (TBEV-FE); convalescence is prolonged, and neurological sequellae (residua) sometimes including pareses are quite common. Major sequelae such as atrophic paralysis of the neck and shoulder are rare in CEE (Ackermann and Rehse-Küpper 1979; Kunz 1981; Holmgren and Forsgren 1990), whereas they are relatively frequent and occasionally combined with a chronic and progressive course (e.g., Kozhevnikov's epilepsy, progressive neuritis of the shoulder plexus, dispersed sclerosis, progressive muscle atrophy) in RSSE (TBEV-S: Zlotnik et al. 1976; Asher 1979; Pogodina et al. 1986; Gritsun et al. 2003).

Several thousand cases of TBE are recorded in Europe each year, with considerable inter-annual variation (Korenberg and Kovalevski 1999; Gritsun et al. 2003; Petri et al. 2010). In some European countries, TBE is quite frequent: for instance, on average, 368 cases (140 to 744 in individual years) a year were reported in Czechland between 1970 and 1999, corresponding to the incidence of 4.2 (1.4–7.4) per 100,000 inhabitants, and it peaked at 1,029 patients (10.0 per 100,000) in the year 2006. In the years 2004–2007, only a few countries have had a higher incidence of TBE than Czech Republic (5.0–10.0): Slovenia 10.2–18.6, Estonia 10.4–13.5, Lithuania 6.5–13.5, Latvia 6.2–10.8, while the TBE incidence was as low as 0.6–1.2 in neighboring Austria, due to a much higher vaccination rate in that country (Mantke et al. 2008).

Diagnosis: serology (ELISA, HIT, CFT, VNT), detection of IgM in early phase or seroconversion in paired serum samples; rarely used is the isolation of the virus from the blood or CSF in cell cultures (e.g., PS pig embryo kidney cells) or in mice, and detection of the virus RNA by using RT-PCR.

Therapy: specific immunoglobulins can be applied to infected persons but they are only effective when inoculated immediately, i.e., within 1–2 days after infection, otherwise they could be even detrimental.

Prevention and control include mapping and surveillance of natural foci of TBE, pasteurization of milk (CEE virus may persist in some dairy products at +4°C for more than 60 days: Grešíková-Kohútová 1959), protection from tick exposure (clothing, repellents), vector tick control, and vaccination. "FSME-Immun" and "Encepur" vaccines (Loew-Baselli et al. 2011; Petri et al. 2010) consist of purified inactivated virus grown in chicken embryo cells produced by methods largely based on a study of Czech virologists (Daneš and Benda 1962). A mass vaccination campaign of Austrian population living in endemic foci led to a significant decline of TBE from 612 cases recorded in 1982 to 89 cases diagnosed in 1990 (C. Kunz, pers. comm.), and a similar 5-to-10 times decrease of TBE incidence has been reported in other European countries after frequent vaccination of population.

European distribution: Fig. 1 Outside Europe, TBEV occurs in the Asian part of Turkey, Asian Russia (Siberia, Far East), Kazakhstan, Kirghizia, Armenia, Azerbaijan, north-eastern China, Japan, and Korean peninsula.

Flavivirus louping ill (LIV)

Synonym: Negishi virus. Prototype strain of LIV is LI-31. Louping-ill virus is very closely related to TBEV, in fact indistinguishable from it by conventional serological and cross-protection tests (Clarke 1962, 1964; Calisher 1988; Calisher et al. 1989; Kopecký et al. 1991; Shiu et al. 1991; Holzmann et al. 1992; Venugopal et al. 1992; Hubálek et al. 1995) and with difficulties by nucleotide sequence homology of the E gene (Gao et al. 1993; Venugopal et al. 1994; Fig. 2 in Gould et al. 2003, Fig. 3 in Weaver 2006; Grard et al. 2007, Fig. 1 in Jääskeläinen et al. 2010). LIV is antigenically and genomically much closer to CEEV than CEEV is related to RSSEV; LIV should thus not be regarded as a separate virus, in that RSSEV and CEEV are considered subtypes of one virus (TBEV). Therefore, Hubálek et al. (1995) and Grard et al.

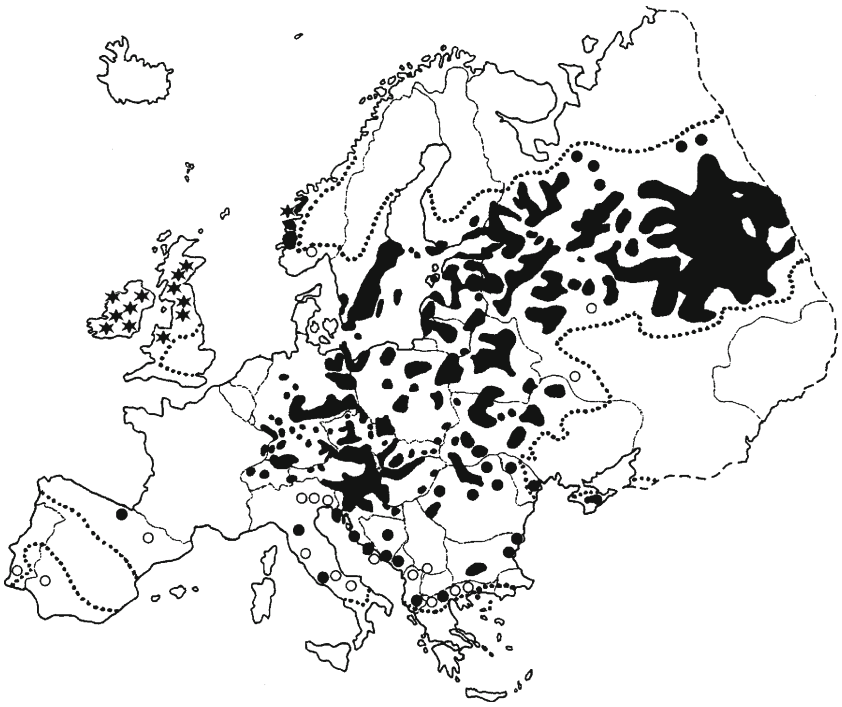

Fig. 1 European distribution of natural foci of tick-borne encephalitis (CEE and RSSE) and louping ill (*asterisks*). Explanation: *black dots* and *black areas*, TBE virus isolation or the virus disease. The *dotted line* shows the limits of the *Ixodes ricinus* plus *I. persulcatus* area

(2007) suggested arrangement of LIV as another subtype of TBEV, and not as a separate virus.

Louping-ill has long been recognized as a disease of sheep in Scotland. For instance, it was recorded in the 1795 Statistical Account or by Walter Scott in 1891 (Davidson et al. 1991). The virus was first isolated from sheep brain in Selkirkshire, Scotland in 1929 (prototype strain Moredun LI-31: Pool et al. 1930) and it is, in fact, the very first arthropod-borne virus isolated in Europe.

Principal vector of LIV is the tick *I. ricinus* (MacLeod and Gordon 1932); LI is also transmissible by the goat and sheep milk (Reid et al. 1984; Reid and Pow 1985), analogically as the other TBEV subtypes.

Vertebrate hosts are e.g., wood mouse (*A. sylvaticus*), common shrew (*S. araneus*), mountain hare (*Lepus timidus*), sheep, and red grouse (*Lagopus lagopus scoticus*: Reid 1990; Gilbert et al. 2000). LIV infection is fatal to suckling rat (i.c., i.p.), lamb (i.c., not s.c.), sometimes rhesus

Fig. 2 European distribution of Tyuleniy (*circles*) and Meaban (*squares*) flaviviruses. (*Slanted area*: antibodies to TYUV)

Fig. 3 European distribution of Uukuniemi (*circles*), Grand Arbaud (*square*), and Ponteves (*asterisk*) viruses. (*Slanted area*: antibodies to UUKV)

monkey (i.c., i.n.: Zlotnik et al. 1976). No symptoms are seen in adult *M. agrestis* (i.c., s.c.), *Cervus elaphus* (s.c.), and *Capreolus capreolus* (s.c.), although meningoencephalitis was demonstrated histologically in the deer (Reid et al. 1982), and LIV was isolated from a roe deer (Reid et al. 1976). LIV occasionally affects also cattle, pig (piglets), goat (kids), horse, dog, hare, and red grouse (with a mortality rate of 70–80 % especially in juvenile birds: Reid et al. 1978, 1980); interestingly, the grouse chicks die when they eat infected ticks. Typical course of LI in sheep is biphasic, with fever and weakness, followed by meningoencephalitis with cerebellar ataxia, generalized tremor, jumping (to "loup" means to leap in vernacular Scottish), vigorous kicking, salivation, champing of jaws, progressing to paralysis, coma and death (lethality 40–60 %). The histopathology shows (sheep, pig, rhesus monkey, or mouse) a diffuse meningoencephalitis with perivascular cuffing, neuronal degeneration, and destruction of Purkinje cells, similar to TBE (Reid 1990). Concurrent tick-borne fever (*Anaplasma phagocytophilum* infection) and external stress enhance the disease course (Reid 1990).

Natural foci of LI are "boskematic" (pastoral: Rosický 1959)—rough, poorly drained hill pastures, heather moorlands with bracken and moor-grass; principally a sheep-tick or sheep-tick-grouse cycle (Reid 1990; Smith and Varma 1981). Unfortunately, spring lambing on hilly pastures coincides with the period of peak seasonal activity of the vector in endemic foci.

The human illness is usually biphasic; the febrile phase, after a short period of improvement, is followed by high fever and symptoms of meningoencephalitis, headache, weakness, stiff neck, conjunctivitis, retrobulbar pain, photophobia, myalgia, arthralgia, dysarthria, excessive sweating, nausea, vomiting, insomnia, drowsiness, confusion, tremors, nystagmus, and ataxia. Additional symptoms are similar to that of TBE.

Nineteen naturally acquired human cases and 26 laboratory infections with LIV have been described in Great Britain between 1934 and 1990 (Davidson et al. 1991), including one fatal encephalitis in a butcher from northern Scotland (Williams and Thorburn 1962). LIV transmission to man is obviously infrequent in the U.K. because the vector ticks only occasionally bite people in endemic areas (similarly as with Lyme borreliosis). It is primarily an occupational disease, affecting shepherds, crofters, veterinary personnel, forestry workers, butchers and laboratory personnel. However, human cases of LI with a milder symptomatology might remain underreported.

Diagnosis: as for TBE.

TBE vaccine is also protective aginst LIV. Control of LI is mainly based on vaccination of sheep; the inactivated LI vaccine is commercially available and in general use. Tick control by dipping the sheep with residual acaricides is also practicized. The methods of environmental control of ticks such as pasture rotation, cutting or burning grass and bush vegetation, and drainage are effective but economically less feasible (Smith and Varma 1981).

European distribution: Norway is the only country of the continental Europe where a typical LIV strain was isolated (Gao et al. 1993) (Fig. 1). LIV does not occur outside Europe.

Flavivirus Tyuleniy (TYUV)

Flavivirus Tyuleniy (TYUV) is related to the Australian Saumarez Reef virus by CFT, VNT and nucleotide sequence of the envelope gene (Marin et al. 1995), while less similar to TBEV by CFT and HIT. TYUV is a member of the Seabird tick-borne virus subgroup of tick-borne flaviviruses.

The virus was first isolated from *Ixodes uriae* collected in nesting grounds of *Uria aalge* on Tyuleniy Island near Sakhalin, Sea of Okhotsk (Asian Russia) in 1969 (Lvov et al. 1971), and simultaneously off the western U.S. coast (Clifford et al. 1971). In Europe, the agent was revealed in fact even earlier—in 1967 as "Murman" virus under similar conditions on the Kola Peninsula, northern Russia (Bekleshova et al. 1970).

Principal vector is *I. uriae* (TST, TOT). Mosquitoes (*Aedes communis*, *Aedes punctor, Aedes excrucians)* may possibly act as secondary (or mechanical) vectors; successful experimental TYUV transmissions by *Aedes aegypti*, *Culex pipiens*, and *Hyalomma asiaticum* have been reported (Lvov and Ilyichev 1979; Lvov et al. 1971, 1973a, b, c, d).

Vertebrate hosts are seabirds *U. aalge*, *Eudyptula minor*, and the suslik *Citellus undulatus*. Antibodies are often present in additional seabirds *Uria lomvia, Lunda cirrhata*, *Fratercula arctica, Fulmarus glacialis, Phalacrocorax urile, Phalacrocorax aristotelis, Larus argentatus, Larus fuscus, Larus marinus, Larus ridibundus*, *Rissa tridactyla* (French coast: Chastel et al. 1985a, b), and in some mammals (*Callorhinus ursinus*, *Alopex lagopus, Lutreola lutreola*). Antibodies were detected in 22–33 % of cattle in the N.-European Russian taiga and tundra zones (Lvov et al. 1989).

Animal disease is unknown, but experimentally inoculated (i.c. or s.c.) birds show clinical symptoms: encephalitis with pareses and occasional death in *R. tridactyla, L. argentatus,* and *U. lomvia* (Berezina et al. 1974). The virus is not pathogenic to adult rabbit (i.p.). Febrile illness with adynamia and anorexia was observed in rhesus monkeys infected aerogenically.

Natural foci of TYUV are seabird colonies on steep rocks.

Three TYUV cases of febrile illness with malaise, laryngitis, lymphadenopathy, arthralgia, and skin petechiae were documented in biologists collecting samples in seabird colonies in the Murmansk region, 1972–78 (Voinov 1978).

European distribution: Fig. 2 Outside Europe TYUV occurs in Asian Russia (Far East–Sea of Okhotsk); coastal West USA (Oregon) and Canada. Migratory seabirds play a role in the exchange of TYUV complex flaviviruses between the northern and southern hemispheres (Lvov and Ilyichev 1979).

Flavivirus Meaban (MEAV)

MEAV is a member of the Seabird tick-borne virus subgroup of tick-borne flaviviruses, Tyuleniy antigenic complex (Calisher et al. 1989). Prototype: Brest/Ar/T707 (*Ornithodoros maritimus*, France, 1981). Closely related to the Australian Saumarez Reef virus by CFT, HIT and even VNT (Chastel et al. 1985a, b), while more distantly related to TYUV (unrelated by VNT), TBEV and other flaviviruses.

First isolated from argasid ticks collected in seagull colonies on Méaban and Penfred islands, Brittany (France) in 1981–82 (Chastel et al. 1985a, b).

Principal vector is the soft tick *O. maritimus* (TST).

Vertebrate hosts are unknown, but antibodies have been detected in gulls *L. argentatus* and *L. fuscus*. Meningoencephalitis in suckling mouse caused by MEAV is characterized by perivascular cuffing and diffuse neuronal necrosis (Chastel et al. 1985a, b).

Natural foci of MEAV occur in seabird colonies.

Animal and human disease caused by MEAV has not been reported, and no antibodies to MEAV were detected by HIT in 562 human sera collected in Brittany (Chastel et al. 1985a, b).

European distribution: Fig. 2 Long-distance migratory *Sterna paradisaea* and *Sterna hirundo* terns could have contributed to the dispersal of MEAV and Saumarez Reef viruses or their common progenitor between Australia and France (Chastel et al. 1985a, b).

Family *Bunyaviridae*

Orthobunyavirus Bahig (BAHV)

Tete antigenic group. Prototype: EgB-90 (*Oriolus oriolus* blood, Egypt, 1966). European topotype: ISS.U.45 (*F. montifringilla* blood, Italy, 1968). Related to Matruh virus by CFT and HIT (indistinguishable by CFT), less to Tete virus.

Originally isolated from the blood of *O. oriolus* caught at Bahig village near Alexandria, Egypt, in 1966 (Watson et al. 1972). In Europe, first reported from migrating birds in N. Italy (Balducci et al. 1973).

Arthropod vector is *H. marginatum* (TOT).

Vertebrate hosts are passerine birds of the genera *Oriolus, Muscicapa, Sylvia, Phylloscopus, Phoenicurus, Luscinia, Chloris,* and *Fringilla* (Balducci et al. 1973; Watson et al. 1972).

Human and animal disease caused by this virus has not been reported.

European distribution: central and northern Italy. Outside Europe: Egypt, Cyprus. BAHV was isolated from larval *H. marginatum rufipes* collected on a northward migrating *Oenanthe oenanthe* in Egypt (Converse et al. 1974) which indicates a possible means of dispersal.

Orthobunyavirus Matruh (MTRV)

Tete antigenic group. Prototype: EgAn 1047–61 (*Sylvia curruca* blood, Egypt, 1961). European topotype: ISS.U.60

(*F. coelebs* blood, Italy, 1968). Related to BAHV by CFT and HIT.

The virus was first isolated by J.R. Schmidt from migrating passerines in Burg el Arab, Matruh Governorate, Egypt, 1961 (Theiler and Downs 1973). In Europe, it was recovered from migrating birds in North Italy (Balducci et al. 1973).

Arthropod vector is probably *H. marginatum*.

Vertebrate hosts are passerine birds of the genera *Phylloscopus, Sylvia, Saxicola, Phoenicurus, Luscinia, Lanius*, *Serinus*, *Carduelis* and *Fringilla* (Italy: Balducci et al. 1973), and *C. coturnix*.

Human and animal disease caused by this virus has not been reported.

European distribution: northern Italy. Outside Europe: Egypt, Cyprus.

Phlebovirus Grand Arbaud (GAV), Phlebovirus Ponteves (PTVV)

Uukuniemi antigenic group. GAV prototype: Argas-2 (*Argas reflexus*, Camargue, France, 1966). PTVV prototype: Larves-6 (*A. reflexus*, France, 1966). Both viruses are related, producing one-way reaction in cross-CFT. Also related to UUKV by CFT.

The viruses were isolated only once from argasid ticks collected in a pigeon house in South France, 1966 (Hannoun et al. 1970).

Arthropod vector is *A. reflexus* (TST, TOT in PTVV).

Vertebrate host is probably pigeon.

Animal and human disease caused by either GAV or PTVV has not been reported.

European distribution: Fig. 3. Outside Europe: unknown.

Phlebovirus Uukuniemi (UUKV)

Uukuniemi antigenic group. Synonyms: Poteplí virus; Sumakh virus. Prototype: S-23 (*I. ricinus*, Finland, 1960). Topotypes: Poteplí PO-63 (*I. ricinus*, Bohemia, 1963), Sumakh (*Turdus merula* heart and lungs, Azerbaijan, 1968). Hemagglutinin is produced, but not readily in all strains.

The virus was originally isolated from *I. ricinus* collected from cattle at Uukuniemi, southeast Finland in 1959 (Oker-Blom et al. 1964), later (1963) in Central Bohemia as “Poteplí” virus (Kolman et al. 1966).

Arthropod vectors are the ticks *I. ricinus* (TST, TOT: Samoilova and Voinov 1980), less commonly *I. persulcatus*. The virus was also isolated occasionally from mosquitoes *Culex modestus, Aedes vexans*, *A. punctor*, *A. communis* and *Aedes cataphylla* (Lvov et al. 1987, 1989; Vinograd et al. 1971)—but mosquitoes are obviously only mechanical vectors.

Vertebrate hosts are forest rodents (*Myodes glareolus, A. flavicollis*: Kožuch et al. 1970a, b; Wróblewska-Mularczykowa et al. 1970; Vinograd et al. 1981) and birds, largely ground-feeding passerines (*T. merula, Turdus philomelos, T. iliacus, T. pilaris, Erithacus rubecula, Prunella modularis, Sylvia communis, O. oenanthe, S. vulgaris, C. corone, P. pica, F. coelebs, Coccothraustes coccothraustes, Emberiza citrinella, Streptopelia turtur,* and *Phasianus colchicus*: Gaidamovich et al. 1971; Hubálek 1994; Lvov and Ilyichev 1979; Saikku 1974; Saikku and Brummer-Korvenkontio 1973; Vasilenko et al. 1975a, b; Vinograd et al. 1971, 1975). Viremia and long-term persistence of the virus was demonstrated in experimentally infected birds of many species. Antibodies were also detected in cows and reptiles. Fatal meningoencephalitis with myositis occurs in suckling mouse but no symptoms are observed in adult mouse (any route incl. s.c., i.n.) or adult rat (i.c.); also pathogenic to suckling but not adult *M. arvalis, A. flavicollis* or *M. glareolus* (i.c., usually not i.p.: Kožuch et al. 1970a, b) and suckling rat (i.c., not i.p.), and non-pathogenic for rhesus monkey inoculated i.p. (but lymphocytic meningitis appeared when UUKV was given i.c.: Grešíková et al. 1970).

Animal and human disease caused by UUKV has not been reported. Antibodies were detected infrequently (≤5 % persons examined) in a few areas (Kolman et al. 1973; Málková et al. 1980; Molnár et al. 1976; Sekeyová et al. 1970; Vasilenko et al. 1975a, b) while only exceptionally at a higher frequency (e.g., 13–14 % in western Belarus and Hungary: Voinov 1978; Molnár et al. 1980), and much more often these serosurveys for UUKV were negative.

European distribution: Fig. 3. Outside Europe: Azerbaijan, Asian Russia. Antibodies in Tunisia. Migratory birds play a role in the widespread distribution of UUKV; e.g., several strains of the virus have been isolated from immature *I. ricinus* collected on migratory passerines (Traavik 1979).

Phlebovirus Zaliv Terpeniya (ZTV)

Uukuniemi antigenic group. Prototype: LEIV-21C. Distantly related to UUK virus by CFT.

Originally isolated from adult *I. uriae* collected in rocky breeding grounds of marine birds (*U. aalge* etc.) on Tyuleniy Island (Sakhalin region) and Commodore Islands (Kamchatka region), Russia in 1969 (Lvov et al. 1973a, b, c, d). In Europe, first isolated under similar conditions in the Murmansk region, North Russia in 1970 (Lvov et al. 1973a, b, c, d, 1989).

Arthropod vectors are *I. uriae* (TST, TOT), rarely *I. signatus*. Occasional isolations from *Ae. communis* mosquitoes in N.-European tundra (Lvov et al. 1987, 1989).

Vertebrate hosts are *U. lomvia*, *R. tridactyla*. Antibodies were also detected in *L. marinus* and *U. aalge*. Some

mortality (acute viral encephalitis) has been observed in chickens inoculated i.c. or s.c. (Chastel 1988).

Animal and human disease caused by ZTV has not been reported. Antibodies rarely occur in farmers who have lived near Cap Sizun (Chastel 1988).

European distribution: Fig. 4. Outside Europe: E. and N. Asian Russia (Sakhalin, Kamchatka, Taimyr), NW. Canada and USA.

Phlebovirus St. Abb's Head (SAHV)

Uukuniemi antigenic group. A non-registered virus (isolates GM710 and M349) that involves a number of closely related (SAHV-like) strains. Prototype: M-349 (*I. uriae*, North Scotland, 1979).

First isolated from adult *I. uriae* and the blood and organs of moribund juvenile kittiwakes (*R. tridactyla*) collected on breeding grounds off N. Scotland, 1979 (Nuttall et al. 1981) and NE. England (Eley and Nuttall 1984).

Arthropod vectors are *I. uriae* (TST) and *I. rothschildi* (Nuttall et al. 1984a).

Main vertebrate host is kittiwake *R. tridactyla*. An illness in juvenile kittiwakes has repeatedly been observed. Antibodies have also been detected in *U. aalge, Alca torda,* and other marine birds (Nuttall 1984; Nuttall et al. 1984a). A relatively low mortality of suckling mice was observed at i.c. inoculation (Nuttall et al. 1984b; Moss and Nuttall 1985).

Human disease caused by SAHV has not been reported.

European distribution: Fig. 4.

Labuda and Nuttall (2004) list a number of additional uukuvirus-like isolates from *I. uriae* ticks collected from European colonial seabird habitats, that may belong either to SAHV or ZTV, or some of them possibly to a novel virus: Arbroath ARB2, (Scotland), Ellidaey ELL-1,-2,-4 (Iceland), Flatholm (Iceland), Foula F89-1 (Shetland Islands), Great Saltee Island GS80-4,-10,-11 (SE. Ireland), Isle of May M320/79, M326/79, M34-81, M35-81 (Scotland), Marsden (England), Rost Islands NorV-697,-707,-820-868 (Norway), Runde Island Ru E82 (Norway), Soay (Scotland–St. Kilda).

Nairovirus Soldado (SOLV)

Hughes antigenic group. Prototype: TRVL-52214 (*Ornithodoros capensis/denmarki*, Trinidad, 1963). European topotypes: EgAr-3608 (*O. maritimus,* N. Wales, 1974) and Brest-Ar/T13 (*O. maritimus*, France, 1977). A remarkable antigenic heterogeneity of SOLV isolates has been found by CFT; in fact, some European (French, Irish) isolates differ from the prototype strain more than eightfold in reciprocal titres (Chastel et al. 1983). SOLV is distantly related to Zirqa and Punta Salinas viruses of the Hughes serogroup by CFT, VNT, and IFA (Converse et al. 1976; Yunker et al. 1977). The virus is very stable at pH 3.

SOLV was originally isolated from mixed nymphal *O. capensis* and *O. denmarki* ticks infesting *Anous stolidus* colonies on Soldado Rock near Trinidad, 1963 (Jonkers et al. 1973). In Europe, it was recovered from *O. maritimus* infesting *L. argentatus* nests on Puffin Island (N. Wales: Converse et al. 1976), Ireland (Keirans et al. 1976), England (Nuttall et al. 1986), and Cap Fréhel and Cap Sizun (Brittany, France: Chastel et al. 1979, 1981a, b, 1988a, b; Quillien et al. 1986).

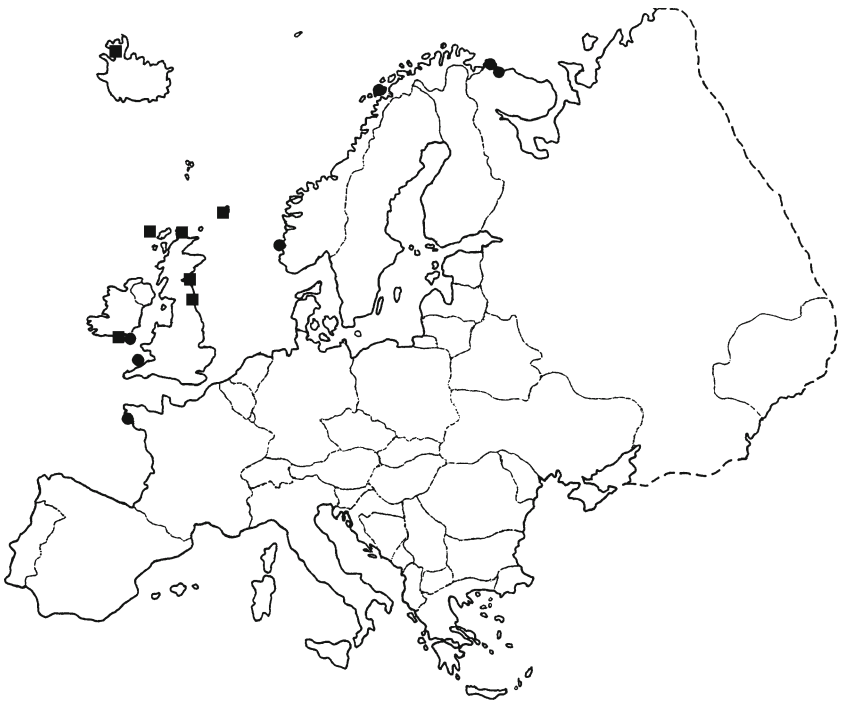

Fig. 4 European distribution of Zaliv Terpeniya (*circles*) and St. Abb's Head (*squares*) viruses

Arthropod vector is *O. maritimus* (TST; the mean infection rate of vector ticks can be as high as 20 %: Johnson et al. 1979) in Europe, while *O. capensis* elsewhere.

Vertebrate hosts are seabirds *Sterna fuscata*, *L. argentatus,* and *R. tridactyla* (Chastel et al. 1990). Antibodies were also detected in *Larus cachinnans*, *Larus cirrhocephalus*, *Phalacrocorax aristotelis,* and other species. Mortality due to SOLV was observed in young seabirds such as *S. fuscata* or *L. argentatus* (Converse et al. 1975; Chastel et al. 1990). Infected *O. capensis* have transmitted the virus to domestic chicks and caused their death on days 5 to 8 post-feeding (Converse et al. 1975).

Natural foci are seabird colonies (usually on rocky offshore islands).

Ornithologists bitten by *O. capensis* in the Seychelles experienced severe pruritus persisting for a few days (Converse et al. 1975); the etiology has remained unclear in that a possible cutaneous reaction to tick bites could not be excluded. A case of febrile illness with persistent rhinopharyngitis and pruritus due to SOLV was observed in a scientist who had been repeatedly bitten by *O. maritimus* in Morocco (Chastel et al. 1981a, b). However, antibodies rarely occur in farmers who live near Cap Sizun (Chastel 1988). The related Zirqa and Punta Salinas viruses may cause fever with headache, pruritus, and erythema in people in the Arabian Gulf and Peru, respectively (Converse et al. 1975, 1976).

European distribution: Fig. 5. Outside Europe: Trinidad, Ethiopia, Senegal, Seychelles, South Africa, Morocco, USA (Hawaii, Texas). Seabird migrations account for the widespread distribution of SOLV (Converse et al. 1975). A number of additional Hughes group SOLV-like isolates from *I. uriae* ticks collected from European colonial seabird habitats were reported (Labuda and Nuttall 2004): Ellidaey ELL81-3b (Iceland), Foule F80-1 (Shetland Islands), Great Saltee 59972, GS80-3 (Ireland), Grimsey G82-1b (Iceland), Inner Farne IF80-3,-4 (England), Isle of May (Scotland).

Nairovirus Puffin Island (PIV)

Hughes antigenic group. Prototype: 9617 (*O. maritimus*, Wales, 1974); other similar isolates are EgArt 608, 3615, 3616 (Wales), and Petticko Wick (Scotland: Labuda and Nuttall 2004). A non-registered virus composed of strains closely related to SOLV but distinguishable by IFA and VNT (Gould et al. 1983). The virus is very sensitive at pH 3.

PIV was first isolated from argasid ticks collected in *L. argentatus* nests on Puffin Island, N. Wales in 1974 and originally referred to as SOLV, but re-identified as a new virus later (Gould et al. 1983).

Arthropod vector is *O. maritimus*, but several Icelandic and British isolates have been recovered also from *I. uriae*.

Vertebrate hosts are *L. argentatus* and *F. arctica*. Antibodies were also detected in *U. aalge*, *A. torda,* and other seabirds.

Animal and human disease caused by PIV has not been reported.

European distribution: Fig. 5 In addition, antigenically closely related virus strains were isolated in Ireland (GS-80-3:

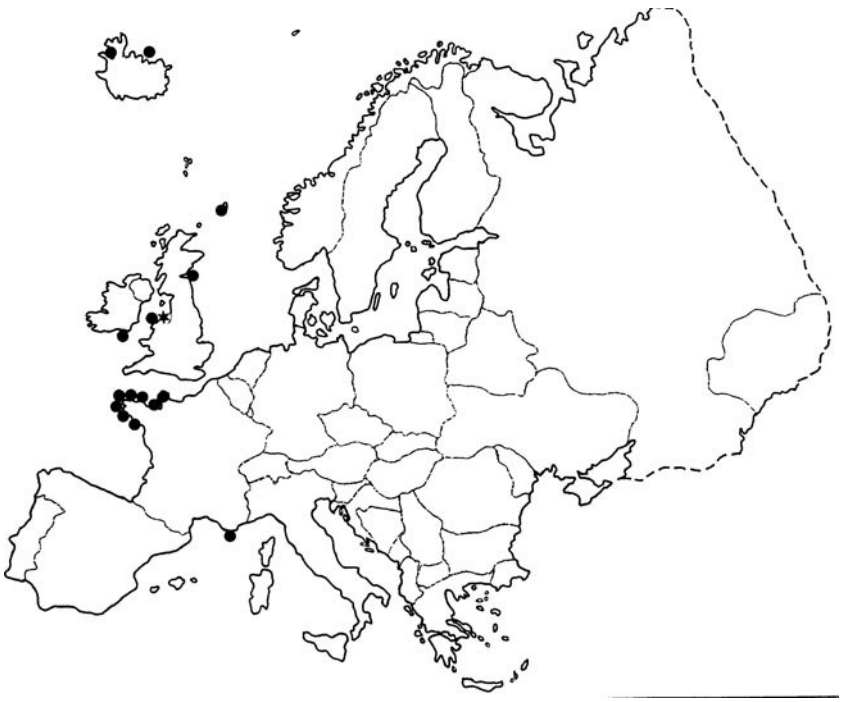

Fig. 5 European distribution of Soldado (*circles*) and Puffin Island (*asterisks*) nairoviruses

Nuttall et al. 1984a), Britain (Nuttall et al. 1986) and Iceland (GRIMS82-1b, ELL-3b: Moss et al. 1986).

Nairovirus Avalon (AVAV)

Sakhalin antigenic group. Synonym: Paramushir virus. Distantly related to Sakhalin virus (SAKV) by CFT (Main et al. 1976a, b). Prototype: CanAr-173 (*I. uriae*, Newfoundland, 1972). Topotype: LEIV-2268Ku ("Paramushir": *I. signatus*, Paramushir Island, Far East, 1969).

First isolated from engorged adult and nymphal *I. uriae* collected in a *L. argentatus* nest on Great Island, Newfoundland, Canada in 1972 (Main et al. 1976a, b). "Paramushir" virus was isolated from *I. uriae* and *I. signatus* collected from seabird colonies in the Far East in fact earlier, in 1969 (Lvov et al. 1976). In Europe, several strains of AVAV were isolated from *I. uriae* collected in Cap Sizun, Brittany, France in 1979 (Chastel et al. 1981a, b; Quillien et al. 1986).

The virus is stable at pH 3, but some strains might be acid labile (Quillien et al. 1986); heat sensitive (inactivated at 56°C within 30 m).

Arthropod vectors are *I. uriae* (TST) and *I. signatus.*

The vertebrate host is *L. argentatus* (Main et al. 1976a, b). Antibodies were also detected in *F. arctica*, *Oceanodroma leucorrhoa*, *Larus marinus*. Spontaneous animal disease is unknown. Although fatal to suckling mouse (i.c.), the survival is long and the titres in suckling mouse brain are rather low.

Natural foci are seabird colonies on cliffs.

Three human cases of cervical adenopathy were described in France (Chastel 1985). However, antibodies in humans occur rarely: only 1 % of farmers who had lived near Cap Sizun were seropositive (Quillien et al. 1986).

European distribution: Fig. 6. Outside Europe: Asian Russia (Far East), Canada.

Nairovirus Clo Mor (CMV)

Sakhalin antigenic group. Prototype: ScotAr-7 (*I. uriae,* Scotland, 1973). Closely related to SAKV (prototype LEIV-71c: *I. uriae,* Far East, 1970–Lvov et al. 1972), the difference in titres being only three to fourfold in cross-CFT (Main et al. 1976a, b). CMV may be regarded as a subtype of SAKV.

First isolated from engorged nymphal *I. uriae* collected in a *U. aalge* colony at Clo Mor, Cape Wrath, Scotland in 1973 (Main et al. 1976a, b).

The virus is very stable at pH 3 (>3 h at 4°C). HA is occasionally produced in suckling mouse brain.

Arthropod vector is *I. uriae* (TOT: Lvov et al. 1972).

Vertebrate hosts are unknown (possibly seabirds, but antibodies have not yet been detected in them). Fatal to suckling mouse (s.c.) but not to adult mouse (s.c.) or chicks (i.c.). Suckling mice are relatively insensitive for the isolation attempts (Nuttall et al. 1984b). Moreover, CMV is poorly immunogenic in mouse at i.p. or i.c. inoculation.

Human disease caused by CMV has not been reported; no antibodies have been detected in humans.

European distribution: Fig. 6. Outside Europe: Asian Russia (northern Far East). Two strains similar to CMV were reported (Labuda and Nuttall 2004) from *I. uriae* ticks in seabird colonies: Old Copper Mine (England–Lundy) and Shiant Islands M325 isolate (Scotland).

Nairovirus of Crimean-Congo hemorrhagic fever (CCHFV)

Synonyms: Crimean hemorrhagic fever (CHF) virus; Congo (CON) virus. Prototype: Khodzha (human blood from a fatal case, Uzbekistan, 1967). African topotype: V-3011 (human blood, Zaire, 1956–registered in 1969). European topotype: Drozdov (human blood, southern Russia, 1967).

The disease (hemorrhagic fever) was first mentioned by Tadjik physician Abu-Ibrahim Djurdjani in the 12th century (Shapiro and Barkaghan 1960). It has been extensively studied since the 1944/45 epidemic (more than 200 human cases, *c.* 10 % were fatal) in the Crimean peninsula and called "Crimean hemorrhagic fever". Mikhail P. Chumakov and co-workers demonstrated viral etiology of the disease by experimental infection of a volunteer with an ultrafiltrate of homogenized nymphal *H. marginatum* ticks collected from local hares in 1945 (Chumakov 1974). CHF virus was first isolated from patients in Astrakhan, Rostov and Uzbekistan in 1967 (Butenko et al. 1968; Chumakov et al. 1971), and from ticks in Crimea in 1972–73 (Chumakov 1974), while CON virus was recovered earlier by G. Courtois from a patient in Zaire (Congo) in 1956 (Simpson et al. 1967). It was recognized that CONV is identical to CHFV (Casals 1969), and Harry Hoogstraal proposed the combined name of the virus and disease–CCHF (Hoogstraal 1979).

Arthropod vectors (and also a reservoir of CCHFV) are metastriate ixodid ticks—*H. marginatum* (TST, TOT), *H. rufipes* (TOT, Africa), *H. turanicum* (Asia), *H. truncatum* (TST, TOT), *H. asiaticum, Hyalomma anatolicum, H. excavatum, H. detritum, H. nitidum, H. impeltatum, H. impressum, H. lusitanicum* (Spain), *H. punctata* (Europe), *Rhipicephalus bursa* (Europe), *R. sanguineus* (Europe), *R. rossicus* (South Russia, TOT: Kondratenko 1976), *R. pumilio, R. pulchellus, R. turanicus, D. marginatus* (Europe, TOT: Kondratenko 1976), *D. daghestanicus, Amblyomma variegatum*, *Boophilus annulatus* (syn. *B. calcaratus*: Bulgaria, Russia), *B. decoloratus* and *B. microplus* (Pakistan). Much less frequent vectors are prostriate ticks (subfamily *Ixodinae*): *I. ricinus* (few CCHFV isolations in Crimea, Moldavia, Bulgaria and Hungary). Occasional vectors outside Europe can be soft ticks *Argas persicus* (Uzbekistan) and *Ornithodoros (Alveonasus) lahorensis* (Iran).

Fig. 6 European distribution of Avalon (*circles*) and Clo Mor (*asterisks*) nairoviruses

Vertebrate hosts are leporids, hedgehog, other small mammals, cattle, horse, goat, sheep. There is inapparent course of the CCHF infection in mammals, and birds are refractory to experimental infection. Fatal to suckling rat (i.c. but not i.p.) and newborn cotton rat (i.c., i.p.). No mortality in adult rat (i.p., s.c.), rabbit (i.p., s.c.; some symptoms after i.c.), hare *Lepus europaeus* (i.v., s.c.), *Citellus pygmaeus* (i.c., s.c.), rhesus monkey i.p. (rash only), sheep (s.c., i.p.), calf (i.v.), donkey and horse (i.v., s.c.). Plaques (and/or indistinct CPE) produced in primary *Cercopithecus* kidney cells. Usually no multiplication in BSC-1, HEp-2, and primary mouse embryo (David-West 1971, 1972).

Natural foci of CCHF are typically xerothermic, mostly open habitats with shrub and dispersed or solitary trees.

CCHF is transmitted mostly by bite of an infective tick, at removal of feeding ticks, but also during shearing of sheep with attached infectious ticks, slaughtering of infected animals (livestock-to-human transmission), or by direct contact with a human patient, e.g., at nursing and care for patients (human-to-human transmission). CCHF is highly contagious, and many hospital, household and laboratory infections (including fatal) have been described. For instance, 6 % of the human CCHF cases recorded in Bulgaria were nosocomial. A much greater proportion of nosocomial and family infections occur in the Middle East, Central Asia, and Pakistan, usually with a high mortality rate (Hoogstraal 1979). CCHF may be an occupational disease in cattle breeders, butchers, livestock industry, health professionals (nosocomial spread), laboratory workers (aerosol).

Human disease: CCHF is characterized by an abrupt onset with fever (3–16 days, often biphasic), chills, general weakness, severe headache, myalgia, neckache, back pains, generalized arthralgia, hyperemia of the face, neck and chest, conjunctivitis, pharyngitis, abdominal and epigastric pains, nausea, anorexia, vomiting, stiffness, diarrhea, photophobia, lymphadenopathy, hepatomegaly, hepatitis, dizziness, psychotic signs (depression, sleepiness, lassitude), bradycardia, hemorrhagic manifestations (from petechial rash on the trunk to large hematomas on the mucous membranes and skin, bleeding from mucous membranes—gums, nose, intestine and lungs or kidney; sometimes bleeding into brain), liver failure, pulmonary failure, hemorrhagic shock. Laboratory findings include increased levels of transaminases, leukopenia, thrombocytopenia and coagulopathy. Long convalescence (common problems are asthenia, hair loss, rapid fatigability, sweating, headache, poor vision and hearing), but without residua. Fatality rate is 3–30 % (but in nosocomial infections up to 50 %).

In south-eastern Europe, several outbreaks of CCHF have been recorded since the 1950s: e.g., 1,568 cases were notified in Bulgaria from 1953 to 2008, with a mean fatality rate of 17 %. Since 1999 (but especially in 2006–07), a reactivation of natural foci and re-emergence of CCHF occurred in Kosovo (119 cases during 1995–2001), southern Russia (regions Stavropol, Astrakhan, Rostov, Volgograd, Kalmykia, Dagestan:—a total of >1,300 patients were diagnosed with CCHF from 1999 until 2007, the fatality rate being 3–5 %. Albania reported eight CCHF cases in 2001, additional cases in 2003–2006. A surprising, continuous epidemic process started in the Asian part of Turkey in 2002,

and until 2009, a total of 4,430 human cases were reported from 680 settlements mainly in the Tokat and Sivas provinces (but as many as 2,615 cases were notified solely in the last 2 years 2008 and 2009), with a mean overall fatality rate of 5 %; in addition, 16 % of healthy population have antibodies to CCHF virus in Turkey at present (most often farmers and village residents). This exceptional epidemiological upsurge of CCHF in Turkey (largely in north-east Anatolia in the Asian part of the country) has been associated ecologically with fragmentation and use of agricultural land and the formation, by this way, of optimal habitats for *H. marginatum* vector ticks (Maltezou and Papa 2010). Some other recent epidemics outside Europe: Iran 248 cases between 2000 and 2004; Mauretania 38 cases (11 fatal) in 2003. In 2009, human cases of CCHF were also reported from Georgia, Kazakhstan, Tajikistan, Iran, Pakistan, and Afghanistan.

Diagnosis: RT-PCR, detection of antibodies or antigen (ELISA, IFA), isolation of the virus (extreme risk).

Treatment: in acute phase (if diagnosed very early) ribavirin though its efficacy has not been unequivocally confirmed in clinical studies. Specific immunoglobulins can be used prophylactically or therapeutically, but only in the first days after infection (Vasilev et al. 1991).

Prevention: a vaccine of Bulgarian provenience (inactivated, suckling mouse brains; not commercial, a small scale production) has been successfully applied to several hundred persons in the Rostov region and Bulgaria (Vasilenko et al. 1975a, b).

European distribution: Fig. 7. Outside Europe: Asian part of Turkey, Armenia, Azerbaijan, Kirghizia, Kazakhstan, Turkmenia, Uzbekistan, Tadjikistan, Mongolia, China, Afghanistan, Pakistan, Iran, Iraq, Saudi Arabia, United Arab Emirates, Oman, Kuwait, Ethiopia, Somalia, Senegal, Guinea, Uganda, Zaire (Congo), Nigeria, Central Africa, Mauritania, Upper Volta, Kenya, Zimbabwe (Rhodesia), S. Africa, Madagascar. Antibodies have been detected in India, Egypt, and Tanzania. Livestock movements and migratory birds play an important role in the transport of infected vector ticks to other areas. For instance, CCHF virus was isolated from nymphal *H. marginatum* removed from *Corvus frugilegus*, *Passer montanus, Galerida cristata,* and *Tockus erythrorhynchus* in Rostov, Astrakhan, Kirghizia and Senegal, respectively (Hoogstraal 1979; Wood et al. 1978; Zeller et al. 1994a, b).

Bhanja Bunyavirus (BHAV)

This virus is, together with two other African tick-borne viruses Kismayo (Butenko et al. 1979) and Forécariah (Boiro et al. 1986), a member of Bhanja group that has not yet been assigned to a recognized genus of the family *Bunyaviridae*. Synonym or subtype: Palma virus (PoTi-4.92 strain, isolated from male *H. punctata* in Portugal, 1992: Filipe et al. 1994); the mean cross-PRNT titre differences among European, Indian and African strains of BHA have been found as great as four to tenfold (Hubálek and Halouzka 1985).

BHAV was isolated first from *Haemaphysalis intermedia* (syn. *H. parva*) ticks that had been collected from a paralyzed goat in Bhanjanagar (district Ganjam, Orissa State, India) in 1954 (prototype strain IG-690), but the record was

Fig. 7 European distribution of Crimean-Congo hemorrhagic fever nairovirus. The *dotted line* shows the northern limits of the *Hyalomma marginatum* area in Europe

published much later (Shah and Work 1969). In Europe, the first isolation was from adult *H. punctata* collected in Italy, 1967 (European topotype ISS.IR.205: Verani et al. 1970a, b), then in Croatia (Vesenjak-Hirjan et al. 1977) and Bulgaria (Pavlov et al. 1978).

The virus is transmitted by metastriate ixodid ticks of several species—in Europe *H. punctata, Haemaphysalis sulcata,* and *D. marginatus*; elsewhere *H. intermedia, Boophilus decoloratus, B. annulatus, B. geigyi, A. variegatum, H. marginatum, H. detritum, H. dromedarii, H. truncatum, R. bursa,* and *Rhipicephalus appendiculatus.* Experimental transmission including TOT was demonstrated in *H. asiaticum* (Gaidamovich et al. 1976).

Vertebrate hosts for BHAV are sheep, goat (Verani et al. 1971), cattle; in Africa, BHAV was also isolated from the four-toed hedgehog (*Atelerix albiventris*) and striped ground squirrel (*Xerus erythropus*). Antibodies were detected in dogs, *C. elaphus, C. capreolus,* and *Sus scrofa* (Punda et al. 1986). The virus does not usually cause apparent infection in adult animals but is pathogenic for young ruminants (lamb, kid, calf), causing fever and CNS affection (meningoencephalitis), or leucopenia in cattle (Theiler and Downs 1973; Hubálek 1987; Semashko et al. 1976; Camicas et al. 1981; Mádr et al. 1984). Experimental encephalitis was produced in rhesus monkey (Balducci et al. 1970; Verani et al. 1970b). Fatal to suckling mouse i.n. and adult mouse (i.c., i.n., but not s.c., i.v., p.o., or per conjunctivae). Encephalitis in lamb (i.c., but not s.c. or i.v.: Semashko et al. 1976; Mádr et al. 1984) and rhesus monkey (i.c.: Balducci et al. 1970). Not fatal to adult goat (s.c.), rabbit (i.c., i.n., s.c., i.v., i.m., p.o.; a low viremia), and several passerine birds. Faint CPE and plaques produced in BSC-1, RK-13, and primary mouse embryo (David-West 1971) cells, while multiplication without CPE in HEp-2 cells (David-West 1972).

Natural foci of BHAV are boskematic—pastoral steppe or forest-steppe ecosystems in xerothermic areas or in karst habitats at more northern latitudes. Based on a comparison of several known natural foci of BHAV infection, their common and typical features were extracted and bio-indicator species (plants, animals) were selected that can be used for prediction of potential presence of BHAV in other geographic areas within Europe (Hubálek 2009).

BHAV causes in human febrile illness with headache, conjunctivitis, or sometimes meningoencephalitis with photophobia, vomiting, and pareses. About ten natural and/or laboratory infections with BHAV have been described in humans, one of them serious—quadriparesis (Calisher and Goodpasture 1975; Punda et al. 1980; Vesenjak-Hirjan et al. 1980). There is some occupational risk for shepherds and veterinary personnel. Probably an underdiagnosed disease in the Mediterranean and Balkan countries.

European distribution: Fig. 8. Outside Europe: India, Kirghizia, Kazakhstan, Azerbaijan, Armenia, Senegal, Guinea, Nigeria, Cameroon, Central Africa, Kenya, Somalia. Antibodies were detected in Srí Lanka, Pakistan, Iran, Turkmenia, Uzbekistan, Tadjikistan, Uganda, Tanzania, Egypt, and Tunisia. Migratory birds might play a role in the transport of infected immature ticks to distant areas.

Family *Reoviridae*

Coltivirus Eyach (EYAV)

A member of Colorado tick fever (CTF) group. Serologically closely related to North-American CTFV by CFT and VNT; however, CTFV is not neutralized with anti-EYA serum.

First isolated from *I. ricinus* ticks collected at Eyach near Tübbingen, Germany, 1972 (prototype: Eyach-38: Rehse-Küpper et al. 1976), later (1981) from *I. ricinus* and *Ixodes ventalloi* collected on a wild rabbit in NW. France (Chastel et al. 1984). There is a hypothesis that this virus, a descendant of CTF agent, could have been imported from North America with U.S. Army dogs and their *Dermacentor* ticks to a military base situated in Germany after the 2nd WW, and evolved into Eyach virus under the selective pressure of European ecosystem (Hubálek and Rudolf 2011). Another hypothesis suggests that CTFV could have been introduced to Europe with cottontail rabbits, *Sylvilagus floridanus* (Attoui et al. 1998).

The dsRNA of EYAV consists of 12 segments, in contrast to the genus *Orbivirus* with ten segments. Very sensitive to trypsin, acid, and heat (60°C).

Arthropod vectors are ticks *I. ricinus* (TST) and *I. ventalloi*.

Vertebrate hosts are rodents (they reveal prolonged experimental viremias) and lagomorphs (*Oryctolagus cuniculus*). Animal infection has an inapparent course, but meningoencephalitis in suckling mouse (i.c.) has been demonstrated histologically.

Serological data indicate possible association (not yet reliably demonstrated) of EYAV with human neuropathies including five patients with meningoencephalitis (Málková et al. 1980; Fraňková 1981); additional investigation is necessary. The closely related CTF virus (principal vector is *Dermacentor andersoni*) causes acute febrile illness in the mountainous northwestern parts of North America, with a number of cases each year.

Diagnosis: serology (IgM ELISA, IFA, VNT; but not HIT); virus isolation.

European distribution: Fig. 9.

Orbivirus Tribeč (TRBV)

Synonyms (or subtypes): Lipovník (LIP-91, *I. ricinus*, East Slovakia, 1963), Koliba, Cvilín (Libíková et al. 1965,

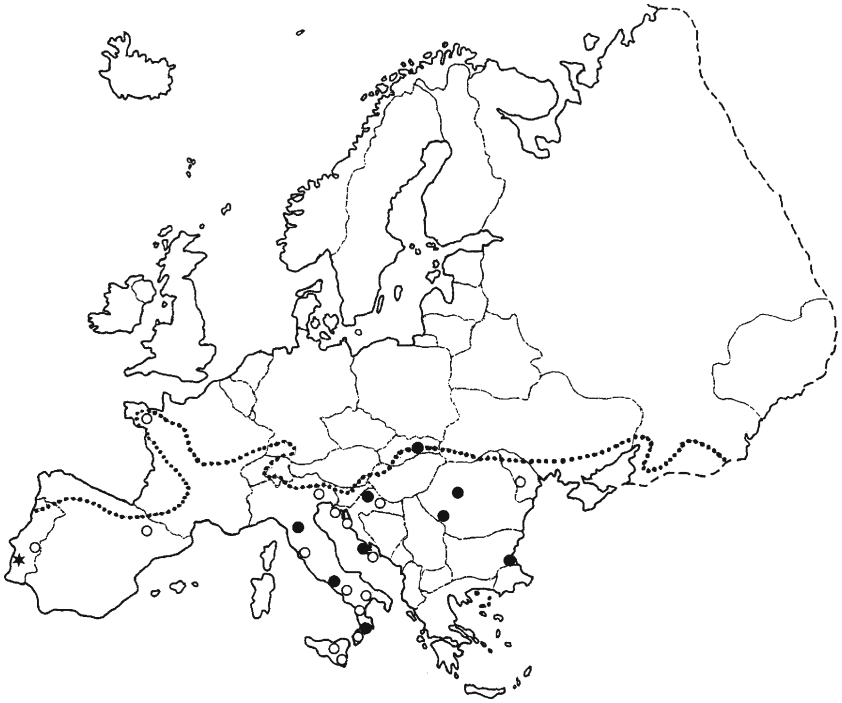

Fig. 8 European distribution of Bhanja virus. Explanation: *black dots*, the virus isolation; *asterisk*, Palma virus (a subtype of BHAV). The *dotted line* shows predicted northern limits of the Bhanja virus area in Europe, based on the presence of bio-indicators (Hubálek 2009) and largely compatible with the range of *Haemaphysalis punctata* and *Dermacentor marginatus* vector ticks

1977), Brezová (subtype: Hubálek et al. 1987a, b), Mircha (strain "634": Vinograd et al. 1977), Kharagysh (Skofertsa et al. 1972). Member of Kemerovo antigenic group, and the Kemerovo subgroup (Belhouchet et al. 2010). Contrary to coltiviruses, orbiviruses of the Kemerovo group have only 10 segments of dsRNA with a total size of 19 kbp. Prototype strain: Tribeč (*I. ricinus*, West Slovakia, 1963). Closely related to the Siberian Kemerovo virus by CFT but distinguishable by VNT (Libíková and Buckley 1971; Libíková and Casals 1971) or RNA-RNA hybridization (Brown et al. 1988). Gene pools of the Kemerovo group and other orbiviruses have a great reassortment potential (because of the segmented dsRNA) and resulting biological variability (Gorman et al. 1978, 1983; Gorman 1983; Brown et al. 1988, 1989). Interestingly, rabbit syncytium virus that occurs in *S. floridanus* rabbit in the USA is also closely related to TRBV.

First 28 strains of TRBV were isolated from *I. ricinus* in three regions of Slovakia in 1963; a few strains had been

Fig. 9 European distribution of Eyach coltivirus

isolated already in 1961 but lost thereafter (Libíková 1964; Libíková et al. 1964, 1965; Grešíková et al. 1965).

Nearly resistant to diethyl ether and sodium deoxycholate but very sensitive to acid (even pH 5–6), alkali (pH 10) and trypsin.

TRBV is transmitted by ticks *I. ricinus* (TST) and *I. persulcatus*, occasionally by *H. punctata* (Topciu et al. 1968).

Vertebrate hosts of TRBV are rodents, e.g., *M. glareolus* and *M. subterraneus* (Grešíková et al. 1965), hare *L. europaeus* (Dobler et al. 2006), goat (Grešíková et al. 1965), European starling *S. vulgaris* and chaffinch *F. coelebs* (Skofertsa et al. 1974, 1976). Antibodies are present very often in grazed ruminants in endemic areas (up to 45–88 % reactors: Hubálek et al. 1986). Animal disease is unknown (inapparent). However, TRBV is fatal to suckling mouse (also s.c.: meningoencephalitis—progressive neuronal and glial damage with perivascular infiltration), suckling rat, and suckling Syrian hamster (i.c., but not s.c.). Meningitis but survival or no symptoms at all in adult mouse inoculated i.c. (but local necrotizing encephalitis demonstrated histologically), while no symptoms in adult mouse given s.c., i.n. or p.o., adult rat (i.c.), rabbit (i.c.), and peripherally inoculated calf or foal. Fever and meningitis are present in rhesus monkey inoculated i.c. (Grešíková et al. 1966).

The virus causes an occasional febrile illness or aseptic meningitis in humans—e.g., at least 15 patients with the CNS infection (meningitis) revealed seroconversion against TRBV in Czechland (Fraňková 1981; Málková et al. 1986; Hubálek et al. 1987a, b). Antibodies occur in human population, interestingly at a higher frequency among patients with multiple sclerosis (Libíková et al. 1978). There is potential occupational risk for forestry workers. The disease caused by TRBV is probably underdiagnosed. Additional studies are necessary to evaluate the public health importance of TRBV.

Diagnosis: serology (CFT, VNT; but not HIT because these viruses do not produce hemagglutinin). Therefore, HIT (and ELISA) cannot be normally used in diagnostic serology.

European distribution: Fig. 10 Outside Europe TRBV was isolated exceptionally in northern Africa. Migratory birds have been implicated in the dispersal of Kemerovo serogroup viruses over vast distances. For instance, the Siberian Kemerovo virus was isolated from a southward migrating *Phoenicurus phoenicurus* in Egypt (Schmidt and Shope 1971; Brown et al. 1988).

Orbivirus Okhotskiy (OKHV)

Kemerovo antigenic group, the Great Island (GI) subgroup. Prototype: LEIV-70C (*I. uriae,* Tyuleniy Island—Far East, 1970). Antigenically and genetically closely related to other Kemerovo group and especially GI subgroup viruses; probably identical with CWV because it hybridizes to all ten OKHV genes. The GI complex viruses may represent a single viral gene pool, i.e., one species (Brown et al. 1989; Belhouchet et al. 2010).

OKHV was originally isolated from nymphal *I. uriae* collected in rocky breeding grounds of seabirds on Tyuleniy Island, Sea of Okhotsk (Russian Far East) in 1970 (Lvov et al. 1973a, b, c, d). In Europe, it was isolated under similar conditions in the Murmansk region, N. Russia, 1970 (Lvov et al. 1989).

The main arthropod vector is *I. uriae* (TST, TOT), occasionally *I. signatus.*

Vertebrate hosts are seabirds: *R. tridactyla*; antibodies were also detected in *F. glacialis, U. aalge,* and *Phalacrocorax pelagicus.* Avian disease is unknown.

Human disease has not been reported, although antibodies were detected in 12 % of inhabitants on the Commodore Islands.

European distribution: Fig. 11 Outside Europe: coastal Asian Russia (Far East), USA and Canada. Seabirds disperse the GI complex viruses transoceanically and introduce them to new areas and new hosts; the GI members therefore occur both in subarctic and subantarctic regions (Lvov and Ilyichev 1979).

Orbivirus Cape Wrath (CWV)

Kemerovo antigenic group, the GI subgroup. Prototype: ScotAr-20 (CW-20; *I. uriae*, Scotland, 1973). Antigenically and genetically closely related to GI, BAU, MYK, TDM, OKH, Nugget and Yaquina Head viruses; In fact, probably identical with (i.e., a synonym of) Okhotskiy virus because it hybridizes to all ten OKH genes (Brown et al. 1989). Very similar or identical non-registered viruses are Arbroath (ARB-1, Scotland: Moss and Nuttall 1985), Broadhaven (FT-363: Carey and Nuttall 1989; Jacobs et al. 1986; Nuttall et al. 1981, 1990a, b), Wexford (GS-80-9, SE. Ireland: Nuttall et al. 1984a; Carey and Nuttall 1989), Thormodseyjarklettur (Iceland), Scottish strains Mill Door/79, Above Maiden, Colony, Foula, Mill Door, North Clett, and Shiant Islands, Irish Great Saltee Island GS 80-4,-7,-8, Ellidaye ELL-3a and Grimsey (Iceland), English isolates Lundy, Inner Farne IF79-1,-2, and North End, Rost Islands NorV-808,-871,-962, and Vaeroy (Norway—Lofoten), and a number of other strains (Jacobs et al. 1986; Labuda and Nuttall 2004). Some of these viruses can be differentiated in PRNT (Carey and Nuttall 1989), but they reassort readily at a high frequency (Moss et al. 1988; Nuttall et al. 1990a, b; Nuttall and Moss 1989). Only minor variability has also been found in the induced protein profiles among different CWV and CWV-like isolates (Black et al. 1986; Spence et al. 1986). The gene reassortment potential of the isolates confirms the close taxonomic

Fig. 10 European distribution of Tribeč virus. Explanation: *black dots*, the virus isolation; *white circles*, specific antibodies detected

relationship of all the GI subgroup members which may, in fact, represent a single gene pool (Moss et al. 1988; Brown et al. 1989; Nuttall et al. 1990a) and therefore one virus species.

First isolated from engorged female *I. uriae* collected in a colony of the common murre *U. aalge* at Clo Mor on Cape Wrath, Scotland, June 1973 (Main et al. 1976a, b).

The virus is sensitive to trypsin and acid (pH 3) but resistant at pH 5.

Arthropod vector is *I. uriae.*

Vertebrate hosts are marine birds. Antibodies were detected in *U. aalge*, *A. torda,* and *O. leucorrhoa* (Main et al. 1976a, b; Nuttall et al. 1984a, b). However, avian disease is unknown. Suckling mouse is a rather insusceptible system for the CWV isolation attempts (Nuttall et al. 1984a, b).

Human disease has not been reported.

European distribution: Fig. 11. Outside Europe: Alaska.

Fig. 11 European distribution of the Great Island subgroup orbiviruses: Cape Wrath and CW-like (*circles*), Okhotskiy (*squares*), Mykines (*asterisk*), Tindholmur (*asterisk*) and Bauline (*diamond*) viruses

Orbivirus Mykines (MYKV) and Tindholmur (TDMV)

Kemerovo antigenic group, the GI subgroup. Prototype MYKV is DenAr-12 (*I. uriae*, Faeroe Islands, 1974), and TDMV DenAr-2 (*I. uriae*, Faeroe Islands, 1974). Both viruses are distinguishable by CFT, and antigenically related to CWV, GIV, BAUV, Yaquina Head, OKHV and other GI subgroup viruses (Brown et al. 1989).

First isolates originated from female *I. uriae* ticks collected in puffin (*F. arctica*) colonies at Mykines and Tindholmur, Faeroe Islands in 1974 (Main 1978).

Arthropod vectors: *I. uriae*.

Vertebrate hosts: probably *F. arctica*.

Animal and human disease has not been reported.

European distribution: Fig. 11.

Orbivirus Bauline (BAUV)

Kemerovo antigenic group, the GI subgroup. Prototype: CanAr-14 (*I. uriae*, Canada, 1971). European topotype: FI-873 (*I. uriae*, Norway, 1974). The Norwegian isolates FI-873 and FI-962 have been found identical with prototype BAUV by RNA–RNA hybridization (Brown et al. 1989). Antigenically closely related to other members of the GI or Kemerovo subgroups (Brown et al. 1989), and indistinguishable from GI virus (CanAr-41) by CFT; both viruses can be differentiated by VNT (Main et al. 1973). Some BAUV and GIV isolates from Newfoundland have exhibited a remarkable variation in all ten genome segments (Oprandy et al. 1988).

Originally isolated from engorged nymphal *I. uriae* ticks collected during July 1971 in a *F. arctica* colony on Great Island off the SE. coast of Newfoundland, Canada (Main et al. 1973). In Europe, it was isolated from *I. uriae* collected in a seabird colony on Rost Island, Lofoten (Norway) in 1974 (Brown et al. 1989; Saikku et al. 1980).

Vertebrate hosts are unknown; antibodies were detected in *F. arctica* and *O. leucorrhoa* birds.

Animal and human disease has not been reported.

European distribution: Fig. 11. Outside Europe: Canada (Newfoundland). Documented transoceanic flights of puffins from NW. Europe to Newfoundland and vice versa contribute to the dissemination of the GI subgroup viruses over wide geographical areas (Lvov and Ilyichev 1979; Main et al. 1973).

Family *Orthomyxoviridae*

Thogotovirus Thogoto (THOV)

Thogoto antigenic group. Prototype: Ken-IIA (mixed metastriate tick spp., Kenya, 1960). African topotype: IbAr-2012 (*Boophilus* spp., Nigeria, 1964); European topotype: SiAr-126 (*R. bursa*, Sicily, 1969). THOV shares only 15–20 % nucleotide homology with influenza orthomyxoviruses. Virions are spherical, 80–120 nm, enveloped, contain ss(−) RNA arranged in six segments with a total size of 10 kbp, and one surface glycoprotein. Some strains form HA in the liver and blood serum of SM or in Vero cells, whereas not in suckling mouse brain.

First isolated from a pool of *B. decoloratus* and *Rhipicephalus* spp. ticks collected on cattle in Thogoto Forest near Nairobi, Kenya in 1960 (Haig et al. 1965). In Europe, it was first isolated from ticks collected on ruminants in Sicily, 1969 (Albanese et al. 1971, 1972; Srihongse et al. 1974) and then in Portugal in 1978 (Filipe and Calisher 1984).

Arthropod vectors are metastriate ticks only—*B. decoloratus*, *B. annulatus, A. variegatum*, *R. appendiculatus, Rhipicephalus sanguineus* (Portugal), *R. bursa* (Sicily), *Rhipicephalus evertsi,* other *Rhipicephalus* spp., *Hyalomma truncatum,* and *H. anatolicum*.

Vertebrate hosts are cattle, camel, and man (isolations in Africa). Antibodies were also detected in sheep and goat. THOV causes leucopenia in cattle, and fever, and abortion in sheep (Davies et al. 1984). Fatal to, and highly hepatotropic or pantropic in, adult mouse (Filipe et al. 1986) and adult Syrian hamster (i.p.). No symptoms in suckling hamster and rabbit (i.p.). CPE is produced in primary mouse embryo and lamb testis cells; faint CPE in HEp-2 cells (David-West 1971, 1972).

Natural foci are boskematic—pastoral xerothermic ecosystems.

Human disease: two cases have been described, one with bilateral optic neuritis and another as a fatal meningoencephalitis with hepatitis although complicated by a sickle-cell disease (Theiler and Downs 1973; Moore et al. 1975). THOV is probably contagious from man to man. Antibodies occur rarely in human sera in Europe: e.g., only 1 % seropositive persons were detected in Portugal (Filipe et al. 1985).

European distribution: Fig. 12. Outside Europe THOV occurs in Nigeria, Kenya, Uganda, Ethiopia, Cameroon, Central Africa, Egypt, Iran. Tick-infested domestic animals (e.g., camels) and migratory birds could disseminate the virus over a wide geographic range (Calisher et al. 1987).

Thogotovirus Dhori (DHOV)

Prototype: IG-611313 (*Hyalomma dromedarii*, India, 1961). European topotype: PoTi-461 "Vidigueira" (male *H. marginatum*, Portugal, 1971). Synonyms: Astra (Butenko and Chumakov 1971), Batken (LEIV-306 K: *H. marginatum*, collected on sheep in Kirghizia, 1970: Lvov et al. 1974). Nucleotide sequence data suggest that DHOV is distantly related to influenza viruses but their envelope proteins (HA, neuraminidase) differ significantly. Virions are spherical,

Fig. 12 European distribution of Dhori (*circles*) and Thogoto (*squares*) viruses

80–120 nm, enveloped, contain ss(−)RNA arranged in 7 segments with a total size of 10 kbp, and one surface glycoprotein. HA is also produced in Vero cells, and HIT can use goose, sheep, monkey or human RBC.

DHOV was first isolated from *Hyalomma dromedarii* ticks collected on camels in Dhori, Gujarat State, India in 1961 (Anderson and Casals 1973). In Europe, it has been isolated several times from *Hyalomma marginatum* and twice from *Hyalomma scupense* collected at Astrakhan, South Russia since 1969 (as "Astra" virus: (Butenko and Chumakov 1971; Butenko et al. 1987; Bannova et al. 1974; Smirnova et al. 1988) and in Crimea (one strain —"Batken"); additional two strains were obtained from *H. scupense* near Astrakhan (Smirnova et al. 1988) and another one in southern Portugal, 1971 (Filipe and Casals 1979).

Arthropod vectors are metastriate ticks *H. dromedarii*, *H. marginatum* (Europe), *H. scupense* and *D. marginatus*. Occasional isolations of DHOV were reported from *Ornithodoros lahorensis* and mosquitoes (*Anopheles hyrcanus, Aedes caspius, Culex hortensis*).

Vertebrate hosts are camel, horse, bats (Kirghizia), but animal disease is unknown (asymptomatic). Antibodies have also been detected in goats, sheep and cattle (Filipe et al. 1985). DHOV is hepatotropic, and causing diffuse necrosis of neurons in mouse (Filipe et al. 1990). No symptoms were observed in inoculated adult or young rabbit (i.c., i.p., s.c.). No CPE or plaques (but multiplication) produced in BSC-1, L, human embryo kidney cells; CPE formed in monkey kidney 6619-1 cells (Smirnova et al. 1988).

Natural foci: boskematic (pastoral xerothermic and semi-desert ecosystems).

Human disease: acute illness with severe fever, headache, general weakness, retrobulbar pain, with encephalitis in *c.* 40 % of patients and a long, 2-month convalescence period. Five cases of severe laboratory infection (due to aerosol) have been described (Butenko et al. 1987). The virus could also be contagious from man to man.

European distribution: Fig. 12. Seroprevalence rate among humans is relatively high in Astrakhan (4–9 %) but low in Portugal (0.8 %: Filipe et al. 1985). Outside Europe DHOV occurs in India, Egypt, Armenia, Azerbaijan, Kirghizia, Uzbekistan, and antibodies were detected in Pakistan.

Family *Asfarviridae*

Asfivirus of African swine fever (ASFV)

The only DNA arbovirus occurring in Europe. There are several antigenic types, while no recognized prototype strain of ASFV. Hemadsorption-inhibiting antibodies are isolate specific, but HA is not produced. Interestingly, neutralizing antibodies do not appear in vertebrates. The virus is sensitive to dodecyl sulphate and heat (60°C) while less sensitive to putrefaction, formaldehyde and alkali.

History: originally isolated by R.E. Montgomery from the blood of a sick pig in Kenya, 1910, and in Europe ASFV was first isolated in 1957 (Karabatsos 1985).

Arthropod vectors are soft ticks *Ornithodoros moubata porcinus* (TST, TOT) in Africa, and *O. erraticus* in SW. Europe.

Vertebrate hosts are *S. scrofa* (domestic and wild swine), in Africa also common warthog *Phacochoerus africanus* (main reservoir), bushpig *Potamochoerus porcus,* giant forest hog *Hylochoerus meinertzhageni* (Jori and Bastos 2009). The wild suids are the reservoir of ASFV with usually inapparent infection (except for *S. scrofa*). ASF is a pantropic, highly contagious disease of pigs with fever, cough, anorexia, skin cyanosis, incoordination, diarrhea; destruction of lymphoreticular elements, vasculitis, widespread hemorrhages, thromboses, infarction, and abortion (Schlafer and Mebus 1984). Lethality is 100 % with virulent strains in naive commercial pig populations, while some strains may produce mild disease and carriership. Cattle, sheep, goat, dog and rabbit (s.c., i.v.) are insusceptible though the virus recovery has been sometimes reported in rabbit and goat. CPE is produced in primary porcine leucocyte, bone marrow and kidney cells.

European epizootics of ASF occurred in Portugal (1957 and 1960: Filipe 1980), SW. Spain (since 1957: Oleaga-Perez et al. 1990), Sardinia, Malta, recently in the Caucasus region (since 2007) including southern Russia (North Ossetia, Krasnodar territory, 2008–2011), and temporarily in France (1964), Italy (1967, 1983: Swaney et al. 1987), Belgium, and the Netherlands.

Natural foci: mainly tropical and subtropical pastoral ecosystems. Principally a wild hog/pig-*Ornithodoros* cycle. Moreover, circulation in pig pens in rural habitats.

Human disease has not been reported.

European distribution: Fig. 13. Occasionally introduced into southern Europe, Belgium, and the Netherlands. Outside Europe: many African countries; temporarily Brazil and some Caribbean islands (Cuba, Haiti).

Conclusions

Several "European" tiboviruses cause very serious human (CEEV, RSSEV, CCHFV) or animal (LIV, ASFV) diseases. Other arboviruses play definite role in human or animal pathology though the disease is usually either less serious or infrequently reported (TYUV, BHAV, AVAV, EYAV, TRBV, DHOV, THOV). In general, three groups of tibovirus diseases can be recognized according to main clinical symptoms produced: (i) febrile illness—usually with a rapid onset, fever, sweating, headache, nausea, weakness, myalgia, arthralgia, sometimes polyarthritis and rash; (ii) the CNS affection—meningitis, meningoencephalitis, or encephalomyelitis with pareses, paralysis, and other sequelae; (iii) hemorrhagic disease. The other European arboviruses are "orphans" without a proven medical or veterinary significance (BAHV, MTRV, MEAV, GAV, PTVV, ZTV, SAHV, UUKV, SOLV, PIV, AVAV, CMV, OKHV, CWV, MYKV, TDMV, BAUV). However, certain arbovirus diseases of free-living vertebrates (but also those of domestic animals and even man) may often pass unnoticed or misdiagnosed and eventually, they might potentially appear as emerging diseases. In addition, active search for new

Fig. 13 European distribution of African swine fever virus

tiboviruses or for new, pathogenic variants of the known tiboviruses in Europe should continue.

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PRÁCE 40

Estrada-Pena A., Hubálek Z., **Rudolf I.** 2014. Tick-transmitted viruses and climate change. In *Viral Infections and Global Change*, First Edition. Edited by Sunit K. Singh. ISBN: 978-1-118-29787-2 Published Online: NOV 2013 DOI: 10.1002/9781118297469.ch31.

Stručná charakteristika: byla připravena jedna z kapitol knihy týkající se distribuce patogenních virů přenášených klíšťaty ve světle globálních změn (např. změny klimatu). Důraz je kladen především na výskyt nových tzv. emergentních a opomíjených patogenů (např. virus Henan, Bhanja).

Hlavní přínos práce: velmi zdařilá kniha širokého autorského kolektivu, která reflektuje současné poznatky zejména o emergenci infekčních onemocnění včetně nových nákaz (SARS-CoV, henipaviry) v souvislosti s globálními změnami environmentálními či socio-ekonomickými.

Příspěvek autora k dané práci: autor se podílel rovným dílem na přípravě kapitoly o klíšťatech a patogenech, jež přenášejí.

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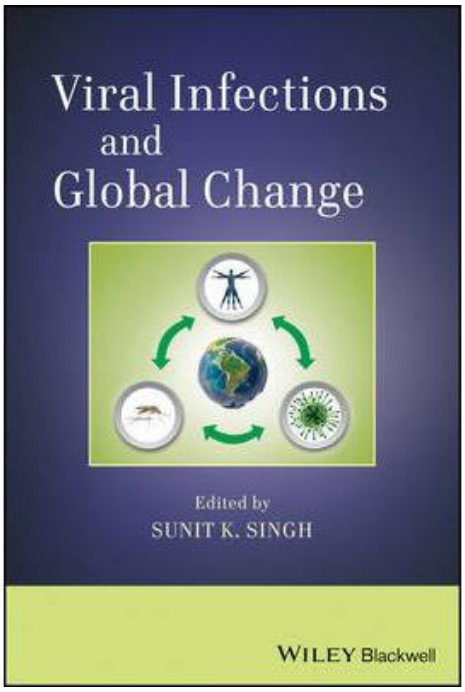

Viral Infections and Global Change

By (author) Sunit K. Singh

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Emerging, Reemerging Viral Infections and Climate Change is a timely look at the impacts of global warming on the spread of infectious disease. As average world temperatures continue to rise, current climate change scenarios suggest that there will be a significant increase in the areas suitable for vector-borne viral transmission to humans. Viral Infections and Climate Change offers detailed descriptions of the epidemiology, molecular pathogenesis and host pathogen interactions of a variety of these viral threats, as well as discussion of a host of other factors in the spread of infectious disease. The introduction of microbes and vectors through increased trans-boundary travel, and the expanding prevalence of drug and pesticide resistance are just a few of the trends generating concern about emerging and re-emerging viral infections.With up to date information on the clinical aspects as well as the basic science of major human viral hemorrhagic fevers, Emerging, Reemerging Viral Infections and Climate Change will be a useful resource for professionals in biology medicine, and veterinerary science working in ecology, environmental management, climatology, neurovirology, virology, and infectious disease.

31

TICK-TRANSMITTED VIRUSES AND CLIMATE CHANGE

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TABLE OF CONTENTS

Viral Infections and Global Change, First Edition. Edited by Sunit K. Singh.

31.1 INTRODUCTION

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans that are distributed from Arctic to tropical regions of the world. Globally, the recognized number of distinct and epidemiologically important diseases transmitted by ticks has increased considerably during the last 30 years. For example, more than 10 newly recognized spotted fever rickettsioses have been identified since 1984 (Paddock et al., 2008; Parola et al., 2005). In the United States, the list of national notifiable diseases included six tick-borne diseases, namely, Lyme disease (*Borrelia burgdorferi* s.l. infection), human granulocytic anaplasmosis (HGA, *Anaplasma phagocytophilum* infection), human babesiosis (*Babesia* spp.), human monocytic ehrlichiosis (*Ehrlichia chaffeensis* infection), Rocky Mountains spotted fever and Powassan disease, most of which have increased steadily in average annual incidence (Bacon et al., 2008). Although advances in molecular technology have contributed to the identification of these pathogens, rapidly expanding pathogen diagnosis and increasing incidence have raised concerns about the accuracy of case counts and epidemiology reports (Mantke et al., 2008). The problem of analyzing the incidence of tick-borne pathogens in humans is the concurrency of factors affecting the whole system such as climate, driving the life cycle of the ticks, the availability, occurrence and seasonal patterns of competent reservoirs, and social habits, leading the contact with tick-infested areas need to be considered. The systems of tick-borne pathogens are very complex in nature, and we should regard them as layers of information, each one increasing the complexity of the previous one until the whole system is covered.

While not specifically mentioned in the preceding text, the virus transmitted by tick bites remains a health problem in many parts of the world. Several events that occurred during the final decades of the twentieth century and the beginning of the twenty-first century suggest a rise of tick-borne viral infections worldwide. These events include recent national and regional epidemics of known diseases such as tick-borne encephalitis (TBE) in Central and Eastern Europe, Kyasanur forest disease (KFD) in Karnataka state in India, and Crimean–Congo hemorrhagic fever (CCHF) in northern Turkey and the southwestern regions of the Russian Federation (Maltezou et al., 2010; Pattnaik, 2006; Randolph, 2008a). Some of them, like TBE, may be also transmitted by milk intake, while others, like CCHF, may be transmitted to humans at abattoirs.

We want to review here some findings relating climate and the behavior in nature of some important tick-transmitted viruses, like the etiologic agents of TBE and CCHF. Our specific point in this review is that climate may be probably behind some of the recent (re) emergence of the reported active foci of the disease, driving the dynamics and the abundance patterns of ticks. However, a note of caution is issued about the lack of suitable data on the dynamics of the hosts and about the changes that climate may operate in social habits, which are difficult to quantify (but see Sumilo et al., 2007; Zeman et al., 2010). The effects of climate on tick-transmitted viruses are indirect and difficult to quantify. A simple

approach might not be enough to capture the many levels at which climate operates driving these infections. Of course this is not the first time the topic has been reviewed. The interested readers will find a text on comparative tick bionomics and viruses (Sonenshine, 1974) and a comprehensive review by Randolph (2008b) focused not only on tick-transmitted viruses but on general tick-borne disease systems, as influenced by climate and other factors. We will also summarize some findings related to other tick-transmitted viruses as associated with human disease. We will use the abbreviations TOT, for transovarial transmission (in arthropods), and TST, for transstadial transmission (in arthropods).

31.2 TICKS IN NATURE

Ticks spend most of their life cycle in the environment, and all tick life cycle stages are dependent on a complex combination of climate variables for development and survival. In summary, ticks must develop from one stage to the next in the life cycle, following a sum of degree-days. While ticks are molting, there is a resulting mortality because of relatively unsuitable climate conditions. After adequate cuticle hardening, ticks quest for a host. Such an activity period results in further mortality because of water losses. Ticks are sensitive to changes in several limiting abiotic factors, including temperature, which affects the timing and speed of development, and atmospheric water deficit, which affects mortality. Changes in these variables shape the probabilities of a tick population to persist. Although surveillance and reporting of changes in the distribution of tick populations are generally inadequate, some well-documented reports support the slow but apparently continuous expansion of the historical frontiers of some tick species into areas where they were previously absent (reviewed by Gray et al., 2009). With this rationale, warmer temperatures have been suggested, together with host movements, as the main driver of some tick geographical range changes (Danielová et al., 2006; Lindgren et al., 2000; Ogden et al., 2004). However, the potential influence of changing rainfall patterns has largely been ignored although this may have a greater effect than temperature on the ability of tick populations to establish in new areas. Finally, there is little doubt that human-induced changes in abiotic (climate, land cover, habitat structure) and biotic (distribution and abundance of tick hosts) conditions have occurred over the past few decades, and there is equally indisputable evidence for the increase in recorded human cases of some tick-borne diseases (Randolph, 2009).

Host availability may modulate the dynamics of tick populations. Though many animal species can serve as tick hosts, there are several determinants of host suitability, and the specificity of tick–reservoir host–pathogen relationships is key to our understanding of the processes conditioning the transmission of pathogens by ticks (Randolph, 2009). Shelter and protection from environmental conditions are critical to tick survival, because questing and diapausing ticks are vulnerable to extreme temperature and humidity. The concerns about climate change added fuel to a debate about how predicted climate changes may alter tick–host–pathogen relationships and particularly tick potential for invasion of new areas and pathogen transmission. However, our efforts to disentangle such complex systems have so far scratched only the surface and are far from providing a complete answer to the many questions about the epidemiology of these processes. Invasive events (the transportation of an exotic tick species into an area far from its native range) are also well documented and seem to be related to unrestricted domestic animal movements or overabundance of certain wild hosts. The spread of ticks is a controversial issue because of a lack of empirical data and its importance in managing the further spread of prominent pathogens affecting human and animal health (Wilson, 2009).

31.3 FAMILY *FLAVIVIRIDAE*

31.3.1 Tick-borne encephalitis virus

Over the past decades, TBE has become a growing public health concern in Europe and Asia and is the most important viral tick-borne disease in Europe. It is also important in the Far East and in other parts of Asia. Protective vaccination is indicated for persons inhabiting or visiting natural foci of TBE. For this purpose, it is necessary to know where TBE virus (TBEV) occurs, where vectors are a potential hazard, and where as a consequence autochthonous TBE cases have been registered. Unlike Lyme borreliosis-endemic regions, TBE risk areas are distributed in a patchwork pattern, sometimes the situation remains stable, and sometimes changes occur due to altered climatic conditions or other factors. Adequate reviews about the topic exist (i.e., Süss, 2003; 2011) as well as comprehensive analyses of the human incidence rates in several zones of Eastern Europe (Sumilo et al., 2007; 2008).

There are three recognized TBE subtypes (Calisher, 1988; Calisher et al., 1989; Clarke, 1964; Dobler, 2010; Gritsun et al., 2003; Lindquist and Vapalahti, 2008; Rubin and Chumakov, 1980; Votyakov et al., 1978): (i) Western or European subtype (TBEV-W), also called Central European encephalitis virus (CEEV—topotype strains are Hypr and Neudoerfl) or sometimes "ricinus" subtype, whose varieties include Spanish sheep encephalitis (SSE), Turkish sheep encephalitis (TSE), and Greek goat encephalitis ("Vergina") viruses (Hubálek et al., 1995); (ii) (Ural-)Siberian subtype (TBEV-S: the prototype strains are Aina and Vasilchenko), sometimes called "persulcatus" subtype, causing Russian spring–summer encephalitis (RSSE:); and (iii) Far Eastern subtype (TBEV-FE with prototype strain Sofyin, isolated from human brain in Khabarovsk, 1937). A taxonomic and nomenclatural confusion around TBEV has repeatedly been emphasized (Calisher, 1988; Clarke, 1964; Holzmann et al., 1992; Stephenson, 1989). In addition, TBEV is very closely related to louping-ill virus (LIV), which should be regarded in fact as the fourth (or, historically, the first?) subtype of TBEV (see following text). According to Ecker et al. (1999), variation in amino acids within a subtype is up to 2% and between subtypes 5–6% (Lindquist and Vapalahti, 2008).

TBEV (its RSSEV subtype) was first isolated in 1937 (Chumakov and Zeitlenok, 1939), and CEEV (strain "256") from *I. ricinus* ticks was collected near Minsk, Belarus, in 1940 (Levkovich and Karpovich, 1962; Votyakov et al., 1978). Further isolations of CEEV were reported in Czechland from human patients and *I. ricinus* ticks in 1948–1949 (Gallia et al., 1949; Krejčí, 1949; Rampas and Gallia, 1949). Principal arthropod vectors are ticks of the genus *Ixodes*: *I. ricinus* for CEEV (TST, TOT: Benda, 1958b; Řeháček, 1962) and *I. gibbosus* (a marginal vector in the Mediterranean). Mean prevalence rate of CEEV in ticks in natural foci can reach 1%, but it is usually much lower, at about 0.1%. Occasional vectors are other tick species such as *Ixodes hexagonus*, while only sporadically metastriate tick species *Haemaphysalis inermis*, *H. concinna*, *H. punctata*, *Dermacentor marginatus*, *D. reticulatus* (Kožuch and Nosek, 1971; Křivanec et al., 1988; Naumov et al., 1980; Riedl et al., 1971), and *Hyalomma marginatum* (Crimea). The main vector for RSSEV is *I. persulcatus* (infection prevalence rates can reach frequently >2%; TST, TOT: Chunikhin, 1990), less often *Ixodes ovatus*, *Dermacentor silvarum*, *D. reticulatus*, *D. marginatus*, *H. concinna* (TOT), *Haemaphysalis longicornis*, and *H. japonica* (Naumov et al., 1980).

Competent vertebrate hosts of TBEV are small forest mammals—especially rodents and insectivores (*Apodemus flavicollis*, *A. sylvaticus*, *Myodes glareolus*, *M. rufocanus*, *Microtus agrestis*, *Sciurus vulgaris*, *Talpa europaea*, *Sorex araneus*, *Erinaceus concolor*), further goat, sheep, and rarely cattle. The role of some forest passerines and other birds as hosts of TBEV has not yet been fully elucidated; the virus was isolated occasionally from a number of other vertebrate species. Experimental viremia has been demonstrated in many mammalian, avian, amphibian, and reptilian species. Encephalitis with ataxia, jumping,

tremor, and convulsions can affect lambs, kids, or, exceptionally, dogs. CEEV infection is usually subclinical in adult ruminants and pig; goats, sheep, and cows excrete virus in the milk (Benda, 1958a; Grešíková, 1958a,b; Smorodintsev et al., 1953; van Tongeren, 1955). TBEV (especially TBE-S and TBE-FE virus subtypes) occasionally kills birds of some species, for example, *Carduelis flammea*, *Passer domesticus*, and *Fulica atra*, and amphibians *Rana temporaria* and *Bufo bufo*.

Natural foci of TBE (and other tick-borne diseases) have been classified (Rosický, 1959) as "theriodic" (situated in deciduous and mixed forest ecosystems, often game preserves, where the main hosts of adult female vector ticks are deer and other wild mammals), "boskematic" (pastoral, where the main vectors of adult female vector ticks are grazed domestic ruminants), mixed "theriodic–boskematic," or "mountain" (Rosický and Bárdoš, 1966). Urban foci of CEE have also been described in Eurasia. In general, most natural foci of TBE are situated in forest (less pastoral) ecosystems.

There are two basic modes of human infection with TBE—by the bite of an infective tick or by consumption of infected raw (unpasteurized) goat (less often sheep or cow) milk or unpasteurized dairy products (Grešíková, 1972; Smorodintsev et al., 1953). Whereas the tick-transmitted cases are sporadic, the milk-borne infections usually affect whole families or population groups in outbreaks. For instance, a large milk-borne TBE epidemic occurred in Rožňava, East Slovakia, in 1951, when 660 persons were infected and 274 of them had to be hospitalized (Blaškovič, 1954). As much as 76% of human infections have been alimentary in Belarus (Ivanova, 1984). The virus may resist in milk at 60 °C for more than 10 m and partially even the pasteurization at 62 °C for 20 m, and it is not inactivated at pH 2.8 within 24 h/4°C. In addition, many laboratory infections (usually by infectious aerosol) have been reported in unvaccinated personnel.

TBEV circulates in a series of interactions between virus, vector ticks, and tick hosts and is able to persist in a given habitat over long periods of time (Nuttall, 1999). The occurrence of vector ticks and suitable vertebrates on which ticks can become infected is crucial for virus existence in a given area. The following mechanisms of virus transmission between ticks occur: (i) feeding/cofeeding (Alekseev and Chunikhin 1990; Labuda et al., 1993a, b), (ii) TOT, and perhaps (iii) sexual transmission. Cofeeding transmission is especially effective, and the virus can be transmitted through this mechanism from a feeding vector tick to cofeeding ticks even on immune hosts, while TOT is considerably less efficient. Studies have shown that tick saliva contains factors that modulate host inflammatory, coagulation, and immune response to improve tick blood feeding and pathogen transmission (Alekseev et al., 1991; Jones et al., 1989; Labuda et al., 1993a; Randolph, 2009). This so-called saliva-assisted transmission (SAT) was reviewed by Nuttall et al. (2008). Inoculation of salivary glands extracts and TBEV into laboratory animal hosts resulted in enhanced transmission from hosts to nymphal ticks when compared with pathogen inoculation alone (Alekseev et al., 1991; Labuda et al., 1993b). SAT helped to explain the mechanism behind the equally novel observation of TBEV transmission between cofeeding ticks in the absence of a systemic infection (Alekseev and Chunikhin, 1990; Labuda et al., 1993a,b; Randolph, 2009).

The non viremic (cofeeding) transmission imposes constraints because it requires cofeeding by at least two tick stages in synchrony in their seasonal activity (Randolph et al., 2000). The long and slow life cycle typical of temperate tick species, caused by low temperature-dependent developmental rates and overwinter diapause, slows the pace of pathogen transmission. As tick phenology is reset each year by winter conditions (Randolph et al., 2002), the critical stages (larvae and nymphs for TBEV) may emerge from diapause in more or less synchrony in the spring, depending on whether temperatures rise sufficiently rapidly to cross the threshold for larval activity (c. 10 °C mean daily maximum) soon after the threshold for nymphal activity (c. 7 °C mean daily maximum) (Randolph and Sumilo, 2007). The variability

of thermal conditions associated with seasonal synchrony between tick stages has been identified as the key determinant of the focal distribution of TBEV across Europe, allowing the predicted risk of TBE to be mapped (Randolph et al., 2000).

Altogether, this information suggests that climate exerts an extreme control of the natural cycles of TBEV and delineates both their intensity (in terms of field tick prevalence rates) and their geographical distribution. According to the prevalent hypothesis outlined before, the climate at the beginning of the spring exerts a regulatory action on the synchrony of the active immature ticks, conditioning the necessary coexistence of nymphs and larvae on the same hosts. Because of the short time of feeding for both larvae and nymphs, small changes in the temperature in that period may promote a lack of synchronicity of a few days, enough to prevent the "backward" transmission of the virus. Therefore, the extreme lability of the TBEV foci would be primarily driven by very small changes in spring temperatures. The system is thus very local in its nature. These events have not yet been captured by a process-driven model, which could be a welcomed addition to our array of epidemiological tools, necessary to understand the TBEV epidemiology and design intervention for its prevention.

At a continental scale, it has been reported that *I. ricinus* and TBEV reach higher altitudes in the Czech mountains in a consistent pattern after the year 2000, higher than reported for the years 1970–1980 (Daniel et al., 2008; Danielová et al., 2006; Materna et al., 2008). The tick has been reported to spread north in Sweden (Eisen, 2008; Lindgren et al., 2000; Lindgren and Jaenson, 2006), Norway (Skarpaas et al., 2006), Finland (Jääskeläinen et al., 2006), as well as Germany (Hemmer et al., 2005; Süss et al., 2008) and west in Austria (Holzmann et al., 2009). Nearly all these data were collected along the fringes of tick distribution and do not apply to the core areas. It must be however realized that the series of cases in humans are not a direct mirror of the "activity" of TBE foci and that the mechanisms regulating the later are far more complex. This is why it has been proposed to check the active foci by direct examination of the ticks collected in the field by a PCR system (Gaumann et al., 2010).

A picture of the number of cases of TBE in European countries has been provided by Süss (2008). It has been speculated that host abundance, changes in social habits, economic fluctuations, environmental changes, and to a lesser extent climate changes have increased the incidence of TBE (Lindgren and Gustafson, 2001; Sumilo et al., 2006, 2007, 2008; Zeman and Benes, 2004). It is anyway difficult to correlate series of human clinical cases against basic climate features because climate has several collateral effects, not only affecting tick life cycle but also hosts and, most importantly, social habits. This has been demonstrated in a series of data for TBE cases in the countries of the Baltic Sea (Sumilo et al., 2007) and the Czech Republic (Zeman and Benes, 2004). Even if we regard the epidemiology of TBE from 1976 to 2007 in general, most questions remain to be answered (Süss, 2008). Thus, the political turnaround and the resulting socioeconomic changes and changes in the behavioral pattern of the exposed population in certain countries of the former Eastern Bloc at the beginning of the 1990s certainly are a significant influence factor. However, this does not explain the increase in the number of TBE cases since the 1990s in Sweden, Italy, Hungary, Czechland, Finland, and Germany. As a result, the TBE incidence in the German risk areas shows the same trend as in the Baltic States; the political turnaround, however, only took place in the eastern part of the country, where TBE incidence is very low compared to southern Germany and the influence on the total number of registered cases consequently is very low (Süss, 2008).

As mentioned earlier, it seems that a kind of chaotic system emanates from the several layers of complexity emanating from the epidemiology of TBEV. The first one is the basic layer of the impacts of climate on the tick populations, which may be extremely local as mentioned before. A process-driven model (i.e., Dobson et al., 2011) may be an excellent starting point to handle such impacts. Analysis of the long-term trends of climate and its impact on

the suitability for *I. ricinus* has been already presented (Estrada-Peña and Venzal, 2006) based in a long series of climate data. Figure 31.1 shows a different kind of analysis, based on the relationships between the sites where the tick *I. ricinus* has been collected and reported and the long-term climate found at these sites. It thus represents the "mean" expected climate suitability for the tick in its distribution range. This index is not correlated with tick abundance, since it depends in local factors. It only provides with an estimation of how suitable climate factors have been in the last 30 years. An analysis of the trends in climate for the period 2000–2010 shows that climate has become clearly more favorable in wide regions of Northern Europe (Figure 31.1). Therefore, using such a basic and primary approach, climate has obvious effects on tick available range to be colonized. These figures, however, are not aimed to provide answers about the seasonal patterns of the tick stages.

A second layer of complexity has also prominent role on the local epidemiological patterns. Such a layer is related to the abundance of populations of reservoir host, their seasonality, and the abundance of hosts that feed large numbers of adult ticks (like ungulates). Both factors would affect the system by providing respectively a larger nonsystemic (cofeeding) transmission and an increased abundance of engorged females that produce more eggs and more ticks for the next year population. The TOT from tick to eggs is known anyway to contribute to the system (Matser et al., 2009). Finally, the peculiar human habits as operating in each country and the impact of climate on those would certainly manifest the third layer of complexity, distorting the previous, natural ones and surfacing into the reported pattern of human incidence rates. We should not discard that every layer of the system may respond to the impact of climate in different ways affected by local constraints.

31.3.2 Louping ill virus

LIV is very closely related to TBEV, in fact indistinguishable from it by conventional serological and cross protection tests (Calisher, 1988; Calisher et al., 1989; Clarke, 1962, 1964; Holzmann et al., 1992; Hubálek et al., 1995; Kopecký et al., 1991; Madrid and Porterfield, 1974; Rubin and Chumakov 1980; Shamanin et al., 1990; Shiu et al., 1991; Stephenson, 1989; Tsekhanovskaya et al., 1993; Venugopal et al., 1992) but also by nucleotide sequence homology of the E gene (Gao et al., 1993; Gould et al., 2003; Grard et al., 2007; Jääskeläinen et al., 2010; Venugopal et al., 1994). Several authors therefore suggested arrangement of LIV as another subtype of TBEV, while not as a separate virus (Grard et al., 2007; Hubálek et al., 1995).

The geographical distribution of LIV involves United Kingdom, Ireland, and Norway (the only country of continental Europe where a typical LIV strain was isolated: Gao et al., 1993). Natural foci there represent most often pastoral heather habitat ("tick–sheep cycle"). LIV does not occur outside Europe. The "louping-ill (LI)" disease of sheep has long been recognized in Scotland. The virus was first isolated from sheep brain in Selkirkshire, Scotland, in 1929 (prototype strain Moredun L1–31: Pool et al., 1930), and it is, in fact, the very first arthropod-borne virus isolated in Europe. The principal vector of LIV is the tick *I. ricinus*; LI is also transmissible by goat and sheep milk similarly as the other TBE subtypes. Vertebrate hosts are rodents (*A. sylvaticus*), insectivores (*S. araneus*), mountain hare (*Lepus timidus*), sheep, and red grouse (*Lagopus lagopus scoticus*: Reid, 1990).

Natural foci of LI are "boskematic" (pastoral: Rosický, 1959)—rough, poorly drained hill pastures and heather moorlands with bracken and moor grass—principally a sheep–tick or sheep–tick–grouse cycle (Reid, 1990; Smith and Varma, 1981).

The human illness is usually biphasic; the febrile phase, after a short period of improvement, is followed by high fever and symptoms of meningoencephalitis, headache,

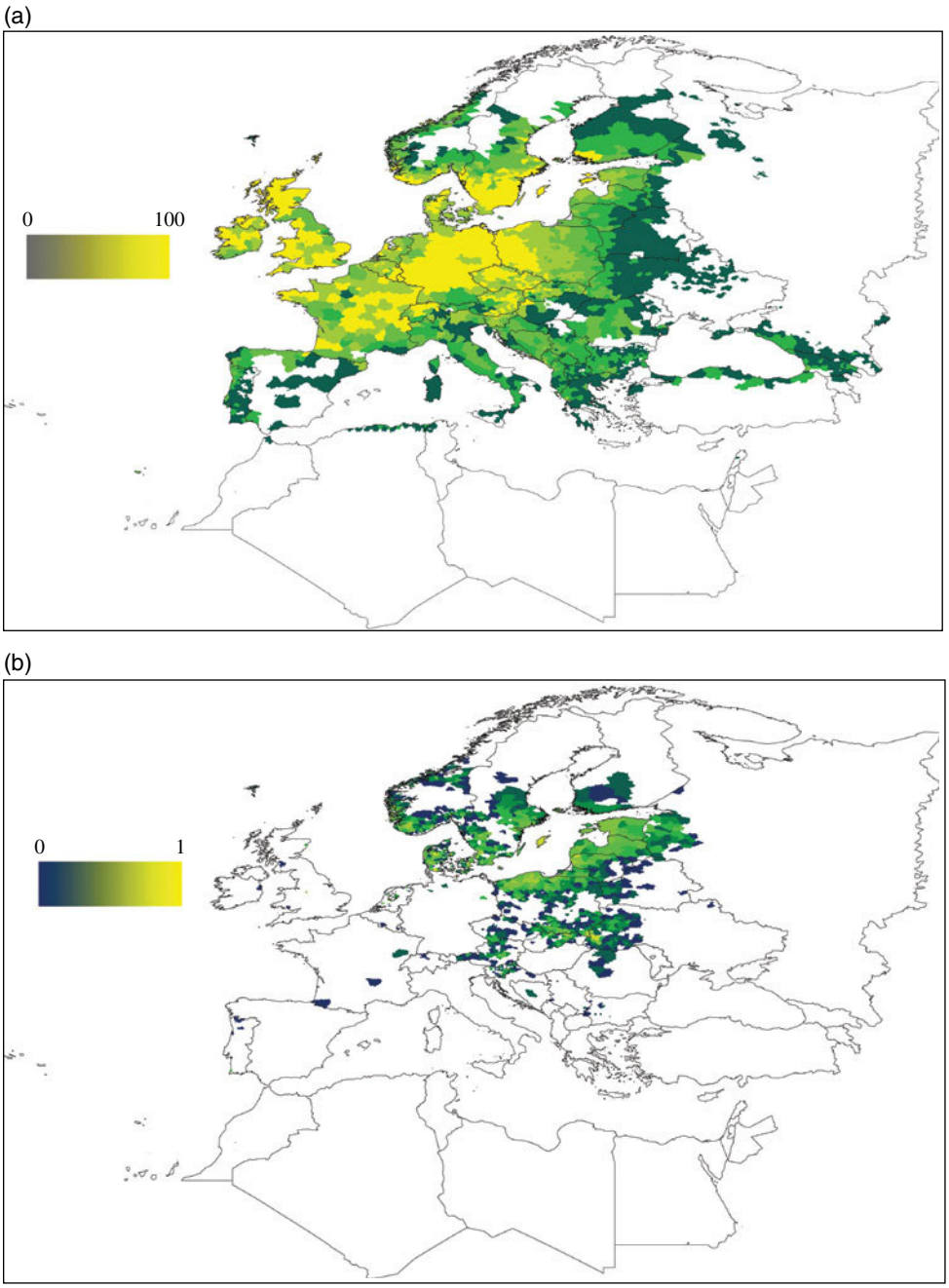

Figure 31.1. Predicted climate suitability for the tick *I. ricinus* in the western Palearctic. (**a**) Predicted climate suitability (0–100) was evaluated by a model trained with more than 4000 occurrence tick points and using Maxent as modeling software. The map is based on previous developments by Estrada-Peña et al. (2006). The ramp of colors shows the probability to find permanent populations of the tick, as driven only by climate conditions, including a set of remotely sensed monthly average temperature and monthly average vegetation stress (NDVI, a proxy for tick water stress) between the years 2000 and 2010. (**b**) Changes in climate suitability for *I. ricinus* in the period 2000–2010 (from 0, the minimum, to 1, the maximum) based in the same model. It is based on the modeling of climate suitability separately for each year, then evaluating the trend of such an index along the period 2000–2010. Both maps (**a** and **b**) are not a depiction of tick abundance but of the appropriateness of the climate for the development of the tick (**a**) and how such a factor evolved in time (**b**). Figures reproduced from Estrada-Peña et al. (2012). For color detail, please see color plate section.

weakness, stiff neck, conjunctivitis, retrobulbar pain, photophobia, myalgia, arthralgia, dysarthria, excessive sweating, nausea, vomiting, insomnia, drowsiness, confusion, tremors, nystagmus, and ataxia. Symptoms of LI in humans are usually milder than in TBE. Nineteen naturally acquired human cases and 26 laboratory infections with LIV have been described in Great Britain between 1934 and 1990 (Davidson et al., 1991), including one fatal encephalitis. LIV transmission to man is obviously infrequent in the United Kingdom because the vector ticks only occasionally bite people in endemic areas (similarly as with Lyme borreliosis). It is primarily an occupational disease, affecting shepherds, crofters, veterinary personnel, forestry workers, butchers, and laboratory personnel. However, human cases of LI with a milder symptomatology might remain underreported. Meningoencephalitis was demonstrated histologically in the deer (Reid et al., 1982), and LIV was isolated from a roe deer (Reid et al., 1976). LIV occasionally affects also cattle, pig (piglets), goat (kids), horse, dog, hare, and red grouse (with a mortality rate of 70–80% especially in juvenile birds: Reid et al., 1978; 1980). Typical course of LI in sheep is biphasic, with fever and weakness, followed by meningoencephalitis with ataxia, generalized tremor, jumping (to "loup" means to leap in vernacular Scottish), vigorous kicking, salivation, and champing of jaws, progressing to paralysis, coma, and death (lethality 40–60%). Concurrent tick-borne fever (*A. phagocytophilum* infection) and external stress enhance the disease course (Reid, 1990).

31.3.3 Powassan virus

This virus (and its variant genetic lineage "deer tick virus" (DTV) is a member of the TBE complex. The complete nucleotide sequence of the genome was determined (a total of 10 839 nucleotides). Powassan virus (POWV) was first isolated from the brain of a child who had died from encephalitis in Powassan, Ontario (Canada), in 1958, and the next year, the virus was also isolated from *Dermacentor andersoni* tick (Theiler and Downs, 1973). Its geographical distribution is North America (northern United States, Canada) and Russian Far East.

Vectors are the ixodid ticks *Ixodes cookei*, *I. marxi*, *I. scapularis* (DTV), and *D. andersoni*, while in Asian Russia, the tick *I. persulcatus* is the major vector, and probably a minor role is played by the species *D. silvarum*, *H. concinna*, *and H. neumanni* (Gritsun et al., 2003). Because POWV is secreted in milk of experimentally infected goats, it can also be transmitted by drinking raw milk and eating raw milk products.

Vertebrate hosts of POWV/DTV are small- and medium-sized mainly forest mammals, especially rodents such as woodchucks *Marmota monax, Peromyscus leucopus* (DTV), and *Tamiasciurus hudsonicus*, also skunk *Mephitis mephitis*, raccoon *Procyon lotor*, fox, and, in the Far Eastern Russia, for example, *Apodemus peninsulae*, *A. agrarius*, and *M. rufocanus*. Animal disease has usually an inapparent course. However, experimental inoculation of adult laboratory mice and *Macaca mulatta* monkeys and horse (Little et al., 1985) causes their encephalitis and death.

Powassan is an encephalitis with fever, headache, prostration, meningitis, spastic pareses, and rarely paralyses and sometimes results in death (fatality rate about 10%); neurological sequelae often persist. In general, it is an infrequent disease in North America. For instance, only 27 cases (without fatalities) were reported in the United States between 1958 (first human case) and 1998, but since the late 1990s, the incidence of human disease seems to be increasing (Hinten et al., 2008; Pesko et al., 2010); anyway, "the disease is probably underrecognized" (Hinten et al., 2008). In Far Eastern Russia, POWV co-circulates with TBEV, and 14 cases of POW disease were described between 1973 and 1988 (Gritsun et al., 2003).

31.3.4 Omsk hemorrhagic fever virus

The virus belongs to the so-called TBE antigenic complex, being related to Kyasanur forest disease virus (KFDV) and readily distinguishable from TBEV. Omsk hemorrhagic fever virus (OHFV) was first isolated in 1947 from human blood and *D. marginatus* ticks (Gritsun et al., 2003). Primary vectors are *D. reticulatus* (TST, TOT) and *Ixodes apronophorus*. Alimentary transmission (consumption of raw milk of goats and sheep or drinking contaminated water) has also been described, as well as direct contact—for example, in muskrat trappers (Theiler and Downs, 1973). Its geographical distribution are steppe ecosystem with lakes in southern and western Siberia—specifically the regions Omsk, Novosibirsk, Kurgan, and Tyumen.

The vertebrate hosts are rodents (muskrat *Ondatra zibethica*, imported to Siberia from Canada in 1928 and 1935–1939; *Arvicola terrestris*; *Microtus gregalis*), possibly also frogs and some birds (Růžek et al., 2010). The animal disease produces occasional epizootics (e.g., mass dying of muskrats in Siberia in the period 1946–1970).

The Omsk hemorrhagic fever is characterized by high fever (accompanied by chills, sometimes biphasic), headache, severe myalgia, cough, nausea, nasal bleeding, pharyngitis, conjunctivitis, hyperemia of the face, petechial rash, hemorrhages, and encephalitis (occasionally) with pareses, with a case fatality rate of 1–3% and long convalescence. Between 1946 and 1958, 972 human OHF cases were reported; thereafter the incidence declined remarkably. During 1988–1998, a total of 172 cases were reported from western Siberia (Gritsun et al., 2003). However, mild cases might have been misdiagnosed or not reported. Seasonal peaks of OHF occur in September and October.

31.3.5 Kyasanur Forest disease virus

This virus also belongs to the TBE antigenic complex. Very closely related to KFDV (in fact, its variants or subtypes) are the viruses Alkhumra virus (ALKV; its overall genomic homology with KFDV is 89%) and Nanjianyin virus (occurring in China). KFD was first recognized as a new hemorrhagic zoonotic disease in Shimoga district of Karnataka (then Mysore) State, India, in 1957 (Theiler and Downs, 1973). ALKV was first isolated from the blood of a butcher in Jeddah, Alkhumra district of Saudi Arabia, in 1995 (Madani, 2005; Pattnaik, 2006). KFD occurs in forest ecosystems in India and West China (province Yunnan, *Nanjianyin* virus) while ALKV in semidesert habitats in Saudi Arabia.

The major hematophagous vector (and reservoir) of KFDV is the tick *Haemaphysalis spinigera*, less important seem to be *H. turturis*, other *Haemaphysalis* spp., and possibly *Ornithodoros savignyi* in ALKV. Direct contact with infected sheep and goats and drinking raw milk seem to be important modes in the transmission of ALKV (Madani et al., 2011; Pattnaik, 2006), and mosquito bites were reported as the only risk factor in one-fifth of 78 Alkhumra hemorrhagic fever (AHF) patients examined, while only 3% of them reported history of tick bites (Madani et al., 2011). There is an occupational risk with ALKV infection (e.g., at slaughtering sheep).

The vertebrate hosts are monkeys, rat *Rattus blanfordi*, striped forest squirrel *Funambulus tristriatus*, insectivores (*Suncus murinus*), and bats (*Rhinolophus rouxii*) in KFD and probably sheep and goat with ALKV in Saudi Arabia. In animals, occasional epizootics like mass dying of primates (KFDV) may be observed. For instance, high mortality due to KFD, significantly reducing population density of local monkeys, was observed in the black-faced langur (*Semnopithecus entellus*) and the red-faced bonnet monkey (*Macaca*

radiata) in the Kyasanur Forest in 1957 and later: 1965–1966 and 1969–1975 (Theiler and Downs, 1973, Pattnaik, 2006).

KFD and AHF present fever (often biphasic course), headaches (severe headache initiates the 2nd phase), malaise, myalgia, arthralgia, anorexia, backache, nausea and vomiting, diarrhea, abdominal pain, erythema on face, conjunctivitis, retro-orbital pain, bradycardia, pharyngitis, meningoencephalitis (in about 20% of cases), neck stiffness, impaired sleep, mental disturbance, hepatitis, hemorrhagic manifestations (nasal and gastrointestinal bleeding), leukopenia and thrombocytopenia, and elevated liver enzyme levels. Fatality rate is 2–15% (but in AHF can be as high as 25%). Convalescence is long—up to 4 weeks (Madani, 2005; Madani et al., 2011). Big outbreaks of KFD occurred in the Indian state Karnataka (then Mysore) in 1957 (hundreds of human cases) and later on in 1986 (213 cases with 14 fatalities). The KFD foci activated in the 1990s, and hundreds of human cases have been reported annually since 2001, with a spike of 915 cases in the year 2003 (Pattnaik, 2006). In Saudi Arabia, about 60 human cases of AHF in Jeddah and Makkah provinces occurred until 2003 (Pattnaik, 2006; the fatality rate was 25%). Additional c. 90 human cases were reported in Makkah province and a number of patients also in Najran, in the south border of Saudi Arabia, with Yemen during 2003–2009 (Madani et al., 2011).

31.4 FAMILY *BUNYAVIRIDAE*

31.4.1 Crimean-Congo hemorrhagic fever virus

CCHF is a serious human disease mainly transmitted by ticks of the genus *Hyalomma*. Since the first outbreak of CCHF described in Europe in 1945, several subsequent outbreaks have been reported worldwide in both newly discovered foci and foci at which the virus was known to be present. Interest in the disease increased after the recent epidemic in Turkey and new viral records reported in areas near Turkey such as the Balkans and Russia (Ergonul and Whitehouse, 2007). Studies have focused in outlining the probable routes for virus introduction into Western Europe from the original foci of the disease in Eastern Europe and Turkey (Gale et al., 2010). The finding of Crimean–Congo hemorrhagic fever virus (CCHFV) in Western Europe (Estrada-Peña et al., 2012a) encouraged the studies aimed to assess the endemic potential of the virus in Europe. These results demonstrated that the virus is not restricted to Eastern Europe, as obviously known, and that a viral strain circulates in southwestern Mediterranean. This increased the concerns about the spread of the virus into northern latitudes (Estrada-Peña et al., 2012b). The virus has the largest known distribution of any other tick-transmitted virus (Ergonul and Whitehouse, 2007). The virus is transmitted to reservoir mammals and humans through the bite of hard ticks, mainly of the genus *Hyalomma* (Hoogstraal, 1979). Humans may also become infected through direct contact with the blood or tissues of infected humans or livestock (Hoogstraal, 1979). Some other tick species from the genera *Dermacentor*, *Amblyomma*, *Rhipicephalus*, and *Haemaphysalis* have been found to harbor the virus in the field or have been artificially infected in the laboratory, but there is little evidence of their involvement in natural transmission or maintenance of foci (Watts et al., 1988). All natural reports linking the transmission of the virus by way of an infected vector have involved ticks of the genus *Hyalomma* (Watts et al., 1988). It would appear that additionally *Hyalomma* ticks are also necessary for the maintenance of active foci of the virus in the field, even within periods of silent activity. The principal species implicated

in transmitting CCHFV in Eurasia are *H. marginatum*, *H. turanicum*, *H. anatolicum*, and *H. scupense* (including the former *H. detritum*, now considered a synonym of *H. scupense*, Guglielmone et al., 2009).

The tick genus *Hyalomma* is widespread in different ecological areas of the Palearctic and Afrotropical regions. The tick vector has three active stages. The immatures (larvae and nymphs) commonly feed on the same hosts, which are many species of small mammals and birds. It is thus a two-host tick, although it may behave as a three-host tick under some conditions (Hoogstraal, 1979). Large ungulates serve as hosts for the adults. Tick females contribute to the infection by TOT of the virus to the eggs. Feeding on infected reservoir hosts or through the nonsystemic (cofeeding) transmission of the virus might also infect ticks. The nonsystemic transmission may occur when uninfected ticks feed in near proximity to infected ones, which pass the virus with the saliva without host systematic infection (Gordon et al., 1993). It is well established that the immature tick stages (and not the adult) of *H. marginatum* infest birds and medium-sized mammals, while adults feed on large ungulates (Hoogstraal, 1979). As for any other species, certain conditions of temperature and humidity are needed for molting of immature stages of *H. marginatum* to adults (Estrada-Peña et al., 2011). Some species, like *H. scupense* (one- or two-host biology) and *H. anatolicum* (two- or three-host biology), prefer to feed on the same large ungulates (mostly cattle) during all developmental stages and then adopt a nidiculous life cycle, protecting them from extreme environmental conditions.

The virus has been reported to survive throughout the life of the tick and passes transstadially and transovarially. The long survival of the virus in ticks is important from the epidemiological point of view. However, there is still a dearth of knowledge regarding host exposure rates and host immune responses particularly in populations of short-lived birds, insectivores, and lagomorphs. Such animals have a high population turnover shown to be important in other tick-borne pathogens (i.e., TBEV) where such hosts develop antibodies to exposure in the nest during their first few days of life. The epidemiological potential, relating climate, ticks, and reservoirs of the active foci, is a very important part of the enzootic ecology of CCHFV. Recent reports of an increased incidence of CCHF stimulated speculation about the presumed effects of climate on the historical geographical range of *H. marginatum* ticks in the Palearctic region (Karti et al., 2004; Maltezou et al., 2010) and the probable spread of the pathogen. The tick is presumed to be the most prominent vector of the virus to humans in a large region extending from the Balkans in Europe to Pakistan and Afghanistan in the Middle East (Ergonul and Whitehouse, 2007). In an expert consultation organized by the European Center for Disease Control in 2008, a short-term priority was recognized to be "endemic regions in countries with CCHF in southeastern Europe should be further mapped on national and international levels, and the degree of CCHF risk in all countries should be estimated."

We ignore basic epidemiological parameters for the transmission of CCHFV and how changes in transmission rates among ticks and competent reservoir hosts affect virus circulation and geographical range. It is known that the tick larvae molt into nymphs while attached to the bird, lengthening the duration of host attachment (12–26 days) and so enabling the passive transport of the immature *Hyalomma* ticks by migrating birds over long distances (Hoogstraal et al., 1961). As an example, an adult male *Hyalomma rufipes* tick was identified on a horse in the Netherlands during a survey of ticks (Nijhof et al., 2007). As that horse was not imported, Nijhof et al. (2007) speculated that the tick was introduced as a nymph by a migratory bird from Africa. *H. rufipes* is endemic in many regions of Africa and has been recorded on migratory birds in spring in Europe (Molin et al., 2011). However, the species is not known to have permanent populations in Europe because it is an Afrotropical

tick, which needs high temperatures for adequate molting (Estrada-Peña and Venzal, 2007). Every year, literally millions of passerine birds reach the European continent, parasitized by ticks coming from the northwestern coast of Africa and which serve as vectors of CCHFV, a pathogen that is known to exist in the area where the birds rest before the entry into southwestern Europe. How the climate could affect the flight of the migratory birds, how the ticks attached may enter at higher rates, and how many infected ticks may spread over the continent each year are key variables that have not yet been evaluated.

Ticks can disperse large distances only while on their hosts (Randolph, 1998). Therefore, the capacity for a population to spread depends on the availability and invading abilities of the potential hosts in combination with other factors that deeply affect the behavior of the host, such as habitat fragmentation and physical barriers to migration. The potential effects of the climate trend on the geographical range of arthropods are commonly evaluated by climate-matching models, a set of methods based on the recorded distribution that assess the potentially available range for a species according to its preferences for a group of explanatory variables (an example is provided in Figure 31.1). Process-driven models focus on each part of the life cycle and are regarded as an essential tool for research on tick-borne pathogen transmission rates (Randolph and Rogers, 2000). Efforts to build process-driven models have been focused on *I. ricinus* (Dobson et al., 2011), but until recently a process-driven model of the life cycle of *H. marginatum* was unavailable (Estrada-Peña et al., 2011).

It is now known that clinical cases of CCHF are not reported everywhere the tick vector exists, making evident that a complement of epidemiological factors are necessary to fire up a new focus or for reemergence of former ones. Studies have been carried out in South Africa (Swanepoel et al., 1983), Tanzania, and African countries from Senegal in the west to Kenya in the east (Hoogstraal, 1979). The field investigations that followed recognition of the disease included antibody sera collected from humans and livestock and a survey of the prevalence of the virus in questing and feeding ticks. Further studies were carried out in west Africa, mainly in Senegal and Mauritania (Chapman et al., 1991; Wilson, 2009; Zeller et al., 1994a, b, 1997). These studies highlighted the clear correlation between antibodies to the disease in livestock and humans and the distribution of ticks of the genus *Hyalomma* (Wilson et al., 2009). Humidity in Senegal varies from 200 mm in the Sahelian zone in the north to more than 1400 mm in the sub-Guinean zone in the south, and this is reflected in changing composition of the tick species across the country. Bioclimatic zones differed in the intensity with which CCHFV was transmitted. Evidence of infection in sheep was greatest in the northern, arid, sparsely vegetated zone of Senegal and decreased consistently toward the southern, moister, forested zone. The specific identity of the tick vectors that maintain CCHFV transmission in Senegal is unknown (Wilson et al., 2009) although their results indicated that *Hyalomma* species are important in the maintenance of local or regional foci of the disease. Further studies (Sylla et al., 2008) focused also on the effect of climate variables along a north–south gradient in Senegal as a marker for the dominant tick species, and in turn the serological prevalence of CCHFV in humans.

Such a kind of climate transition affecting the main vectors of CCHFV is harder to outline for other areas in Africa, because of the wide variety of habitats and species with well-varied climate preferences. Figure 31.2 includes the reported distribution of several species in the genus *Hyalomma* in both western Palearctic and Africa. Records in the Mediterranean basin correspond to *H. marginatum*, the main vector of the virus in the area. The other records correspond to several species of *Hyalomma* as reported in the Afrotropical region. Such a kind of detailed distribution is missing for other areas where *Hyalomma* ticks are known to be present. It has been however reported that warmer scenarios would favor the distribution of *Hyalomma* in South Africa (Estrada-Peña, 2003). In the western

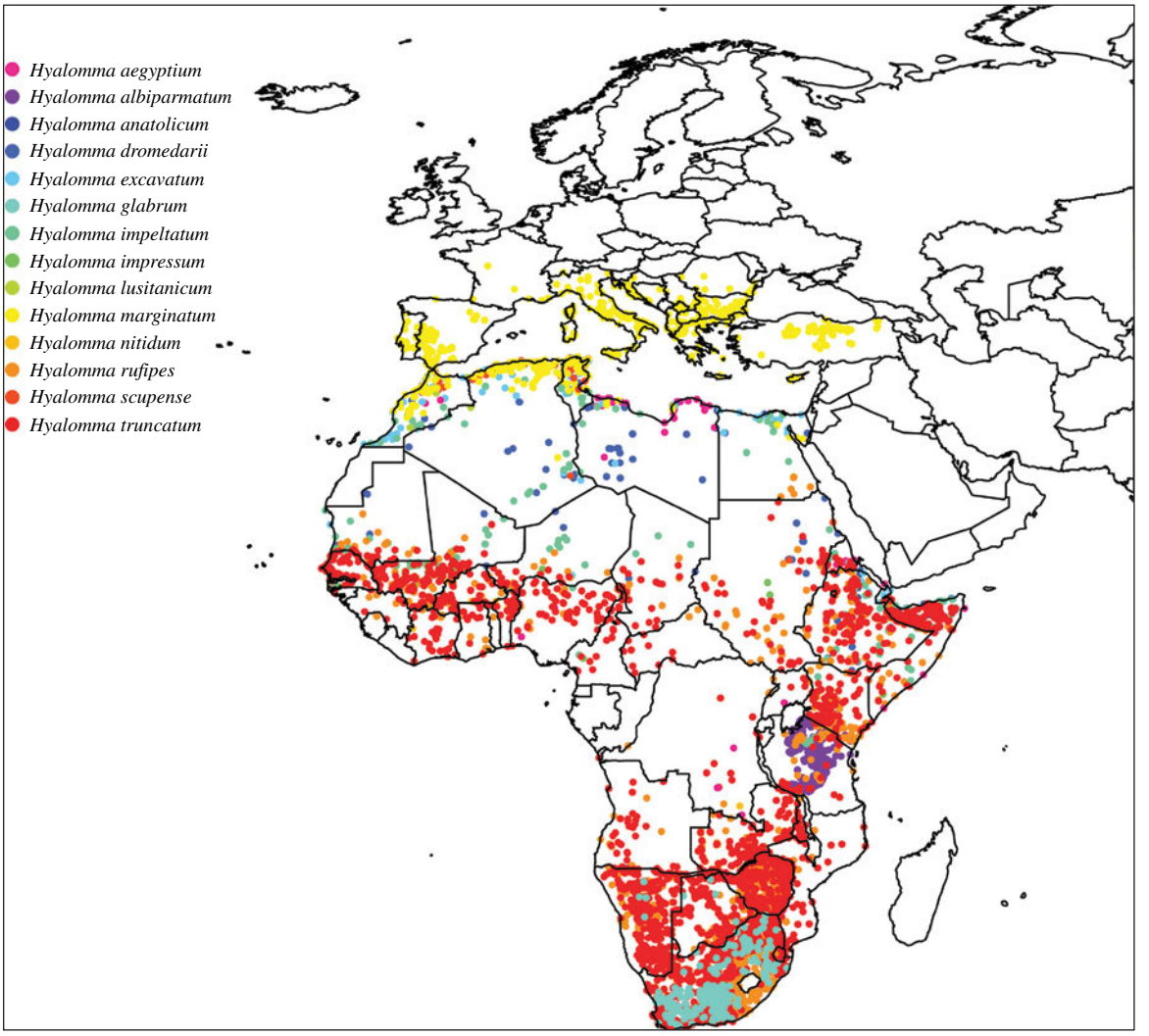

Figure 31.2. The distribution of the most prominent species of the genus *Hyalomma* in Africa. Only those records with accurate georeferences where included, that is, with a pair of coordinates or with an unambiguous name of a locality. The compilation is not intended to be exhaustive, and it provides only general information on the distribution of those species. For color detail, please see color plate section.

Palearctic, studies suggest that scenarios of warmer climate would increase the northern distribution limit of the tick (Figure 31.3) because it would improve the colder conditions in winter and would rise the number of days with temperature above the minimum threshold necessary for completion of development by the tick (Estrada-Peña et al., 2012b).

The recent epidemic of CCHF in Turkey began with some isolated cases in Tokat Province (Gozalan et al., 2004). The human health authorities soon realized that more clinical cases were being reported from neighboring sites and then later over a large territory in the country (Yilmaz et al., 2009), largely coinciding with the expected distribution of the tick *H. marginatum* in an early paper about the dynamics of the infection in that country (Estrada-Peña et al., 2007). The very focal nature of CCHF in Turkey

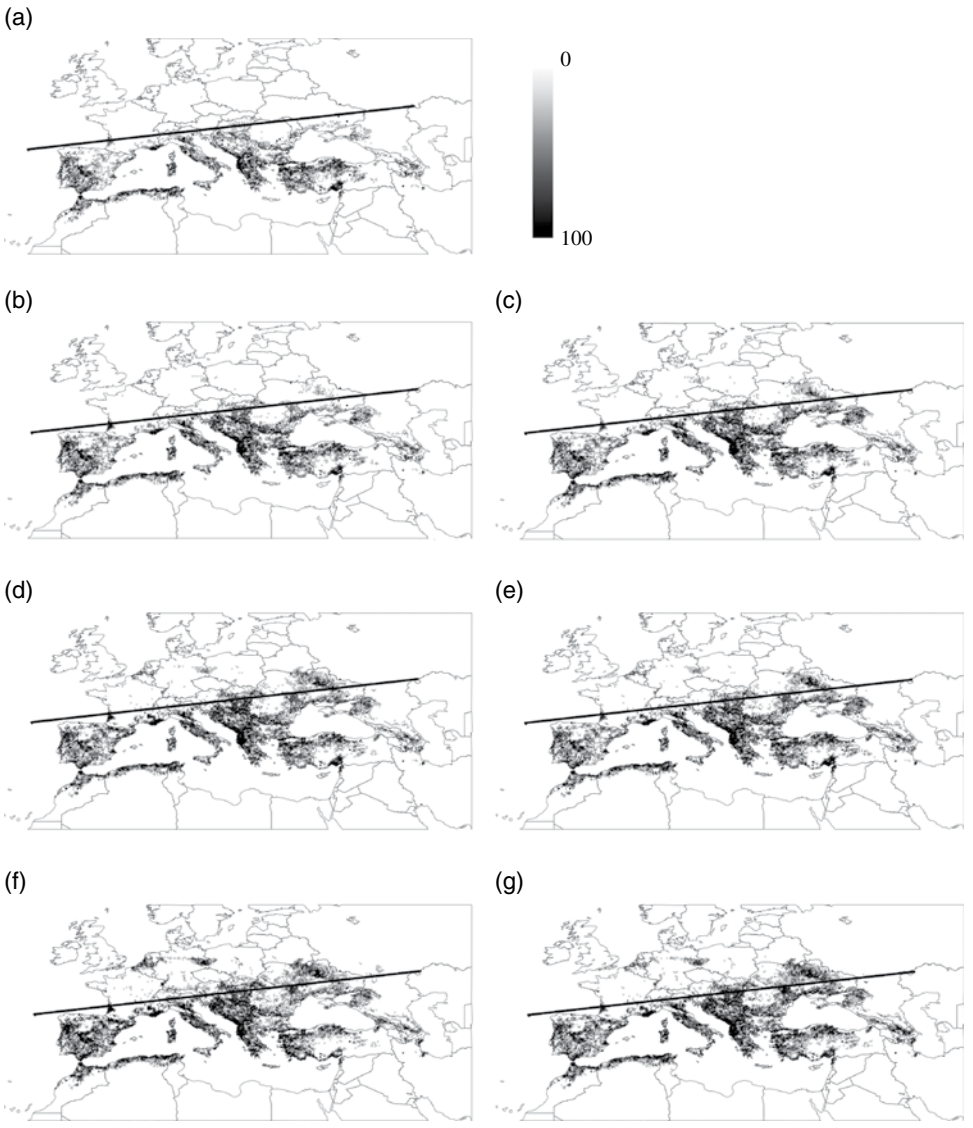

Figure 31.3. Changes in the computed probability of occupancy for *H. marginatum* in the western Palearctic. The measure is unitless, combining the net growth rate of the tick in the site and the connectivity of vegetation patches due to host movements. Data on probability of occupancy range from 0 to 100 (with data and methods reported by Estrada-Peña et al. 2012c). (**a**) Results for the current climate (1961–2000). (**b–g**) Recruitment was computed for climate projections for the years 2020 (**b, c**), 2050 (**d, e**), and 2080 (**f, g**) and scenarios **a** (**b, d, f**; hard growth, high emissions) and **b** (**c, e, g**; low growth, fewer emissions). The black line in every figure marks the approximate latitudinal level of the northern limit of the ticks under current climate conditions.

exhibited a strong correlation of the highest incidence in human cases with the presence of particular land use categories, significantly small and highly mosaic agricultural localities. This was consistent with the most common transmission mechanism reported, the bite of an infected tick, and with the increased densities of ticks in fragmented sites, which in turn provide an environment for higher exposure in humans.

Similar to other tick-borne diseases, climate trends have been commonly linked to outbreaks of clinical cases. Ticks are highly sensitive to small changes in climate, and long-term, sustained, and small differences in key climate variables may drive a serious change. An assessment of the effects of climate on the presence of human clinical cases of the disease in Turkey was carried out (Estrada-Peña et al., 2010). The analysis included monthly values of several climate variables and concluded that climate was not different in sites with active foci of the disease as compared with sites where *H. marginatum* is common but human cases are not reported. They concluded that it is not possible to predict, based solely on climate grounds, where new cases could appear in a reasonably near future. Climate is not the sole factor driving the virus amplification, even if climate in autumn/winter may have a strong regulating role on the survival of tick populations (Hoogstraal, 1979). It is noteworthy that *Hyalomma* endemic areas with mildest autumns and winters in the Mediterranean basin have historically been and are currently free of the disease, so the impact of a warmer climate seems not to be the only factor determining the occurrence of the disease.

There is however evidence that a fragmented landscape, with many small patches existing within a matrix of unsuitable tick habitat, may lead to isolated populations of both ticks and hosts, producing an amplification cycle whereby ticks feed on infected hosts (Estrada-Peña et al., 2010). For CCHFV eco-epidemiology, the degree of habitat patchiness contributes to the increased contact rate among reservoir hosts, humans, and ticks. It also leads to the occurrence of isolated amplification foci, with consequently higher tick exposure to humans (Estrada-Peña et al., 2009). Isolated fragments surrounded by a matrix agricultural land have commonly the poorest diversity of mammals. Although *Hyalomma* ticks can move horizontally, they require a vertebrate host to move over significant distances. Because these host populations are relatively isolated, there are few local movements of hosts and therefore new "naive" animals carrying uninfected ticks are not diluting the prevalence rates in the isolated patch; however, the immune response of such isolated populations against the ticks and the pathogen may seriously decrease the population of infected ticks. These aspects have never been studied for CCHFV.

Several scenarios are of concern regarding the possible spread of CCHFV to new foci or the reemergence of former ones. One is the impact that a warmer and drier climate may have on the distribution range of the ticks in Africa, where they already are occupying a climate niche consistent of warm and dry environments. The second is the probable spread of active foci in western Palearctic or the importation of infected ticks from the western coast of Africa into southwestern Europe. The second has been partially confirmed by the finding of CCHFV in *H. lusitanicum* ticks in southern Spain (Estrada-Peña et al., 2012b). The details around the findings suggest that the virus strain has been circulating in the area since a long time ago, because it has been detected in a tick that does not use migratory hosts (i.e., it is resident and restricted to some areas in Southern Europe). However, no clinical cases have been reported in such area in Spain nor in the near Portugal. All these extremes must to be confirmed before confirming the hypotheses of an old existence of the virus in Western Europe.

31.4.2 Henan virus

Henan fever bunyavirus (HNFV) is a new, emerging bunyavirus, not yet assigned to a genus (Xu et al., 2011; Yu et al., 2011). It is also called Huaiyangshan virus (HYSV: Chen et al., 2012) or severe fever with thrombocytopenia syndrome virus (SFTSV: Yu et al., 2011). The virus is distantly related to tick-borne Uukuniemi bunyavirus

(Xu et al., 2011; Yu et al., 2011). It extends in China (mainly northeast and central provinces); the natural foci are situated in woody and hilly areas.

The main tick vector is *Haemaphysalis longicornis*. However, the virus is also transmitted from person to person by contacting patient's blood (Bao et al., 2011; Liu et al., 2012). The vertebrate hosts are unknown, possibly rodents. Newborn mice are highly susceptible at experimental inoculation with HNFV: the mice that died developed extensive necrotic areas in liver while no obvious pathologic changes were seen in other organs. HNFV antigen and RNA were present in almost all organs, indicating a systemic infection (Chen et al., 2012).

Human disease consists of severe fever with thrombocytopenia syndrome (SFTS) or fever with thrombocytopenia and leukopenia syndrome (FTLS)—hemorrhagic fever-like disease. The key clinical features include fever, fatigue, diarrhea, abdominal pain, lymphocytopenia, and thrombocytopenia. In a clinical study, 8 of 49 patients (16.3%) with hemorrhagic fever caused by HYSV died; and the fatal outcome was associated with high viral RNA load in blood at admission, higher liver transaminase levels, and pronounced coagulation disturbances (Zhang et al., 2012). Other sources report a 21% case fatality rate among 171 patients by September 2010 (Dr. Wang Yu, personal communication). Human FTLS cases have been observed in China since 2006, and up to 2010, about 300 patients with this syndrome were confirmed (Liu et al., 2012; Xu et al., 2011).

31.4.3 Bhanja virus

This virus (synonym Palma virus, Filipe et al. 1994) is, together with two other African tick-borne viruses Kismayo and Forécariah, a member of Bhanja group that has not yet been assigned to a recognized genus of the family *Bunyaviridae*. Bhanja bunyavirus (BHAV) was isolated first from *Haemaphysalis intermedia* (syn. *Haemaphysalis parva*) ticks that had been collected from a paralyzed goat in Bhanjanagar (district Ganjam, Orissa State, India) in 1954 (Shah and Work, 1969). In Europe, the first isolation was from adult *H. punctata* collected in Italy in 1967 (Verani et al., 1970). The geographical distribution is Southern and partly Central Europe (Italy, Croatia, Bulgaria, Romania, Slovakia, Portugal). Outside Europe it has been reported from India, Kirghizia, Kazakhstan, Azerbaijan, Armenia, Senegal, Guinea, Nigeria, Cameroon, Central Africa, Kenya, and Somalia. Antibodies were detected in vertebrates of many additional countries (Spain, Moldova, Sri Lanka, Pakistan, Iran, Turkmenia, Uzbekistan, Tajikistan, Uganda, Tanzania, Egypt, and Tunisia).

The virus is transmitted by metastriate ixodid ticks: *Haemaphysalis intermedia, H. punctata, H. sulcata, D. marginatus, Rhipicephalus decoloratus, R. annulatus, R. geigyi, Amblyomma variegatum, Hyalomma marginatum, H. detritum, H. dromedarii, H. truncatum, H. asiaticum* (TOT), *Rhipicephalus bursa*, and *R. appendiculatus*. Probable vertebrate hosts for BHAV are sheep, goat, and cattle; in Africa, BHAV was also isolated from the four-toed hedgehog (*Atelerix albiventris*) and striped ground squirrel (*Xerus erythropus*). The virus does not usually cause apparent infection in adult animals but is pathogenic for young ruminants (lamb, kid, calf), causing fever and meningoencephalitis (Camicas et al. 1981; Mádr et al., 1984; Semashko et al., 1976; Theiler and Downs, 1973). Experimental encephalitis was produced in rhesus monkey (Balducci et al., 1970).

Natural foci of BHAV are boskematic (pastoral steppe or forest) steppe ecosystems in xerothermic areas or in karst habitats at more northern latitudes. Based on a comparison of several known natural foci of BHAV infection, their common and typical features were extracted and bio-indicator species (plants, animals) were selected that can be used for prediction of potential presence of BHAV in other geographical areas within Europe (Hubálek, 2009).

In humans, BHAV can cause febrile illness with headache, conjunctivitis, or sometimes meningoencephalitis with photophobia, vomiting, and pareses. About 10 natural and/or laboratory infections with BHAV have been described in humans, one of them serious, with quadriparesis (Calisher and Goodpasture 1975; Punda et al., 1980; Vesenjak-Hirjan et al., 1980). There is some occupational risk for shepherds and veterinary personnel. Probably, this has been an underdiagnosed disease in some countries.

31.4.4 Keterah virus

This virus has not yet been assigned to a genus. Keterah bunyavirus (KETV) was first isolated from larval *Argas pusillus* infesting *Scotophilus* bats in Malaysia, 1966 (Karabatsos, 1985), while an identical virus (Issyk-Kul virus) later from bats and their ticks *Argas vespertilionis* in Kirghizia (Lvov et al., 1973). Its geographical distribution involves Malaysia and Central Asia (Kirghizia, Tajikistan, Uzbekistan).

The vectors are soft ticks (*Argasidae*), possibly also biting midges (*Culicoides schultzei*) and mosquitoes. The vertebrate hosts are bats. The animal disease is asymptomatic in bats; in green monkeys it causes damage to visceral organs but without overt clinical symptoms. A human outbreak with more than 60 cases was described in southern Tajikistan, 1982 (Lvov et al., 1984).

31.5 FAMILY *REOVIRIDAE*

31.5.1 Colorado tick fever virus

The Colorado tick fever virus (CTFV) is transmitted by ixodid ticks (principal vector is *Dermacentor andersoni*, but CTFV has also been isolated from *D. occidentalis, D. parumapertus, D. albipictus*). It may be transmitted by blood transfusion, because the virus causes in humans persistent viremia up to 120 days, being localized in erythrocytes. This virus extends in North America, with natural foci occurring in the Rocky Mountains of the United States and Canada, most often at altitudes of 1200–3000 m above sea level.

The main vertebrate hosts are rodents (reservoirs: mainly *Spermophilus lateralis, Tamias minimus*, *T. amoenus*, *Tamiasciurus richardsoni*, *Erethizon dorsatum*, *Neotoma cinerea*, *Peromyscus maniculatus*). The disease in animals seems to be inapparent but teratogenic in mice. Colorado tick fever (CTF) is usually a biphasic fever disease in humans, with headache, myalgia and arthralgia, conjunctivitis, photophobia, sometimes orchitis, and affection of the CNS (mainly in children); temporary rash occurs less often (5–10% of patients) than in RMSF, and occasionally myopericarditis, pneumonia, and hepatitis occur. Rare complications with this disease have included aseptic meningitis, encephalitis, and hemorrhagic fever. Laboratory findings include leukopenia, thrombocytopenia, and mildly elevated liver enzyme levels. Mortality is low, but the convalescence long (fatigue, lethargy).

31.5.2 Kemerovo virus

Kemerovo virus (KEMV) was first isolated from ixodid ticks and a patient during an expedition to study RSSE in Siberia in 1962 (Chumakov et al., 1963). It is transmitted by *I. persulcatus*. Migratory birds have been implicated in the dispersal of KEMV over vast distances. For instance, KEMV was isolated from a migrating redstart *Phoenicurus phoenicurus* in Egypt in 1961 (Schmidt and Shope, 1971). Its vertebrate hosts are birds and

rodents. The animal disease has an inapparent course but meningoencephalitis was observed in experimentally inoculated monkeys (Grešíková et al., 1966).

31.5.3 Tribeč virus

Tribeč virus (TRBV) is a member of Kemerovo antigenic group and the Kemerovo subgroup (Belhouchet et al., 2010), closely related to the Siberian KEMV by complement-fixation test but distinguishable by virus neutralization test (Libíková and Buckley, 1971) or RNA–RNA hybridization (Brown et al., 1988). Kemerovo group and other orbiviruses have a great reassortment potential (because of the segmented dsRNA), resulting in biological variability (Brown et al., 1988; Gorman, 1983). The reported synonyms and subtypes are Lipovník, Koliba, Cvilín, Brezová, Mircha, and Kharagysh virus. It is known from Slovakia, Czechland, Ukraine, Belarus, Russia, southern Norway, Italy, and exceptionally northern Africa. Natural foci of TRBV infections are both boskematic and theriodic (pastoral and mixed woodland ecosystems).

The first strains of TRBV were isolated from *I. ricinus* in three regions of Slovakia in 1963 (Grešíková et al., 1965; Libíková et al., 1964, 1965). TRBV is transmitted by ticks *I. ricinus* (TST), occasionally by *H. punctata* (Topciu et al., 1968). The vertebrate hosts of TRBV are rodents, for example, *Myodes glareolus* and *Microtus subterraneus*; hare *Lepus europaeus*; goat; European starling *Sturnus vulgaris*; and chaffinch *Fringilla coelebs* (Dobler et al., 2006; Grešíková et al., 1965; Skofertsa et al., 1974). Animal disease is unknown.

TRBV causes febrile illness or aseptic meningitis in humans occasionally; for example, about 15 patients with the CNS infection (meningitis) revealed seroconversion against TRBV in Czechland (Fraňková, 1981; Hubálek et al., 1987; Málková et al., 1986). The disease caused by TRBV is probably underdiagnosed. Additional studies are necessary to evaluate the public health importance of TRBV.

31.6 FAMILY *ORTHOMYXOVIRIDAE*

31.6.1 Thogoto virus

Thogoto virus (THOV) was first isolated from a mixed pool of *Rhipicephalus decoloratus* and *Rhipicephalus* spp. ticks collected on cattle in Thogoto Forest near Nairobi, Kenya, in 1960 (Haig et al., 1965). In Europe, it was first isolated from ticks collected on ruminants in Sicily in 1969 (Albanese et al., 1972) and then in Portugal in 1978 (Filipe and Calisher, 1984). Arthropod vectors of THOV are metastriate ticks only—*R. annulatus*, *A. variegatum*, *R. appendiculatus*, *R. sanguineus*, *R. bursa*, *R. evertsi*, other *Rhipicephalus* spp., *H. truncatum*, and *H. anatolicum*. THOV extends in areas of Kenya, Uganda, Ethiopia, Nigeria, Cameroon, Central Africa, Egypt, Iran, Portugal, and Sicily. Tick-infested domestic animals (e.g., camels) and migratory birds could disseminate the virus over a wide geographical range (Calisher et al., 1987). Natural foci are boskematic—pastoral xerothermic ecosystems.

Vertebrate hosts for THOV are camel and horse. Antibodies were also detected in sheep and goat. The infection course is usually inapparent in animals, but THOV can cause leukopenia in cattle and fever and abortion in sheep (Davies et al., 1984; Theiler and Downs, 1973).

Only two cases of human disease have been described, one with bilateral optic neuritis and another as a fatal meningoencephalitis with hepatitis although complicated by a sickle-cell disease (Moore et al., 1975; Theiler and Downs, 1973). THOV is probably contagious from man to man.

31.6.2 Dhori virus

Dhori virus (DHOV) was first isolated from *H. dromedarii* ticks collected on camels in Dhori, Gujarat State, India, in 1961 (Anderson and Casals, 1973). In Europe it has been recovered several times from *H. marginatum* and twice from *H. scupense* collected at Astrakhan, South Russia, since 1969 (as "Astra" virus (Bannova et al., 1974; Butenko et al., 1971, 1987)) and in Crimea (one strain—"Batken"); additional two strains were obtained from *H. scupense* near Astrakhan (Smirnova et al., 1988) and another one in southern Portugal in 1971 (Filipe and Casals, 1979). The reported Batken virus (Lvov et al., 1974) is a synonym of DHOV. It is known from areas in Portugal, Crimea, Astrakhan (southern Russia), Armenia, Azerbaijan, Kirghizia, Uzbekistan, India, and Egypt, while only antibodies were detected in vertebrates from Pakistan. Natural foci are boskematic, typically pastoral xerothermic ecosystems.

Principal arthropod vectors are metastriate ticks *Hyalomma dromedarii*, *H. marginatum*, *H. scupense*, and *D. marginatus*. Occasional isolations of DHOV were reported from *Ornithodoros lahorensis*. Vertebrate hosts are cattle, camel, horse, and bats (Kirghizia), but animal disease is asymptomatic. Natural foci of DHOV are boskematic (pastoral xerothermic and semidesert ecosystems).

DHOV produces an acute illness with severe fever, headache, general weakness, and retrobulbar pain, with encephalitis in c. 40% of patients and a long 2-month convalescence period. Five cases of severe laboratory infection (due to aerosol) have been described (Butenko et al., 1987). The virus could be contagious from man to man.

31.7 OTHER TICK-TRANSMITTED VIRUSES

Nine additional tick-borne viruses, occasionally causing clinical disease in humans, are only briefly presented in Table 31.1. No details exist about the impact of the climate on the spread of these viruses since they have been poorly investigated.

31.8 CONCLUSIONS

Talking in general terms, we are not yet able to evaluate the fine effects of climate trends on the epidemiology of the most prominent tick-borne viruses. We should keep in mind that these changes do not affect only the dynamics of tick vectors but also the abundance of hosts for immature stages of the tick or their migratory timings in the case of birds (which may be hosts for the immatures of the ticks) or even how climate may affect the densities of hosts in natural conditions. While available models might greatly contribute to understand the behavior of the ticks under variable climate conditions, we need yet to build upon those models to reach the necessary level of complexity. Local processes are not captured yet by these models, adding "noise" to the general background picture of the fine-scale distribution of ticks, their vectors, and the pathogens they transmit. There is an implicit need of further research at both local and regional scales. The first must be to finely capture the molecular details of the tick–pathogen relationships (Randolph, 2009), and the second should focus to describe such relationships under a generalist framework (Estrada-Peña et al., 2012).

TABLE 31.1. Tick-Transmitted Viruses Causing Disease in Humans only occasionally (Charrel et al. 2004; Karabatsos 1985; Labuda and Nuttall 2004; Theiler and Downs 1973)

Virus	Genus	Tick Vectors	Vertebrate Hosts	Geographical Distribution	Human Infection
Flaviviridae					
Tyuleniy	*Flavivirus*	*Ixodes uriae*	Seabirds (*Uria aalge*, *Eudyptula minor*), suslik (*Citellus undulatus*)	Coastal N. Russia (Murmansk), Norway (Lofoten), Asian Russia (Far East), western United States (Oregon), Canada	Three cases (malaise, laryngitis, lymphadenopathy, arthralgia, and skin petechiae) in biologists visiting seabird colonies
Bunyaviridae					
Soldado	*Nairovirus*	*Ornithodoros maritimus, O. capensis*	Seabirds (*Sterna fuscata*, *Larus argentatus*, *Rissa tridactyla*)	Trinidad, Hawaii, Texas, Ethiopia, Senegal, Seychelles, South Africa, Morocco, United Kingdom, Ireland, France, Iceland	Several cases (fever, pruritus, rhinopharyngitis)
Zirqa	*Nairovirus*	*Ornithodoros muesebecki*	Seabirds	Persian Gulf	Several cases (fever, headache, pruritus, erythema)
Punta Salinas	*Nairovirus*	*Ornithodoros amblus*, *Argas arboreus*	Colonial birds	Peru, Tanzania	Several cases (fever, headache, pruritus, erythema)
Dugbe	*Nairovirus*	*A. variegatum*, *R. decoloratus*, *H. truncatum*	Rodents	Tropical Africa	Two cases (encephalitis)
Nairobi sheep disease (syn. Ganjam)	*Nairovirus*	*R. appendiculatus*, *Haemaphysalis* spp.	Sheep, goat, *Arvicanthis abyssinicus*	Kenya, Uganda, South Africa, India	Six cases (fever, arthritis)
Avalon (syn. Paramushir)	*Nairovirus*	*I. uriae*, *Ixodes signatus*	Seabirds (*L. argentatus*)	France (Brittany), Asian Russia (Far East), Canada	Three cases (cervical adenopathy)
Wanovrie	Unassigned	*Hyalomma* spp.	?	India	One case (hemorrhagic fever)
Orthomyxoviridae					
Quaranfil	Unassigned	*A. arboreus*, *Argas reflexus*	Colonial birds (*Ardeola ibis*, pigeon)	Egypt, Yemen, Kuwait, Iran, Iraq, Afghanistan, Nigeria, South Africa	Two cases (fever)

? means not known.

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PRÁCE 41

Hubálek Z., **Rudolf I.**, Nowotny N. 2013. Arboviruses pathogenic for domestic and wild animals. *Adv. Virus Res.* 89: 201–275.

Stručná charakteristika: autoři se pokusili sestavit komplexní review shrnující údaje o arbovirech způsobujících onemocnění u domácích i volně žijících zvířat. Popudem pro vznik review byla neexistence takové práce v posledních desetiletích. Práce popisuje taxonomii, geografické rozšíření, vektory, obratlovčí hostitele, onemocnění a zdravotnické riziko u 50 arbovirů patogenních pro zvířata.

Hlavní přínos práce: jde o ucelený přehled arbovirů patogenních pro zvířata, který může sloužit především veterinářům a expertům z oblasti 'animal and public health'.

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CHAPTER FIVE

Arboviruses Pathogenic for Domestic and Wild Animals

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Contents

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Abstract

The objective of this chapter is to provide an updated and concise systematic review on taxonomy, history, arthropod vectors, vertebrate hosts, animal disease, and geographic distribution of all arboviruses known to date to cause disease in homeotherm (endotherm) vertebrates, except those affecting exclusively man. Fifty arboviruses pathogenic for animals have been documented worldwide, belonging to seven families: *Togaviridae* (mosquito-borne Eastern, Western, and Venezuelan equine encephalilitis viruses; Sindbis, Middelburg, Getah, and Semliki Forest viruses), *Flaviviridae* (mosquito-borne yellow fever, Japanese encephalitis, Murray Valley encephalitis, West Nile, Usutu, Israel turkey meningoencephalitis, Tembusu and Wesselsbron viruses; tick-borne encephalitis,

louping ill, Omsk hemorrhagic fever, Kyasanur Forest disease, and Tyuleniy viruses), *Bunyaviridae* (tick-borne Nairobi sheep disease, Soldado, and Bhanja viruses; mosquito-borne Rift Valley fever, La Crosse, Snowshoe hare, and Cache Valley viruses; biting midges-borne Main Drain, Akabane, Aino, Shuni, and Schmallenberg viruses), *Reoviridae* (biting midges-borne African horse sickness, Kasba, bluetongue, epizootic hemorrhagic disease of deer, Ibaraki, equine encephalosis, Peruvian horse sickness, and Yunnan viruses), *Rhabdoviridae* (sandfly/mosquito-borne bovine ephemeral fever, vesicular stomatitis-Indiana, vesicular stomatitis-New Jersey, vesicular stomatitis-Alagoas, and Coccal viruses), *Orthomyxoviridae* (tick-borne Thogoto virus), and *Asfarviridae* (tick-borne African swine fever virus). They are transmitted to animals by five groups of hematophagous arthropods of the subphyllum *Chelicerata* (order Acarina, families *Ixodidae* and *Argasidae*—ticks) or members of the class *Insecta*: mosquitoes (family *Culicidae*); biting midges (family *Ceratopogonidae*); sandflies (subfamily *Phlebotominae*); and cimicid bugs (family *Cimicidae*). Arboviral diseases in endotherm animals may therefore be classified as: tick-borne (louping ill and tick-borne encephalitis, Omsk hemorrhagic fever, Kyasanur Forest disease, Tyuleniy fever, Nairobi sheep disease, Soldado fever, Bhanja fever, Thogoto fever, African swine fever), mosquito-borne (Eastern, Western, and Venezuelan equine encephalomyelitides, Highlands J disease, Getah disease, Semliki Forest disease, yellow fever, Japanese encephalitis, Murray Valley encephalitis, West Nile encephalitis, Usutu disease, Israel turkey meningoencephalitis, Tembusu disease/duck egg-drop syndrome, Wesselsbron disease, La Crosse encephalitis, Snowshoe hare encephalitis, Cache Valley disease, Main Drain disease, Rift Valley fever, Peruvian horse sickness, Yunnan disease), sandfly-borne (vesicular stomatitis—Indiana, New Jersey, and Alagoas, Cocal disease), midge-borne (Akabane disease, Aino disease, Schmallenberg disease, Shuni disease, African horse sickness, Kasba disease, bluetongue, epizootic hemorrhagic disease of deer, Ibaraki disease, equine encephalosis, bovine ephemeral fever, Kotonkan disease), and cimicid-borne (Buggy Creek disease). Animals infected with these arboviruses regularly develop a febrile disease accompanied by various nonspecific symptoms; however, additional severe syndromes may occur: neurological diseases (meningitis, encephalitis, encephalomyelitis); hemorrhagic symptoms; abortions and congenital disorders; or vesicular stomatitis. Certain arboviral diseases cause significant economic losses in domestic animals—for example, Eastern, Western and Venezuelan equine encephalitides, West Nile encephalitis, Nairobi sheep disease, Rift Valley fever, Akabane fever, Schmallenberg disease (emerged recently in Europe), African horse sickness, bluetongue, vesicular stomatitis, and African swine fever; all of these (except for Akabane and Schmallenberg diseases) are notifiable to the World Organisation for Animal Health (OIE, 2012).

ABBREVIATIONS

BSL biosafety level (CDC, 2009)
CAHS congenital arthrogryposis-hydranencephaly syndrome
CFT complement fixation test
CPE cytopathic effect
HIT hemagglutination inhibition test

i.c. intracerebral
i.m. intramuscular
i.n. intranasal
i.p. intraperitoneal
i.v. intravenous
IFA immunofluorescent antibody assay
p.o. peroral
s.c. subcutaneous
TOT transovarial transmission (in arthropods)
TST transstadial transmission (in arthropods)
VNT virus neutralization test

ARBOVIRUS ABBREVIATIONS

AHSV African horse sickness virus
AINV Aino virus
AKAV Akabane virus
ASFV African swine fever virus
BCRV Buggy Creek virus
BEFV Bovine ephemeral fever virus
BHAV Bhanja virus
BTV bluetongue virus
COCV Cocal virus
CVV Cache Valley virus
EEEV Eastern equine encephalitis virus
EEV Equine encephalosis virus
EHDV Epizootic hemorrhagic disease virus
GETV Getah virus
HJV Highlands J virus
IBAV Ibaraki virus
ITMV Israel turkey meningoencephalitis virus
JEV Japanese encephalitis virus
KASV Kasba virus
KFDV Kyasanur Forest disease virus
KOTV Kotonkan virus
LACV La Crosse virus
LIV Louping ill virus
MDV Main Drain virus
MIDV Middelburg virus
MVEV Murray Valley encephalitis virus
NSDV Nairobi sheep disease virus
OHFV Omsk hemorrhagic fever virus
PHSV Peruvian horse sickness virus
RVFV Rift Valley fever virus
SBV Schmallenberg virus
SFV Semliki Forest virus
SHUV Shuni virus

SINV Sindbis virus
SOLV Soldado virus
SSHV Snowshoe hare virus
TBEV tick-borne encephalitis virus
THOV Thogoto virus
TMUV Tembusu virus
TYUV Tyuleniy virus
USUV Usutu virus
VEEV Venezuelan equine encephalitis virus
VSAV vesicular stomatitis—Alagoas virus
VSIV vesicular stomatitis—Indiana virus
VSNJV vesicular stomatitis—New Jersey virus
WEEV Western equine encephalitis virus
WNV West Nile virus
WSLV Wesselsbron virus
YFV Yellow fever virus
YUOV Yunnan virus

1. INTRODUCTION

Arboviruses (an acronym for "*ar*thropod-*bo*rne *viruses*") form an ecological but not taxonomic grouping and involve viruses of nine families. There are nearly 500 arboviruses known at present (Karabatsos, 1985); however, only some of them can cause disease in endotherm (homeotherm) vertebrates (domestic and wild mammals and birds; arboviruses exclusively pathogenic for human beings were not considered in this review).

Hematophagous arthropods are regarded as biological (in contrast to mechanical) vectors of a pathogen only when they are able to ingest a particular pathogen during feeding on an infected vertebrate host (donor), followed by replication of the pathogen in the vector and subsequent transmission of the pathogen to a new vertebrate host (recipient). Some hematophagous invertebrates reveal transstadial and transovarial transmission (TST and TOT, respectively) of certain arboviruses during their ontogenesis from one life stage to another (larva, nymph), or from an infected female to progeny. Vector species with TOT ability may also act as long-term reservoirs of particular pathogens. Viruses transmitted by arthropods only mechanically (passively), that is, without replication in their body, are not the subject of this review. For instance, lumpy skin disease poxvirus, transmissible mechanically by insects (mosquitoes) among ruminants (cattle), is not regarded as an arbovirus. Particular vertebrates are considered as hosts

of arboviruses when the virus can be isolated from them; many vertebrate species serve as "amplifying hosts" for a virus when the virus has established a sufficiently high- and long-term viremia.

In this review, all arboviruses known to cause disease in homeotherm animals (excluding man-only diseases) are mentioned systematically, describing briefly their taxonomic classification, history, arthropod vectors, vertebrate hosts, animal disease, human disease, biosafety level (BSL) (according to CDC, 2009), and geographic distribution. Additional details about common arbovirus diseases of animals, including clinical symptoms, pathological anatomy, histopathology, diagnosis, therapy, epidemiology, prevention, and control, can be found in general (MacLachlan & Dubovi, 2011) and specialized textbooks (Brown & Torres, 2008; Coetzer & Tustin, 2004; OIE, 2012; Reid, 1990). The virus taxonomy and nomenclature in this review was adopted from King, Adams, Carstens, & Lefkowitz (2012).

Experimental pathogenicity of individual arboviruses for laboratory animals and their cytopathic effects (CPEs) in cell cultures are presented in Tables 5.1 and 5.2, respectively.

Table 5.1 Experimental pathogenicity of arboviruses for laboratory animals (Hubálek & Halouzka, 1996; Karabatsos, 1985; additional sources)

	SM	SM	M	M	H	H	GP	GP	C	CE	Other
	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	s.c.	y.s.	
Togaviridae											
EEEV	2-3	2-4	2-4	4-7	+	nd	2-5	nd	+	+	
WEEV	2-6	4-8	+	+	+	nd	+	nd	+	+	RM, rabbit i.c.+
VEEV	+	+	+	+	+	+	+	+	+	+	Rabbit +, RM (+)
HJV	2	2-3	8-10	–	4-6	nd	nd	nd	2-4	1-2	
BCRV	+	–	–	–	(+)	(+)	nd	nd	+	+	
SINV	2-4	2-4	–	–	–	–	–	–	+	1-3	RM i.c.–
MIDV	2	2	–	–	nd	nd	–	–	–	nd	RM i.c.–
GETV	4-7	4-10	–	–	nd	nd	–	–	(+)	3-4	RM s.c.– rabbit i.p.–
SFV	2	+	3-4	3-6	+	(+)	nd	nd	–	+	RM i.c. (+)

Table 5.1 Experimental pathogenicity of arboviruses for laboratory animals (Hubálek & Halouzka, 1996; Karabatsos, 1985; additional sources)—cont'd

	SM	SM	M	M	H	H	GP	GP	C	CE	Other
	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	s.c.	y.s.	
Flaviviridae											
YFV	5-7	6-7	7-8	8-12	nd	nd	nd	nd	nd	nd	
JEV	3-4	4-5	5-6	6-10	+	–	–	–	–	+	RM i.c.+ s.c.–
MVEV	5-7	6-8	7	13	+	+	–	–	–	2	RM i.c.+ sheep i.c. +
WNV	2-5	4-5	3-5	4-9	+	+	–	–	(+)	2-4	RM, sheep i.c.(+)
USUV	5-6	6-11	6-7	–	–	–	–	–	–	–	
ITMV	4	9-11	6-8	–	–	–	–	–	+	+	Turkey i.c., i.m.(+)
TMUV	4	nd	5-8	nd	nd	nd	–	–	(+)	+	Rabbit i.p.–
WSLV	6-9	+	9-12	+	nd	–	nd	nd	nd	(+)	RM s.c.–
LIV	3-4	3-6	4-7	5-10	+	(+)	9-12	–	–	(+)	Lamb, goat i.c.+
TBEV	3-5	3-6	4-7	5-9	4-8	4-12	7-8	(–)	nd	3-7	RM, lamb i.c.+ s.c.–
OHFV	3-6	3-6	3-6	4-7	4-8	nd	6-10	nd	nd	3-4	RM i.c.+
KFDV	3-5	4-6	5-7	5-8	nd	nd	nd	nd	nd	nd	RM i.c., s.c.(+)
TYUV	3-6	4-8	3-7	–	nd	nd	(+)	–	–	nd	Rabbit i.p.–
Bunyaviridae											
NSDV	2-6	4-10	5-7	(+)	nd	nd	nd	–	nd	–	
SOLV	4-9	–	5-11	–	–	nd	5-8	nd	+	4-5	Rabbit, pigeon i.c.–
BHAV	3-5	5-6	5-8	–	–	–	5-6	–	nd	4-6	RM, lamb i.c.(+)
LACV	2	2-3	5	6	nd	nd	nd	nd	nd	nd	
SSHV	2-3	2-4	3-6	(7-10)	+	+	nd	nd	nd	2-5	Rabbit –
CVV	+	+	+	nd	nd	–	nd	nd	–	nd	Rabbit i.p., s.c.–
MDV	3	nd	4	–	nd	nd	nd	nd	–	nd	
AKAV	2-5	nd	3-6	nd	nd	nd	nd	nd	nd	+	
AINV	2	5-6	3	–	nd	nd	–	–	nd	nd	Rabbit i.v., i.c.–

Continued

Table 5.1 Experimental pathogenicity of arboviruses for laboratory animals (Hubálek & Halouzka, 1996; Karabatsos, 1985; additional sources)—cont'd

	SM	SM	M	M	H	H	GP	GP	C	CE	Other
	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	i.c.	i.p.	s.c.	y.s.	
SHUV	2	2	nd	–	nd	nd	nd	nd	nd	nd	
SBV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
RVFV	2-4	2-6	2-5	2-7	nd	nd	nd	nd	nd	(+)	RMsc –, lamb s.c.+
Reoviridae											
AHSV	2-3	2-5	3-5	(3-8)	nd	(+)	8-12	(+)	nd	3-6	Horse s.c.+
KASV	+	(+)	(+)	–	nd	nd	nd	nd	nd	–	
BTV	3-5	(+)	–	–	+	–	–	–	nd	3-6	Sheep s.c., p.o.+
EHDV	+	+	–	–	–	–	–	–	nd	–	Deer s.c.+
IBAV	3-5	nd	nd	–	nd	nd	–	–	nd	+	Sheep i.v.–
EEV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
PHSV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
YUOV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Rhabdoviridae											
BEFV	5-10	–	–	–	nd	nd	nd	nd	nd	nd	Sheep –
KOTV	11	nd	14	nd	nd	nd	nd	nd	nd	nd	
VSNJV	2-3	2-3	2-3	–	+	–	+	nd	nd	+	
VSIV	4-5	4-5	4-5	–	+	nd	4-5	nd	nd	+	
VSAV	+	+	nd	nd	nd	nd	nd	nd	nd	nd	
COCV	2-3	3-5	3-5	4-10	nd	–	nd	nd	nd	+	
Orthomyxoviridae											
THOV	3	3-4	4-8	(+)	+	3	nd	–	nd	nd	
Asfarviridae											
ASFV	nd	nd	nd	nd	nd	nd	nd	nd	nd	6-7	Pig s.c.+

Table shows the average survival time (days) of laboratory animals inoculated with various arboviruses (inocula established after several mouse passages); +, death; (+), irregular death; (–), irregular encephalitis or pareses, but survival; –, no death; nd, no data. Animals: SM, suckling mouse; M, adult mouse; H, adult Syrian hamster; GP, guinea pig; C, chick (newly hatched); CE, chick embryo (inoculated into yolk sac); RM, rhesus monkey. Inoculation mode: i.c., intracerebrally; i.p., intraperitoneally; s.c., subcutaneously.

Table 5.2 Susceptibility of cell cultures to animal-pathogenic arboviruses (David-West, 1971, 1972; Hronovský, Plaisner, & Benda, 1978; Hubálek & Halouzka, 1996; Karabatsos, 1985; Leake, Varma, & Pudney, 1977; Singh, 1972; Stim, 1969)

	CEC, DEC	BHK-21	VERO	CV-1	GMK	LLC-MK2	PS, SPEV	HeLa	XTC-2	AA	Other
Togaviridae											
EEEV	+	+	+	nd	nd	+	+	+	nd	m	
WEEV	+	+	+	nd	nd	+	+	+	nd	m	
VEEV	+	+	+	nd	nd	+	+	+	nd	+	
HJV	+	+	+	nd	nd	+	nd	nd	nd	nd	
BCRV	+	nd	+	nd	nd	nd	nd	nd	nd	nd	
SINV	+	+	+	+	+	+	+	+	+	m	
MIDV	+	+	+	nd	nd	+	nd	nd	nd	nd	
GETV	nd	+	+	nd	nd	(+)	+	nd	+	m	
SFV	+	+	+	nd	nd	+	+	+	nd	m	
Flaviviridae											
YFV	nd	+	+	+	+	+	+	nd	–	m	
JEV	+	nd	+	nd	nd	+	+	nd	–	+	
MVEV	+	nd	+	nd	nd	+	+	nd	nd	m	
WNV	+	(+)	+	+	+	+	+	(+)	+	(+)	
USUV	+	(m)	+	nd	nd	+	+	m	nd	nd	
ITMV	+	m	+	nd	nd	+	+	nd	nd	nd	
TMUV	+	nd	+	nd	nd	+	+	nd	nd	nd	
WSLV	nd	+	+	nd	nd	+	+	nd	nd	nd	
LIV	p	(+)	(p)	+	+	p	+	(+)	m	nm	
TBEV	p	(+)	(p)	+	+	p	+	(+)	m	nm	
OHFV	(+)	+	nd	+	+	nd	+	+	+	nd	
KFDV	+	+	+	+	+	+	+	+	nd	nm	MK +
TYUV	(+)	p	p	(+)	(+)	(+)	+	–	nd	–	

Continued

Table 5.2 Susceptibility of cell cultures to animal-pathogenic arboviruses (David-West, 1971, 1972; Hronovský, Plaisner, & Benda, 1978; Hubálek & Halouzka, 1996; Karabatsos, 1985; Leake, Varma, & Pudney, 1977; Singh, 1972; Stim, 1969)—cont'd

	CEC, DEC	BHK-21	VERO	CV-1	GMK	LLC-MK2	PS, SPEV	HeLa	XTC-2	AA	Other
Bunyaviridae											
NSDV	nd	+	+	nd	nd	+	nd	nd	nd	(–)	
SOLV	nd	m	–	nd	nd	–	nd	nd	+	nm	
BHAV	m	+	+	+	+	–	+	(+)	–	–	
LACV	nd	+	+	+	+	+	+	nd	+	nd	
SSHV	+	+	+	+	+	+	+	nd	+	nd	
CVV	p	+	+	nd	nd	p	nd	nd	nd	m	
MDV	p	+	+	nd	nd	nd	nd	nd	nd	m	
AKAV	nd	+	+	nd	nd	+	nd	nd	nd	nd	
AINV	nd	nd	nd	nd	nd	nd	p	nd	nd	nd	
SHUV	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	
SBV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
RVFV	+	+	+	nd	nd	nd	nd	nd	nd	nd	
Reoviridae											
AHSV	(+)	+	+	+	+	nd	nd	nd	nd	m	
KASV	nm	nd	+	nd	nd	nd	+	nd	nd	m	
BTV	+	+	+	nd	nd	nd	nd	+	+	m	
EHDV	–	+	nd	nd	+	nd	nd	+	+	m	L929 +
IBAV	+	+	nd	nd	nd	nd	nd	nd	nd	nd	BEK +
EEV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
PHSV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
YUOV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
Rhabdoviridae											
BEFV	nd	+	+	nd	nd	nd	nd	nd	nd	m	BEK +

Table 5.2 Susceptibility of cell cultures to animal-pathogenic arboviruses (David-West, 1971, 1972; Hronovský, Plaisner, & Benda, 1978; Hubálek & Halouzka, 1996; Karabatsos, 1985; Leake, Varma, & Pudney, 1977; Singh, 1972; Stim, 1969)—cont'd

	CEC, DEC	BHK-21	VERO	CV-1	GMK	LLC-MK2	PS, SPEV	HeLa	XTC-2	AA	Other
KOTV	nd	+	+	nd	nd	nd	nd	nd	nd	m	
VSNJV	+	+	+	nd	nd	+	+	+	nd	m	
VSIV	nd	+	+	nd	nd	+	nd	nd	nd	m	
VSAV	nd	+	nd	nd	nd	p	+	nd	nd	nd	
COCV	+	p	+	nd	nd	+	nd	+	nd	nd	
Orthomyxoviridae											
THOV	–	+	+	nd	nd	p	nd	nd	nd	–	LT +
Asfarviridae											
ASFV	+	+	+	nd	nd	nd	+	nd	nd	nd	LT +

Explanations: +, CPE and plaques produced; (+), faint CPE formed; p, plaques produced (under overlay); (p), indistinctive plaques produced, usually no CPE; −, neither CPE nor plaques produced (data on multiplication missing); m, multiplication without CPE/plaques production; nm, no multiplication; nd, no data; AA, *Aedes albopictus* cell line; LT, lamb testis continuous cell line; BEK, bovine embryo kidney primary cells; MK, monkey kidney primary cells.

2. FAMILY *TOGAVIRIDAE*

2.1. Eastern equine encephalitis virus

Taxonomy: genus *Alphavirus*.

History: isolated by C. Ten Broeck et al. from the brain of a horse in Delaware (USA) during an equine epidemic in 1933 (Karabatsos, 1985). Retrospectively evidence was provided that Eastern equine encephalitis virus (EEEV) was the cause of extensive epidemics in horses along the eastern coast of the United States also in the years 1831 and 1845 (Hayes, 1981; Ten Broeck, Hurst, & Traub, 1935).

Arthropod vectors: principal vector is the ornithophilic mosquito *Culiseta melanura*; also important are *Culex erraticus*, *Uranotaenia sapphirina* (feeding on amphibians and reptiles), *Aedes sollicitans*, and *Coquillettidia perturbans* (as bridge vectors, feeding on both birds and mammals), *Cx. pedroi* in Peru, and in Brazil *Aedes taeniorhynchus*, and *Cx. taeniopus* (Theiler & Downs, 1973).

Vertebrate hosts: wild birds (largely passerines) (Fig. 5.1), but probably also reptiles and amphibians, and rodents in South America.

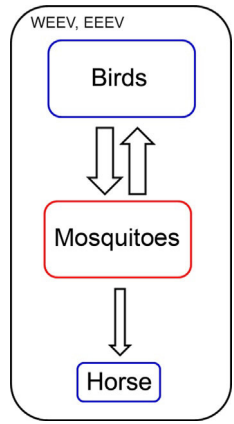

Figure 5.1 Schematic representation of the eco-epidemiological cycle of Eastern and Western equine encephalitis viruses.

Animal disease: encephalomyelitis in equids with a high mortality rate. The disease starts with fever, anorexia, and colic, then the virus attacks the CNS, leading to encephalitis and myelitis associated with abnormal behavior (staggering, imbalance, tendency to walk in circles), somnolence, paralyses, and convulsions before death. Widespread epidemics have occasionally occurred, for example, about 185,000 horses were affected in eastern USA in 1938. In the large 1947 outbreak of EEE in Louisiana, the case–mortality rate was 83% in 14,334 equine cases (Hayes, 1981). A big outbreak of EEE in horses was also observed in Braganca (North Brazil) in 1960. Major EEE epornitics involving at least 50 avian species have also been described (Kissling, Chamberlain, Sikes, & Eidson, 1954), including imported species such as pheasants, emus, or starlings (*Sturnus vulgaris*): in them the virus causes fever, ataxia, trembling, leg and generalized paralysis, and death; direct contact infections have been observed among pheasants and starlings kept captive (Hayes, 1981; Komar, Dohm, Turell, & Spielman, 1999; Tyzzer, Sellards, & Bennett, 1938). EEE is a notifiable disease (OIE, 2012).

Prevention: an EEEV vaccine for immunization of horses (and pheasants) is available (Hayes, 1981).

Human disease: a number of cases described, fatality rate high. BSL-3.

Geographic distribution: North America (mainly in the eastern part, including Canada: Quebec, Ontario, Alberta), Central America (Mexico, Panama, Cuba, Jamaica, the Dominican Republic), and South America (Guyana, Colombia, Peru, Brazil, Argentina). EEEV may be transported from the USA southward by birds migrating in autumn (Stamm & Newman, 1963).

2.2. Western equine encephalitis virus

Taxonomy: genus *Alphavirus*.

History: the virus was first isolated from the brain of an encephalomyelitic horse in California, 1930 (Meyer, Harring, & Howitt, 1931).

Arthropod vectors: mosquitoes *Culex tarsalis* (principal vector), *Culiseta inornata*, *Aedes melanimon* (TOT: Fulhorst, Hardy, Eldridge, Presser, & Reeves, 1994), *Aedes vexans*.

Vertebrate hosts: wild birds (mainly passerines such as the English sparrow *Passer domesticus*) (Fig. 5.1), ground squirrel *Citellus richardsoni*, blacktail jackrabbit *Lepus californicus*, probably also snakes and frogs (Artsob, 1981).

Animal disease: in equids fever, signs of fatigue, somnolence, incoordinated movement of the limbs, grinding of teeth, and encephalomyelitis with paralysis of the lips, inability to swallow and stand (Hayes, 1981); mortality rate (20–30%) is lower than that in EEE. The outbreaks are not as extensive as with EEE, but the 1930 WEE epidemic in the San Joaquin Valley in California involved nearly 6000 equine cases, and the mortality rate was high, about 50%. Mortality caused by WEE in birds is much lower than with EEE, and adult pheasants are resistant (Kissling, Chamberlain, Sudia, & Stamm, 1957). In Canada, WEE is the most important arboviral infection: at least 17 major WEE epidemics in horses have been documented in the country since 1935; especially large outbreaks, involving about 12,000 and 52,000 horses, occurred in Manitoba and Saskatchewan, 1937–1938, with a mortality rate of 28% (Artsob, 1981). WEE is a notifiable disease (OIE, 2012).

Vaccination of horses with a commercially available vaccine is recommended in natural foci of Western equine encephalitis virus (WEEV) (Hayes, 1981); for instance in Canada, the vaccine was introduced in 1938 and reduced the equine morbidity and mortality rapidly (Artsob, 1981).

Human disease: occasional cases described, fatality rate moderate. BSL-3.

Geographic distribution: North America (its western parts, including Canada and Mexico), and sporadically South America (Guyana, Brazil, Uruguay, Argentina). Usually in fresh water swamps ecosystem.

2.3. Venezuelan equine encephalitis virus

Taxonomy: genus *Alphavirus*. Prototype strain: donkey I Trinidad (Theiler & Downs, 1973). About six subtypes have been described, with varying antigenic structure and virulence. Closely related to Mucambo virus.

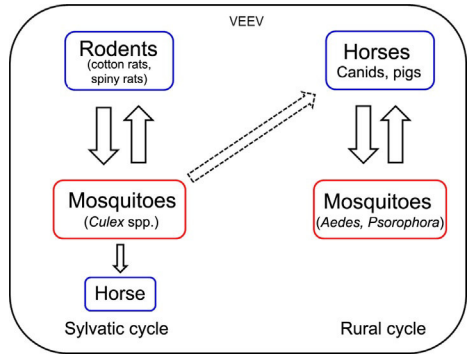

Figure 5.2 Schematic representation of the eco-epidemiological cycle of Venezuelan equine encephalitis virus.

History: first isolated from the brain of a horse during an epizootic in Venezuelan Guajira, 1938 (Kubes & Rios, 1939).

Arthropod vectors: mosquitoes of the genera *Culex*, *Mansonia*, *Anopheles*, and *Aedes*.

Vertebrate hosts: small mammals (wild rodents: *Peromyscus gossypinus*, *Sigmodon hispidus*, *Proechimys*, and *Oryzomys* spp.) (Fig. 5.2), horses (high level of viremia), canids, pigs, occasionally birds, possibly also bats (*Eptesicus fuscus*: experimental viremia).

Animal disease: in horses, either an encephalitic form (with high fever, muscle spasms, incoordination of movements, convulsions, and death) or a milder febrile illness with leucopenia, and diarrhea in some individuals; the virus is also viscerotropic, causing lymphadenitis, splenitis, and necrotic pancreatitis. In addition to equids, Venezuelan equine encephalitis virus (VEEV) causes a mild febrile disease in dogs, pigs, sheep, and goats, associated with anorexia and depression (McConnell & Spertzel, 1981).

Focal outbreaks of VEE occur periodically, but sometimes there are large regional epizootics involving hundreds of horses. For instance, a severe epidemic caused by VEEV occurred in Colombia and Venezuela in 1962 involving thousands of horses, frequently with fatal outcome (Theiler & Downs, 1973). Another large epizootic started in Peru in 1969, and reached Texas in 1971; it killed estimated 200,000 horses. VEE is a notifiable disease (OIE, 2012).

Prevention: both inactivated and attenuated (TC-83) VEEV vaccines are employed in large-scale animal immunization programs of equids in endemic areas of the Americas.

Human disease: many cases described, fatality rate relatively low. BSL-3.

Geographic distribution: South (Venezuela, Colombia, Peru, Ecuador, British Guyana, Guatemala, Argentina) and Central (Trinidad, Honduras, El Salvador, Nicaragua) America, Mexico, Texas, south Florida.

2.4. Highlands J virus

Taxonomy: genus *Alphavirus*, WEE antigenic complex. Prototype strain B-230.

History: first isolated by J.R. Henderson et al. from serum of an asymptomatic adult *Cyanocitta cristata* jay in Florida, 1960 (Karabatsos, 1985).

Arthropod vector: mosquitoes (*Cs. melanura*, *Ae. cinereus*).

Vertebrate hosts: wild birds.

Animal disease: very sporadic cause of encephalitis in horses (Florida). Death in i.m. inoculated young chickens, turkeys, and partridges, decreased egg production in adult turkeys (Karabatsos, 1985; MacLachlan & Dubovi, 2011). However, a spontaneous disease in wild or domestic birds has not yet been reported.

Human disease: unknown. BSL-2.

Geographic distribution: USA (Florida, Maryland, New Jersey, Connecticut).

2.5. Buggy Creek virus

Taxonomy: genus *Alphavirus*, WEE antigenic complex. Very closely related to North-American viruses Fort Morgan and Stone Lakes.

History: isolated from cliff swallows, their bugs, and house sparrows in central-western Oklahoma, 1991 (Hopla, Francy, Calisher, & Lazuick, 1993).

Arthropod vector: cimicid bug *Oeciacus vicarius* (a hematophagous ectoparasite of the colonially nesting cliff swallow).

Vertebrate hosts: passerine birds—cliff swallow (*Petrochelidon pyrrhonota*), house sparrow (*P. domesticus*).

Animal disease: affects fitness in house sparrows that invade cliff swallow colonies: young sparrows in the nests die (O'Brien & Brown, 2012). Infected nestlings exhibit ataxia, torticollis, paresis, and lethargy; histological examination revealed encephalitis, myositis, myocarditis, and hepatitis (O'Brien, Meteyer, Ip, Long, & Brown, 2010).

Human disease: unknown. BSL-2.

Geographic distribution: USA (Oklahoma, Nebraska).

2.6. Sindbis virus

Taxonomy: genus *Alphavirus*, WEE complex. Prototype strain EgAr-339 (*Culex univittatus*, Egypt). Synonyms and subtypes: **Babanki**, **Kyzylagach**, **Whataroa**, **Ockelbo**, and **Karelian fever viruses**.

History: originally isolated from *Cx. univittatus* mosquitoes collected in Sindbis village, Nile Delta, Egypt in 1952 (Theiler & Downs, 1973).

Arthropod vectors: mainly ornithophilic mosquitoes *Culex* spp., but also *Culiseta morsitans*, *Coquillettidia richiardii*, *Mansonia africana*, *Aedes* spp., *Anopheles hyrcanus*.

Vertebrate hosts: largely wild birds, less often rodents, bats, and amphibians.

Animal disease: a few cases of encephalitis in horses, South Africa (Venter et al., 2010).

Human disease: many cases, epidemics in Scandinavia and Karelia ("Ockelbo," "Pogosta," and "Karelian fever")—febrile illness with arthralgia (polyarthritis) and rash. BSL-2.

Geographic distribution: Africa, Israel, Asian Turkey, India, Indonesia, Australia, New Zealand (Whataroa strain), China, central Asia, Azerbaijan, Sweden, Finland, Russia, infrequently Italy (Sicily), Slovakia, and Germany.

2.7. Middelburg virus

Taxonomy: genus *Alphavirus*. Prototype strain Ar 749.

History: first isolated from *Aedes caballus* mosquitoes in Cape Province of South Africa in 1957 (Kokernot, de Meillon, Paterson, Heymann, & Smithburn, 1957).

Arthropod vectors: mosquitoes *Ae. caballus* and other *Aedes* spp., *Mansonia africana*.

Vertebrate hosts: ruminants (sheep, goat).

Animal disease: a few cases of serious horse disease, including encephalitis, were reported from southern Africa (Attoui et al., 2007; Venter et al., 2010).

Human disease: unknown. BSL-2.

Geographic distribution: South Africa, Cameroon, Kenya, Central African Republic, Senegal.

2.8. Semliki Forest virus

Taxonomy: genus *Alphavirus*.

History: first isolated from *Aedes abnormalis* group by K. C. Smithburn and A. J. Haddow in Bundiyama, Uganda, 1942 (Karabatsos, 1985; Theiler & Downs, 1973).

Arthropod vectors: mosquitoes of *Aedes abnormalis* group, *Aedes argentiopunctatus*, *Aedes togoi*.

Vertebrate hosts: wild birds, rodents, insectivores.

Animal disease: in rhesus monkeys, an intracerebral (i.c.) inoculation was followed by fever, paralysis, and death in some individuals; however, the majority of monkeys recovered (Karabatsos, 1985). The virus is abortogenic in pregnant mice inoculated at gestation day 11 (Milner & Marshall, 1984). However, spontaneous disease in wild animals has not yet been reported.

Human disease: a number of cases described; fatality rate low. BSL-3.

Geographic distribution: tropical Africa (Uganda, Cameroon, Mozambique, DR Congo, Nigeria, Central African Republic, Senegal).

2.9. Getah virus

Taxonomy: genus *Alphavirus*. Prototype strain MM2021. Synonym (or subtype): **Sagyiama virus**. Getah virus (GETV) is very closely related to, or possibly identical with, **Ross River virus** (Karabatsos, 1985).

History: first isolated from *Culex gelidus* mosquitoes near Kuala Lumpur (Malaysia), 1955 (Karabatsos, 1985). Disease in animals (horses) was first recognized in Japan, 1978 (Kamada et al., 1980).

Arthropod vectors: mosquitoes *Cx. gelidus*, *Culex tritaeniorhynchus*, *Culex fuscocephala*, *Ae. vexans nipponensis*, *Aedes nigripes*, *Aedes communis*, *Aedes excrucians*.

Vertebrate hosts: horse, pig, wild boar.

Animal disease: in horses (often racehorses), the disease is characterized by depression, anorexia, fever, nasal discharge, urticarial rash, edema of the hind limbs, swelling of the submandibular lymph nodes, and lymphocytopenia (experimentally confirmed); in pigs abortions (Shibata, Hatano, Nishimura, Suzuki, & Inaba, 1991). When pregnant mice or guinea pigs were infected, death occurred in some fetuses, associated with high titers of GETV in fetal brains and muscles (Asai, Shibata, & Uruno, 1991; Kumanomido, Kamada, Wada, Kenemaru, Sugiura, et al., 1988, Kumanomido, Wada, Kanemaru, Kamada, Akiyama, et al., 1988; Kumanomido, Wada, Kanemaru, Kamada, Hirasawa, et al., 1988). Outbreaks of GETV infection were first recorded in racehorses at two training centers in Japan, 1978. Since then, several

outbreaks of the disease have been reported in Japan especially at horse race tracks (e.g., 1991–1997), and one was reported from India in 1990 (Brown & Timoney, 1998).

Human disease: unknown; the very closely related Ross River virus causes epidemic polyarthritis in Australia. BSL-2.

Geographic distribution: Malaysia, Australia, Vietnam, Cambodia, Sri Lanka, India, Korea, China, Mongolia, eastern Siberia, Japan, Philippines. GETV occurs surprisingly in very diverse ecosystems from the tropics to the northern tundra.

3. FAMILY *FLAVIVIRIDAE*

3.1. Yellow fever virus

Taxonomy: genus *Flavivirus*. Prototype strain "Asibi" was isolated from a febrile man in Ghana, 1927 (Theiler & Downs, 1973).

History: YF has been known from the early 1900s and even before (e.g., outbreaks occurring in Cuba investigated by the Walter Reed Yellow Fever Commission around 1900).

Arthropod vectors: mosquitoes, largely *Aedes aegypti* (TOT demonstrated), *Ae. simpsonii*, *Ae. fucifer-taylori*, *Ae. africanus* in Africa, while *Haemagogus spegazzinii* and other *Haemagogus* spp. in the South-American jungle YF.

Vertebrate hosts: primates.

Animal disease: fatal disease (epizootics) among some species of monkeys (*Alouatta* spp.) in South-American tropical forests; the infected monkeys show necrotic lesions in liver and kidneys (similar as observed in humans). Interestingly, African monkey species are resistant.

Human disease: a great number of cases, fatality rate high. BSL-3.

Geographic distribution: tropical Africa and South America, occasionally Central America (1948–1959).

3.2. Japanese encephalitis virus

Taxonomy: Japanese encephalitis antigenic group, genus *Flavivirus*.

History: isolated by T. Mitamura, M. Kitaoka et al. from human brain in Tokyo, 1935 (Karabatsos, 1985).

Arthropod vectors: *Cx. tritaeniorhynchus* (primary vector), *Cx. vishnui* (India), *Cx. gelidus* (Indonesia). TOT demonstrated in *Aedes albopictus*.

Vertebrate hosts: colonial waterbirds—herons and egrets (*Nycticorax nycticorax*, *Egretta garzetta*), also other birds, and pigs (domestic, feral) serving

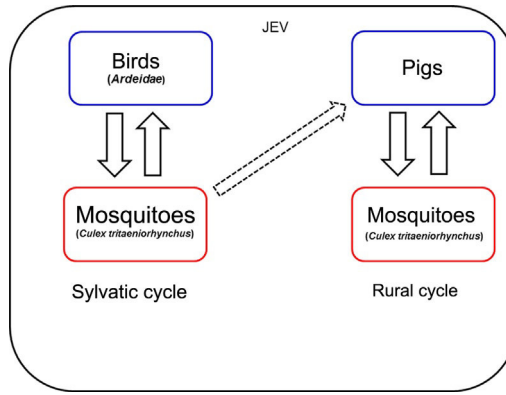

Figure 5.3 Schematic representation of the eco-epidemiological cycle of Japanese encephalitis virus.

as amplifying hosts (Fig. 5.3), possibly also orang-utans (*Pongo pygmaeus*) in Borneo. Bats and fruit bats may also serve as amplifying hosts of Japanese encephalitis virus (JEV) (a long viremia demonstrated experimentally: Mackenzie, David, Williams, & Smith, 2007).

Animal disease: abortions in pigs. Infected pregnant sows can produce mummified fetuses or give birth to stillborn or weak piglets, including such with hydrocephalus; JEV is also associated with infertility in boars, and total reproductive losses in pigs may reach 50% or more. Sporadical encephalitis in horses (with about 5% mortality rate). Horses infected by JEV may develop severe encephalitis, although inapparent infections are more common (Ellis, Daniels, & Banks, 2000; Mackenzie et al., 2007). JE is a notifiable disease (OIE, 2012).

Prevention: JE vaccine is used for immunization of horses in some countries.

Human disease: a great number of cases, fatality rate moderate. BSL-3.

Geographic distribution: Japan, Russian Far East, Korea, China, Taiwan, Thailand, Vietnam, Cambodia, Laos, Malaysia, Indonesia, Papua New Guinea, northern Australia, Guam, Philippines, India—including Nepal, Sri Lanka (Mackenzie et al., 2007).

3.3. Murray Valley encephalitis virus

Taxonomy: Japanese encephalitis antigenic group, genus *Flavivirus*. Synonym: **Alfuy virus**.

History: initially associated with outbreaks of human encephalitis which was given the name "Australian X disease" on the east coast of

Australia in the early twentieth century. After a long period without outbreaks, this epidemic encephalitis reappeared in the Murray Darling River basin in 1951, and again in 1974. Murray Valley encephalitis virus (MVEV) was isolated from human brain by E.L. French during the 1951 epidemic (Karabatsos, 1985; Mackenzie et al., 1994; Theiler & Downs, 1973).

Arthropod vectors: mosquitoes (*Culex annulirostris* and other *Culex* spp.).

Vertebrate hosts: waterbirds, mainly egrets (viremia demonstrated in them).

Animal disease: sporadically fatal encephalitis in horse (Holmes, Gilkerson, El Hage, Slocombe, & Muurlink, 2012), sheep, and monkey (Karabatsos, 1985).

Human disease: a great number of cases, fatality rate moderate. BSL-3.

Geographic distribution: Australia, Papua New Guinea.

3.4. West Nile virus

Taxonomy: Japanese encephalitis antigenic group, genus *Flavivirus*. Several genomic lineages of West Nile virus (WNV) exist, medically most important are the lineages 1 and 2. Prototype strain B-956 belongs to lineage 2, while the Egyptian topotype Eg-101 (child, Egypt, 1950) to lineage 1. The Australian **Kunjin virus** is not a distinct virus species but constitutes lineage 1b of WNV.

History: WNV was originally isolated from the blood of a febrile woman in the West Nile district of Uganda, 1937, later from a child in Egypt, 1950 (Theiler & Downs, 1973).

Arthropod vectors: WNV was isolated mainly from ornithophilic mosquitoes *Culex pipiens* (TOT), *Culex salinarius* (TOT), *Culex modestus*, and *Cq. richiardii*; occasional vectors are *Aedes triseriatus*, *Aedes cantans*, and *Anopheles maculipennis* group (Hubálek, 2000; Murgue, Zeller, & Deubel, 2002), while certain *Hyalomma*, *Argas*, and *Ornithodoros* ticks could play an alternative role as vectors in dry ecosystems of southern Russia (Lvov, Klimenko, & Gaidamovich, 1989).

Vertebrate hosts: wild birds (Fig. 5.4), occasionally certain mammals (Root, 2013), for example, tree squirrels and chipmunks in North America (Padgett et al., 2007; Platt et al., 2007). High- and/or long-term viremia was documented in experimentally infected birds of many species (Komar et al., 2003; Ziegler et al., 2013). WNV persisted in the organs (liver, spleen, CNS) of domestic pigeons for at least 20 days (Semenov, Chunikhin, Karmysheva, & Yakovleva, 1973). Persistent infections of house sparrows

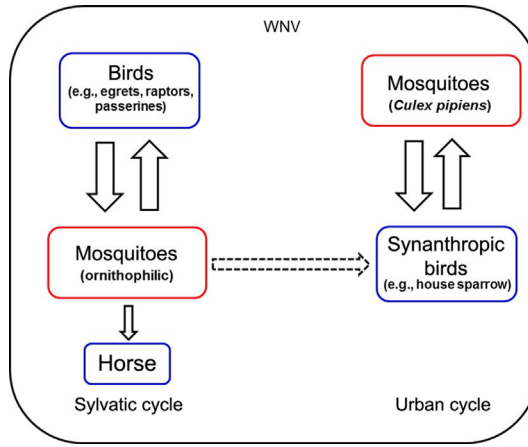

Figure 5.4 Schematic representation of the eco-epidemiological cycle of West Nile virus.

with WNV were also documented (Wheeler, Vineyard, Woods, & Reisen, 2012). Occasional hosts may be amphibians.

Animal disease: West Nile disease (WND) is a febrile illness and (polio) encephalomyelitis in equids, with ataxia, pareses, paralyses (even tetraplegia), and mortality rate up to 25% (Castillo-Olivares & Wood, 2004; Guillon, Oudar, Joubert, & Hannoun, 1968; Oudar, Joubert, Lapras, Hannoun, & Guillon, 1972; Panthier et al., 1966, Schmidt & Mansoury, 1963; Tee, Horadagoda, & Mogg, 2012), often occurring in epizootics (Egypt, Morocco, France, Portugal, Italy, North America). Camelids may also be attacked (alpaca: Dunkel et al., 2004), less frequently dogs (Cannon et al., 2006). Fatal systemic disease in birds such as corvids (markedly susceptible is the American crow, *Corvus brachyrhynchos*), birds of prey, and some other avian groups has been observed in North America, where a highly virulent WNV strain has caused widespread devastating epornitics (Fitzgerald et al., 2003; Foppa, Beard, & Mendenhall, 2011; Komar et al., 2003, Nemeth, Gould, Bowen, & Komar, 2006; Nemeth et al., 2011). WNV sometimes causes clinically manifest disease in domestic geese, feral pigeons, raptors, and other free-living birds with occasional deaths also in the Old World (Bakonyi et al., 2013, 2006; Joyner et al., 2006; Taylor, Work, Hurlbut, & Rizk, 1956). For instance, Mediterranean WNV strains are highly pathogenic for *Alectoris rufa* partridges (Sotelo et al., 2011). Recently, a lineage 2 WNV strain emerged in Hungary (Bakonyi et al., 2006) and dispersed quickly (Bakonyi et al., 2013). The most vulnerable species of birds for this virus strain is the goshawk (*Accipiter*

gentilis), which regularly develops neuroinvasive disease, usually with fatal outcome (Bakonyi et al., 2013; Wodak et al., 2011); other species of birds of prey such as falcons are also highly susceptible. The symptoms of WND in birds include depression, ataxia, paralysis, myocarditis, and multiorgan inflammation with necrosis, for example, necrotic hepatitis, splenitis, pancreatitis.

U.S. reported equine and avian WND cases (CDC data):

Year	1999	2000	2001	2002	2003	2004	2005	2006
Horses	25	63	738	9157	4146	1341	1072	1086
Birds	295	4323	7333	14,122	11,350	7074	5204	4106

Even recently (2012), more than 400 equine cases were recorded in the United States.

European equine WND cases (Hubálek, 2000; ECDC data) have occurred in France (Camargue, 1962–1965: more than 50 horses with encephalitis; 2000: 131 horse cases—21 died; Var, 2003: 4 horses; Camargue, 2004: 18 animals), southern Portugal (an epizootic before 1970; 2010, Lisbon: 1 horse, euthanized); Spain (Andalusia, 2010: 41 horses, 10 died), Italy—Toscana, 1998: encephalomyelitis in 14 horses—6 died, and from the CNS of one animal a WNV lineage 1 strain was isolated, which was very similar to strain ArD 93548 recovered from *Culex neavei* mosquitoes in Senegal, 1993; Emilia-Romagna, 2008: 33 horses (5 died); 2009: 26 horses; Sicily (2010: 4 horses); Veneto (2010: 8 horses), Albania (2010: 1 case), northern Greece (2010: 32 equine cases; 2011: 6 cases), Hungary (2007: 30–40 horses died due to a WNV lineage 2 strain; 2008 and 2009: several cases in horses). Total reported WNV equine cases in Europe, 2010: 166 (11.4% fatal); 2011: 84 (Italy including Sardinia 63, Greece 20, Spain 1). Three equine cases were reported in Croatia, 2012. WND is a notifiable disease (OIE, 2012).

Prevention: commercial vaccines based on WNV lineage 1 are licensed for horses in the United States and in Europe; crossprotection for WNV lineage 2 strains was demonstrated (Minke et al., 2011); they also can be used for immunization of pets and endangered bird species (Boyce et al., 2011; Wheeler et al., 2011).

Human disease: a great number of cases, fatality rate moderate. BSL-3.

Geographic distribution: the most widespread flavivirus, distributed throughout Africa (including Madagascar), Asia, Europe, and Australia

(Kunjin virus); since 1999, WNV lineage 1 is also present in the Americas: in North America, it spread from the East to the West coast within 4 years after its introduction; WNV then dispersed also to Central and South America (up to Argentina in the south). In south-western Europe (southern France, Spain, Portugal) only sporadic cases of WND have been reported so far (all lineage 1), while in central and south-eastern Europe since 2008 WNV lineage 2 (Bakonyi et al., 2006) has been spreading quickly causing cases and outbreaks in Hungary and eastern Austria since 2008 (Bakonyi et al., 2013; Wodak et al., 2011), Greece since 2010 (Papa et al., 2011), Italy and in several Balkan states, 2012. In northern Italy, a lineage 1 strain has caused outbreaks since 2008 (Savini, Monaco, Calistri, & Lelli, 2008), and in southern Russia (wider Volgograd region), a different lineage 2 strain is responsible for widespread WND since 2007 and 2010, respectively; this strain caused also an outbreak in Romania in 2010 (Sirbu et al., 2011). Natural foci of WNV infections are situated usually in wetland ecosystems; revealing principally an avian–mosquito cycle. Migratory birds play a role in the widespread geographic distribution of WNV (Owen et al., 2006; Rappole, Derrickson, & Hubálek, 2000).

3.5. Usutu virus

Taxonomy: Japanese encephalitis antigenic group, genus *Flavivirus*.

History: The virus was first isolated by B.M. McIntosh from *Cx. neavei* in South Africa, 1959 (prototype strain SAAr 1776: Karabatsos, 1985).

Arthropod vectors: largely ornithophilic mosquitoes *Culex* spp. (*Cx. univittatus*, *Cx. perfuscus*), *Coquillettidia aurites*, *Mansonia africana*. In Austria and Italy, RNA of Usutu virus (USUV) was detected in *Cx. pipiens*, *Cx. hortensis*, *Cx. territans*, *Culiseta annulata*, *Ae. vexans*, and *Ae. rossicus* (Weissenböck, Chvala-Mannsberger, Bakonyi, & Nowotny, 2007).

Vertebrate hosts: birds.

Animal disease: highly pathogenic for certain passeriform birds (especially birds of the genus *Turdus*) and birds of prey, causing apathy, inability to fly, incoordination, with encephalitis, carditis, hepato- and splenomegaly, and death; mortality rate in blackbirds is very high, up to 100%.

USUV emerged in Austria in 2001, killing hundreds of wild birds (predominantly blackbirds, *Turdus merula*) in and around Vienna, but also some birds in aviaries (Weissenböck et al., 2002; 2003; 2007; Chvala et al., 2007; Bakonyi, Gould, Kolodziejek, Weissenböck, & Nowotny, 2004);

subsequently, USUV-associated bird (mostly blackbird) die-off was also reported from Hungary (Bakonyi et al., 2007), Switzerland (Steinmetz et al., 2011), Italy (Manarolla et al., 2010), Germany (Becker et al., 2012; Jost et al., 2011), and Czechland (Hubálek et al., 2012). However, retrospective studies showed that USUV emerged in Europe already before 2001 as it was detected in histological samples prepared from seven blackbirds that died in Tuscany region of northern Italy in 1996 (Weissenböck, Bakonyi, Rossi, Mani, & Nowotny, 2013).

Human disease: two cases in immunosuppressed individuals, BSL-2.

Geographic distribution: Africa (Morocco, Senegal, Central African Republic, Nigeria, Uganda, South Africa, Burkina Faso, Cote d'Ivoire; Nikolay, Diallo, Boye, & Sall, 2011), Austria, Hungary, Switzerland, Italy, Germany, Czechland, Spain (in the latter country probably a different virus strain, not yet associated with avian mortality)—mainly in the European lowland river valley ecosystem.

3.6. Israel turkey meningoencephalitis virus

Taxonomy: Ntaya antigenic group, genus *Flavivirus*. Synonym (or subtype): **Bagaza virus** (BAGV, prototype strain DakArB 209).

History: first isolated during an epizootic of meningoencephalitis of turkeys in Israel, 1959 (Komarov & Kalmar, 1960). BAGV was isolated by J.P. Digoutte and F.X. Pajot from *Culex* mosquitoes collected at Bagaza, Central African Republic in 1966 (Karabatsos, 1985).

Arthropod vectors: *Culex* spp. (*Cx. poicilipes*, *Cx. neavei*, *Cx. perfuscus*, *Cx. guiarti*, *Cx. thalassius*, and other species), *Aedes* spp., and possibly also biting midges *Culicoides* spp.

Vertebrate hosts: birds.

Animal disease: a fatal disease of adult turkeys (progressive paralysis associated with meningoencephalitis, mortality rate about 50%) and some other galliform birds such as wild red-legged partridge *A. rufa* (mortality rate 38%) and pheasant *Phasianus colchicus* (mortality rate 8%); wood pigeons (*Columba palumbus*) were less often affected during an BAGV epornitic in southern Spain, 2010 (Gamino et al., 2012). Clinical signs in partridges included incoordination, disorientation, ataxia, and histopathology showed (meningo)encephalitis, carditis, and severe hemosiderosis in the liver and spleen (Aguero et al., 2011; García-Bocanegra et al., 2013; Gamino et al., 2012). Domestic chickens, ducks, and pigeons are resistant.

Human disease: unknown. BSL-2.

Geographic distribution: Israel, South Africa. BAGV: Central African Republic, Cameroon, Mauritania, Senegal, India, and Spain (2010).

3.7. Tembusu virus

Taxonomy: Ntaya antigenic group, genus *Flavivirus*. Synonym: **duck egg-drop syndrome virus** (DEDSV) also called **Baiyangdian virus** (BYDV: Su et al., 2011; Tang et al., 2012); very closely related is also **Sitiawan virus** (STWV: Kono et al., 2000; Liu, Chen, et al., 2012; Liu, Lu, et al., 2012). Tembusu virus (TMUV) is less closely related to Israel turkey meningoencephalitis virus (ITMV).

History: TMUV prototype strain MM 1775 was isolated from *Cx. tritaeniorhynchus* in Kuala Lumpur (Malaysia), 1955 (Karabatsos, 1985; Theiler & Downs, 1973). STWV was first isolated from diseased chickens in Malaysia, 1999 (Kono et al., 2000), and BYDV/DEDSV during outbreaks occurring on duck farms in eastern and southern China, 2010 (Su et al., 2011; Tang et al., 2012).

Arthropod vectors: mosquitoes (mainly *Culex* spp.).

Vertebrate hosts: birds (ducks, chickens).

Animal disease: duck (and chick) egg-drop syndrome—a sudden decline of feed uptake, diarrhea, an uncoordinated gait, accompanied by hemorrhagic ovary, and a marked decrease in egg production; mortality rate 5–15% (Liu, Chen, et al., 2012; Liu, Lu, et al., 2012; Su et al., 2011). The disease has caused heavy economical losses in many Chinese farms. STWV causes encephalitis, torticollis, imbalance, depression, and growth retardation in chicks (Kono et al., 2000).

Human disease: unknown. BSL-2.

Geographic distribution: Malaysia, Thailand, Indonesia, China.

3.8. Wesselsbron virus

Taxonomy: genus *Flavivirus*. Prototype strain SA H-177 (Theiler & Downs, 1973).

History: the virus was isolated by K.C. Smithburn and B. de Meillon from the blood of a febrile man in the South African town of Wesselsbron and by K.E. Weiss et al. from a dead lamb during the same outbreak in 1955 (Karabatsos, 1985; Weiss, Haig, & Alexander, 1956).

Arthropod vectors: mosquitoes *Aedes caballus, Ae. circumluteolus, Ae. lineatopennis*.

Vertebrate hosts: probably rodents (Cape short-eared gerbil *Desmodillus auricularis*).

Animal disease: "Wesselsbron disease" in sheep, clinically similar to Rift Valley fever, with fever, weakness, anorexia, abortions in pregnant ewes (and 20% mortality in pregnant ewes), and death of lambs, congenital arthrogryposis-hydranencephaly syndrome (CAHS) (Coetzer & Barnard, 1977). Sheep show histopathological lesions: hepatitis, hemorrhagic, and degenerative changes in kidney and heart. Wesselsbron virus (WSLV) causes less severe disease (fever) in goat, cattle, and pig (Coetzer & Theodoridis, 1982), and it was detected as a cause of neurological disease in two horses, South Africa (Venter et al., 2010).

Human disease: occasional cases. BSL-3.

Geographic distribution: South Africa, Botswana, Zimbabwe, Uganda, Cameroon, Mozambique, Uganda, Central African Republic, Senegal, Nigeria, DR Congo, Madagascar, Thailand.

3.9. Louping ill virus

Taxonomy: genus *Flavivirus* (ecogroup tick-borne flaviviruses). Synonym: **Negishi virus**. Prototype strain of louping ill virus (LIV) is LI-31. LIV is very closely related to tick-borne encephalitis virus (TBEV), in fact indistinguishable from it not only by conventional serological and crossprotection tests but also by nucleotide sequence homology of E gene. For instance, LIV is antigenically and genomically much closer to the western subtype of TBEV (CEEV) than the latter is related to the eastern subtype of TBEV (RSSEV); thus, the inclusion of LIV as another subtype of TBEV rather than its classification as a separate virus species has been suggested (Grard et al., 2007, Hubálek, Pow, Reid, & Hussain, 1995).

History: louping ill has been for a very long time recognized as a disease of sheep in Scotland; for example, it was recorded in the 1795 Statistical Account, or by Walter Scott in 1891 (Davidson, Williams, & MacLeod, 1991). The virus was first isolated from sheep brain in Selkirkshire, Scotland, in 1929 (prototype strain Moredun LI-31: Pool, Brownlee, & Wilson, 1930).

Arthropod vectors: principal vector of LIV is the tick *Ixodes ricinus* (MacLeod & Gordon, 1932); LIV is also transmissible by goat and sheep milk (Reid, Buxton, Pow, & Finlayson, 1984; Reid & Pow, 1985), similarly to the other TBEV subtypes.

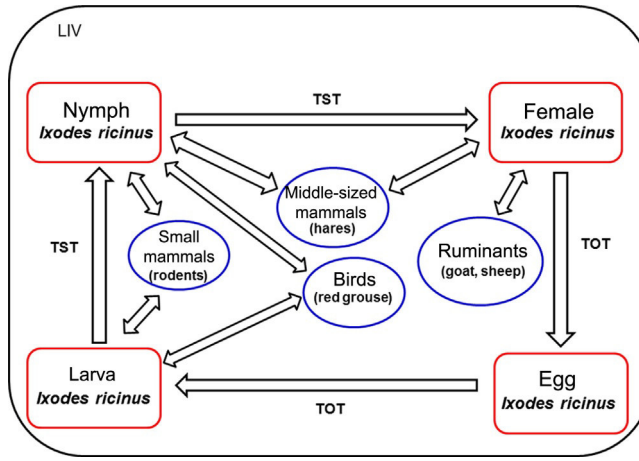

Figure 5.5 Schematic representation of the eco-epidemiological cycle of louping ill virus.

Vertebrate hosts: yellow-necked mouse (*Apodemus sylvaticus*), common shrew (*Sorex araneus*), mountain hare (*Lepus timidus*), sheep, and red grouse *Lagopus lagopus scoticus* (Fig. 5.5) (Buxton & Reid, 1975; Reid, 1990; Reid & Boyce, 1974; Reid, Duncan, Phillips, Moss, & Watson, 1978).

Animal disease: Typical course of LI in sheep is biphasic, with fever and weakness, followed by meningoencephalitis with cerebellar ataxia, generalized tremor, jumping, vigorous kicking, salivation, champing of jaws, progressing to paralysis, coma, and death; mortality rate is 40–60% (Smith & Varma, 1981). LI occasionally affects also cattle, pig (piglets), goat (kids), horse, dog, hare, lama (Macaldowie, Patterson, Nettleton, Low, & Buxton, 2005), and red grouse (with a mortality rate of 70–80% especially in juvenile birds: Buxton & Reid, 1975; Reid & Boyce, 1974; Reid et al., 1978; Reid, Moss, Pow, & Buxton, 1980); interestingly, juvenile grouse die when they eat (through foraging or grooming) infected ticks (Gilbert et al., 2004). Histopathology (sheep, pig, rhesus monkey, or mouse) shows diffuse meningoencephalitis with perivascular cuffing, neuronal degeneration, and destruction of Purkinje cells, similar to TBE (Reid, 1990). Concurrent tick-borne fever (*Anaplasma phagocytophilum* infection) and external stress enhance the course of disease (Reid, 1990). Experimental infection with LIV is fatal to suckling rat (i.c., i.p.), lamb (i.c., not s.c.), sometimes rhesus monkey (i.c., i.n.); Zlotnik, Grant, & Carter, 1976). No symptoms are seen in adult *Microtus agrestis* (i.c., s.c.), *Cervus elaphus* (s.c.), and *Capreolus capreolus* (s.c.), although meningoencephalitis was demonstrated histologically in deer (Reid, Buxton, Pow, & Finlayson, 1982).

Prevention: control of LI is mainly based on vaccination of sheep; an inactivated LIV vaccine is commercially available and in general use. Tick control by dipping the sheep with residual acaricides is also practicised. The methods of environmental control of ticks such as pasture rotation, cutting or burning grass and bush vegetation, and drainage are effective but economically less feasible (Smith & Varma, 1981).

Human disease: a total of about 30 cases (one fatality). BSL-3.

Geographic distribution: British Islands. Norway is the only country of continental Europe where a typical LIV strain was isolated (Gao et al., 1993). Natural foci of LI are "boskematic" (pastoral: Rosický, 1959) characterized by rough, poorly drained hill pastures, heather moorlands with bracken and moor-grass; principally a sheep-tick or sheep-tick-grouse cycle (Reid, 1990; Smith & Varma, 1981).

3.10. Tick-borne encephalitis virus

Taxonomy: genus *Flavivirus* (ecogroup tick-borne flaviviruses). There are three recognized TBEV subtypes: (1) Western or European subtype (TBEV-W), also called Central European (CEEV: topotype strains are Hypr and Neudoerfl) or sometimes "ricinus" subtype (Calisher, 1988; Gritsun, Nuttall, & Gould, 2003; Lindquist & Vapalahti, 2008)—varieties of this subtype are Spanish sheep encephalitis, Turkish sheep encephalitis, and Greek goat encephalitis ("Vergina") viruses (Hubálek et al., 1995); (2) (Ural-)Siberian subtype (TBEV-S: the prototype strains are Aina and Vasilchenko), sometimes called "persulcatus" subtype, causing Russian spring-summer encephalitis; and (3) Far-Eastern subtype (TBEV-FE with prototype strain Sofyin, isolated from human brain in Khabarovsk, 1937). A taxonomic and nomenclatural confusion around TBEV has repeatedly been emphasized (Calisher, 1988; Stephenson, 1989). In addition, TBEV is very closely related to LIV, which, to our opinion, should be regarded in fact as the fourth (or, historically, the first?) subtype of TBEV (see Section 3.9).

History: RSSEV subtype of TBEV was first isolated in the Russian Ural mountains in 1938 (Chumakov & Zeitlenok, 1939), and CEEV (strain "256") from *I. ricinus* ticks collected near Minsk, Belarus, in 1940 (Votyakov, Protas, & Zhdanov, 1978).

Arthropod vectors: principal vectors are the ticks *I. ricinus* (LIV, CEEV) and *I. persulcatus* (RSSEV)—TOT was demonstrated.

Vertebrate hosts: forest rodents (*Apodemus* spp., *Myodes* spp.) and insectivores (*Talpa europaea*, *Sorex araneus*, *Erinaceus concolor*); probably also

certain bird species. Some rodent species (e.g., red vole *Myodes rutilus*: Bakhvalova, Potapova, Panova, & Morozova, 2009) can serve as reservoir hosts due to vertical transmission of TBEV from infected mother to offspring (TOT).

Animal disease: TBEV infection is usually subclinical in adult ruminants; goats, sheep, and cows excrete virus in the milk (Grešíková, 1958; Smorodincev et al., 1953; van Tongeren, 1955). Encephalitis with ataxia, jumping, tremor, and convulsions can affect lambs, kids, or dogs (Klimes et al., 2001; Pfeffer & Dobler, 2011; Tipold, Fatzer, & Holzmann, 1993; Weissenböck, Suchy, & Holzmann, 1998). Horses are susceptible to TBEV infection (Rushton et al., 2013) but they rarely develop clinical (CNS) disease (Luckschander, Kölbl, Enzesberger, Zipko, & Thalhammer, 1999; Waldvogel, Matile, Wegmann, Wyler, & Kunz, 1981). TBEV (especially TBE-S and TBE-FE virus subtypes) occasionally kills birds of certain species, for example, *Carduelis flammea*, *Passer domesticus*, and *Fulica atra* (Naumov & Gutova, 1979; van Tongeren, 1962). The diffuse meningoencephalitis in mammals is characterized by perivascular infiltration, neuronal degeneration and necrosis, and focal glial proliferation.

Human disease: a great number of cases. BSL-3 (BSL-4 for RSSEV).

Geographic distribution: many European countries (especially those in central and eastern Europe), Turkey, Asian Russia (Siberia, Far East), Kazakhstan, Kirghizia, Armenia, Azerbaijan, north-eastern China, Japan, and Korean peninsula. Natural foci of TBE have been classified (Rosický, 1959) according to main mammal hosts of adult ixodid ticks as "theriodic" (situated in deciduous and mixed forest ecosystems, often game preserves), "boskematic" (pastoral), mixed "theriodic-boskematic," and "mountain."

3.11. Omsk hemorrhagic fever virus

Taxonomy: genus *Flavivirus* (ecogroup tick-borne flaviviruses).

History: first isolated by M.P. Chumakov et al. from a febrile boy with hemorrhagic syndrome during an epidemic in Omsk and Novosibirsk regions, Siberia (Russia), 1946–1947 (Karabatsos, 1985). Human cases of OHF have been observed in the area since 1941 (Růžek, Yakimenko, Karan, & Tkachev, 2010).

Arthropod vectors: *Dermacentor reticulatus* tick (TOT demonstrated), but in the steppe habitats *Dermacentor marginatus*, and probably also *Ixodes apronophorus* in wetland areas; mosquitoes could participate in mechanical transmission of the virus (a few isolates of Omsk hemorrhagic fever virus (OHFV) were recovered from them, but their role in natural foci

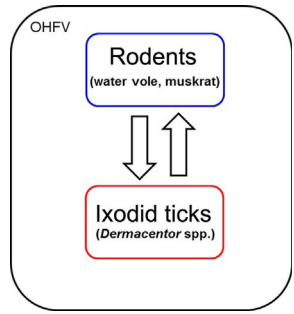

Figure 5.6 Schematic representation of the eco-epidemiological cycle of Omsk hemorrhagic fever virus.

is probably minor). However, OHFV can often be transmitted by direct contact among infected animals (their bodily fluids)—for example, muskrats; moreover, the virus can survive in lake water for at least 2 weeks in summer and for 3 months in winter (Růžek et al., 2010).

Vertebrate hosts: rodents (water vole *Arvicola terrestris*, nonnative muskrat *Ondatra zibethicus* introduced from Canada in the 1930s, *Microtus gregalis*, *M. oeconomus*) (Fig. 5.6); possibly also some amphibians (several OHFV isolates were recovered from frogs).

Animal disease: hemorrhagic fever (it can last 3 weeks), encephalitis and death in wild muskrats (fatality rate up to 80%). The virus is shed with urine, feces, and blood of the infected host. Some birds of prey (Lvov, 1988) can also reveal clinical symptoms and lethality (*Circus aeruginosus*, *Falco tinnunculus*, *Asio otus*), as well as rooks (*Corvus frugilegus*).

Human disease: at least 200 cases in total. BSL-4.

Geographic distribution: Siberia (Omsk, Novosibirsk, Tyumen and Kurgan regions).

3.12. Kyasanur Forest disease virus

Taxonomy: genus *Flavivirus* (ecogroup tick-borne flaviviruses). Kyasanur Forest disease virus (KFDV) is moderately related to OHFV. **Alkhumra virus** (ALKV), sometimes named or spelled as Alkhurma virus, is a subtype of KFDV (Charrel, Zaki, & Attoui, 2001). Prototype strain of KFDV is P-9605 (human, India).

History: first KFDV strains were isolated from an ill man, monkeys, and *Haemaphysalis* ticks during a surprising outbreak in tropical Kyasanur Forest near Baragi (Shimoga district, Karnataka—then Mysore—state) in India,

1957 (Work, Rodriguez, & Bhatt, 1959). ALKV emerged in Saudi Arabia in 1995 (Zaki, 1997).

Arthropod vectors: *Haemaphysalis spinigera* (TOT), *H. turturis*, and other tick species (Pattnaik, 2006). ALKV was detected in *Ornithodoros savignyi* and *Hyalomma dromedarii* ticks (Memish, Charrel, Zaki, & Fagbo, 2010), also suspected of mosquito transmission (especially in ALKV, but no hard data present), and observed to replicate in C6/36 mosquito (*Ae. albopictus*) cells (Madani et al., 2012).

Vertebrate hosts: rodents (rats *Rattus blanfordi* and *R. rattus*, squirrels *Funambulus tristriatus* and *Petaurista petaurista*), shrew *Suncus murinus*; possibly also bats (four isolates from *Rhinolophus rouxi*, frugivorous bat *Cynopterus sphinx*); monkeys may also carry the virus (Fig. 5.7).

Animal disease: an outbreak of fatal disease in a large number of monkeys (black-faced langur *Presbytis entellus* and red-faced bonnet macaques *Macaca radiata*) in the Shimoga district of Mysore state in 1957 (Theiler & Downs, 1973). The monkeys were weak, febrile, thirsty, unable to walk or climb, prostrate. Histopathologically, focal hemorrhages, focal necrosis in parenchymatous organs, tubular necroses, gastrointestinal, and lymphoid lesions were demonstrated, while only restricted pathologic lesions appeared in the CNS (Webb & Chatterjea, 1962). Main epizootics occurred in the years 1964–1966, 1969–1973, 1975, 1982, and 2001–2004. For instance, in the period 1964–1973, 1046 monkeys died in the Kyasanur Forest, and KFDV was isolated from 118 *P. entellus* and 13 *M. radiata*

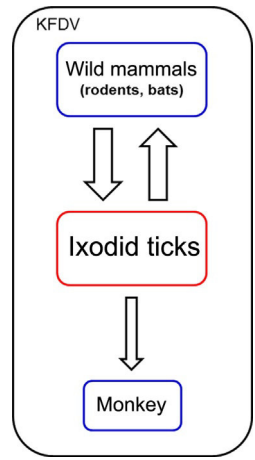

Figure 5.7 Schematic representation of the eco-epidemiological cycle of Kyasanur Forest disease virus.

(Pattnaik, 2006); deaths of 8 *M. radiata* and 2 *P. entellus* were recorded in the same Indian state in 2012.

Prevention: formalin-inactivated KFDV vaccine produced in chicken-embryo fibroblast cells is available.

Human disease: a great number of cases. BSL-4.

Geographic distribution: India (Karnataka state)—rain forest ecosystem, China (Wang et al., 2009), Saudi Arabia (ALKV: Zaki, 1997).

3.13. Tyuleniy virus

Taxonomy: Tyuleniy antigenic group, genus *Flavivirus* (ecogroup tick-borne flaviviruses).

Related to the Australian Saumarez Reef virus by complement fixation test (CFT), virus neutralization test (VNT), and nucleotide sequence of the envelope gene, while less similar to TBEV by CFT and hemagglutination inhibition test (HIT).

History: the virus was first isolated from *Ixodes uriae* collected in nesting grounds of *Uria aalge* on Tyuleniy Island near Sakhalin, Sea of Okhotsk (Asian Russia) in 1969 (Lvov et al., 1971), and simultaneously off the western U.S. coast (Clifford, Yunker, Thomas, Easton, & Corwin, 1971).

Arthropod vectors: principal vector is *I. uriae* (TST, TOT). Mosquitoes (*Ae. communis*, *Ae. punctor*, *Ae. excrucians*) may possibly act as secondary vectors; experimental Tyuleniy virus (TYUV) transmissions by *Ae. aegypti*, *Cx. pipiens*, and *Hyalomma asiaticum* was reported (Lvov, Kostyrko, & Gromashevski, 1973, Lvov et al., 1971).

Vertebrate hosts: seabirds (*U. aalge*, *Eudyptula minor*) and the suslik *Citellus undulatus*.

Animal disease: natural animal disease is unknown, but experimentally inoculated (i.c. or s.c.) birds *Rissa tridactyla*, *Larus argentatus*, and *Uria lomvia* show clinical symptoms: encephalitis with pareses and occasional death (Berezina, Smirnov, & Zelenskiy, 1974). Febrile illness with adynamia and anorexia was observed in rhesus monkeys infected aerogenically.

Human disease: three cases in biologists visiting seabird colonies. BSL-2.

Geographic distribution: TYUV occurs in Asian Russia (Far East—Sea of Okhotsk); coastal West USA (Oregon) and Canada, northern Norway and European Russia (Murmansk, Kola). Migratory seabirds play a role in the exchange of TYUV complex flaviviruses between the northern and southern hemispheres (Lvov & Ilyichev, 1979). Natural foci of TYUV are seabird colonies on steep rocks.

4. FAMILY *BUNYAVIRIDAE*

4.1. Nairobi sheep disease virus

Taxonomy: genus *Nairovirus*. Synonym (or Indian subtype): **Ganjam virus** (GANV).

History: first isolated by R.E. Montgomery from a sheep with acute gastroenteritis in Nairobi (Kenya), 1912 (Theiler & Downs, 1973).

Arthropod vectors: principal vectors are metastriate ticks *Rhipicephalus appendiculatus* (in Africa, TOT demonstrated), *Haemaphysalis wellingtoni*, and *Haemaphysalis intermedia* (India), but Nairobi sheep disease virus (NSDV) has occasionally been isolated also from midges (*Culicoides tororoensis*) in Africa.

Vertebrate hosts: sheep and goat; the rat *Arvicanthis abyssinicus* may serve as a reservoir of the virus in natural foci (Fig. 5.8) (Daubney & Hudson, 1931; Simpson, 1966).

Animal disease: an important disease of ruminants, mainly sheep and goats—fatal hemorrhagic gastroenteritis, starting with high fever, depression, respiration problems, myocarditis and tubular nephritis, often pulmonary edema; mortality rate is very high, 30–90%. Abortions in pregnant ewes and goats, as well as developmental defects have been observed (Parsonson, Della-Porta, O'Halloran, et al., 1981a; Parsonson, Della-Porta, & Snowdon, 1981b; Parsonson, Della-Porta, & Snowdon, 1981c). NSD is a notifiable disease (OIE, 2012). The disease may appear as a result of introduction of naive livestock into an endemic area.

Prevention: attenuated vaccine.

Human disease: occasional cases (at least six reported). BSL-2/3.

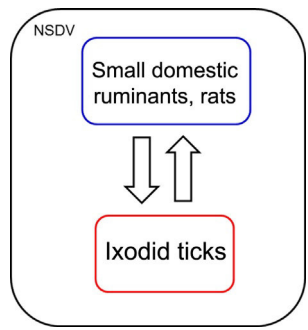

Figure 5.8 Schematic representation of the eco-epidemiological cycle of Nairobi sheep disease virus.

Geographic distribution: Africa (Kenya, Uganda, Nigeria, Central African Republic, DR Congo, South Africa), India (Ganjam strain).

4.2. Soldado virus

Taxonomy: Hughes antigenic group, genus *Nairovirus*. Prototype strain: TRVL-52214 (*Ornithodoros capensis/denmarki*, Trinidad, 1963: Theiler & Downs, 1973). A remarkable antigenic heterogeneity of Soldado virus (SOLV) isolates was identified by CFT; in fact, some European (French, Irish) isolates differ from the prototype strain more than eightfold in reciprocal titers (Chastel, Le Goff, & Le Lay, 1983, Chastel, Le Lay, Le Goff, & Monnat, 1990). The virus is very stable at pH 3.

History: SOLV was originally isolated from mixed nymphal *O. capensis* and *O. denmarki* ticks infesting *Anous stolidus* colonies on Soldado Rock near Trinidad, 1963 (Jonkers, Casals, Aitken, & Spence, 1973). In Europe, it was recovered from *Ornithodoros maritimus* infesting *Larus argentatus* nests on Puffin Island (Chastel, 1988).

Arthropod vectors: soft ticks *O. maritimus* (the mean infection rate of vector ticks can be as high as 20%: Johnson et al., 1979) in Europe, while *O. capensis* elsewhere.

Vertebrate hosts: seabirds *Sterna fuscata*, *L. argentatus*, *R. tridactyla* (Chastel et al., 1990).

Animal disease: Mortality due to SOLV was observed in young seabirds such as *S. fuscata* or *L. argentatus* (Chastel et al., 1990; Converse, Hoogstraal, Moussa, Feare, & Kaiser, 1975). Infected *O. capensis* ticks have transmitted the virus to domestic chicks and caused their death on days 5–8 postfeeding (Converse et al., 1975).

Human disease: exceptional cases. BSL-2.

Geographic distribution: Trinidad, Ethiopia, Senegal, Seychelles, South Africa, Morocco, United States (Hawaii, Texas), Iceland, Scotland, North Wales, Ireland, England, Brittany, and southern France. Seabird migrations account for the widespread distribution of SOLV (Converse et al., 1975). Natural foci are seabird colonies (usually on rocky off-shore islands).

4.3. La Crosse virus

Taxonomy: California antigenic group, genus *Orthobunyavirus*.

Genetic reassortment with all possible combinations of the three RNA segments has been demonstrated among members of California group viruses, and the reassortants appear as results of mixed infections of a vector mosquito (Chandler et al., 1991).

History: La Crosse virus (LACV) was originally isolated by W.H. Thompson from the brain tissue of a dead 4-year-old girl with encephalitis in La Crosse (Wisconsin), 1964 (Karabatsos, 1985).

Arthropod vectors: culicine mosquitoes, mainly *Aedes triseriatus* (TOT documented).

Vertebrate hosts: principal hosts are rodents (chipmunks such as *Tamias striatus*, tree squirrels *Sciurus carolinensis*, *S. niger*) and lagomorphs (*Sylvilagus floridanus*). Experimental viremia has been demonstrated in juvenile lagomorphs.

Animal disease: several cases of encephalitis in young dogs caused by LACV were described in the United States (Black et al., 1994; Tatum et al., 1999); experimental infection of puppies led to fatal CNS disease (Godsey, Amoo, Yuill, & DeFoliart, 1988). Experimental infection of pregnant ewes resulted in abortions and fetal malformation—CAHS (Edwards, Karabatsos, Collisson, & de la Concha-Bermejillo, 1997).

Human disease: many cases, especially in children. BSL-2.

Geographic distribution: USA (most states in, or east of, the Mississippi River valley).

4.4. Snowshoe hare virus

Taxonomy: California antigenic group, genus *Orthobunyavirus*. Very closely related to La Crosse and Tahyna viruses; genetic reassortment among these three agents was demonstrated experimentally.

History: prototype strain "snowshoe hare original" was isolated from the blood of an emaciated snowshoe hare (*Lepus americanus*) in Montana, USA, 1959 (Burgdorfer, Newhouse, & Thomas, 1961).

Arthropod vectors: *Ae. cinereus*, *Ae. vexans*, *Ae. communis*, *Ae. punctor*, *Ae. cataphylla*, *Cs. inornata*, *Cs. impatiens*.

Vertebrate hosts: snowshoe hare *L. americanus*, *Myodes rutilus*, and *Dicrostonyx torquatus* (lemming). Experimental viremia in *L. americanus*, *Citellus lateralis*, *C. columbianus*, *Eutamias amoenus*, *Microtus pennsylvanicus*, and *Neotoma cinerea*.

Animal disease: equine encephalitis accompanied with fever, ataxia, and circling was described in Canada; the horses usually recovered within 1 week (Heath, Artsob, Bell, & Harland, 1989; Lynch, Binnington, & Artsob, 1985). Perivascular cuffing and neuronal necrosis occur in experimentally inoculated mice.

Human disease: sporadic cases. BSL-2.

Geographic distribution: northern USA, Canada, northern European, and Asian Russia. Natural foci: tundra and taiga biomes.

4.5. Cache Valley virus

Taxonomy: Bunyamwera antigenic group, genus *Orthobunyavirus*. Prototype strain CV 633 (Theiler & Downs, 1973). Very closely related are **Tlacotalpan**, **Maguari**, and **Fort Sherman viruses** (in fact, subtypes of Cache Valley virus (CVV): Calisher, Sabattini, Monath, & Wolff, 1988).

History: first CVV strain was isolated from *Cs. inornata* mosquito in Cache Valley, Utah in 1956 (Holden & Hess, 1959). Teratogenicity (CAHS) of this virus for ruminants was described much later, in Texas, 1987 (Chung, Livingston, Edwards, Crandell, et al., 1990; Chung, Livingston, Edwards, Gauer, & Collisson, 1990, Chung, Livingston, Jones, & Collisson, 1991, Crandell & Livingston, 1988; De la Concha-Bermejillo, 2003).

Arthropod vectors: mosquitoes of several genera (*Cs. inornata*—TOT demonstrated, *Cx. tarsalis*, *Ae. taeniorhynchus*, *Ae. canadensis*, *Ae. vexans*, *Anopheles quadrimaculatus*).

Vertebrate hosts: probably wild (less so domestic) ruminants (e.g., white-tailed deer), horse.

Animal disease: in sheep, the majority of infections are subclinical, but in pregnant ewes (especially when infected during the first trimester of gestation) CVV can cause embryonic death, stillbirths, mummification of fetuses, abortions, and CAHS—malformation of fetuses with arthrogryposis, torticollis, scoliosis, lordosis, hydranencephaly, hydrocephalus, microcephaly, porencephaly, cerebellar, and muscular hypoplasia (Chung, Livingston, Edwards, Crandell, et al., 1990, Chung, Livingston, Edwards, Gauer, et al, 1990; De la Concha-Bermejillo, 2003; Edwards, 1994). The congenital malformations induced by CVV were reproduced experimentally (Chung, Livingston, Edwards, Gauer, et al., 1990).

Human disease: occasional cases. BSL-2.

Geographic distribution: United States (Texas, Utah, Michigan, Nebraska, Maryland, Pennsylvania, Virginia, South Carolina, Indiana, Illinois, North Dakota), Canada (Saskatchewan), Mexico, Jamaica, Trinidad. The Maguari subtype occurs in Brazil, Guyana, French Guiana, Trinidad, Colombia, Ecuador, Argentina, and the Fort Sherman subtype in Panama.

4.6. Main Drain virus

Taxonomy: Bunyamwera antigenic group, genus *Orthobunyavirus*. Prototype strain BF 5015 (Theiler & Downs, 1973).

History: first isolated by R.P. Scrivani from *Culicoides variipennis* midges in Kern County, California, 1964 (Karabatsos, 1985).

Arthropod vectors: the principal vector are biting midges *C. variipennis*. Experimental transmission was successful in *C. variipennis* and *C. nubeculosus*. Occasional vectors can be mosquitoes.

Vertebrate hosts: lagomorphs (*L. californicus*).

Animal disease: Main Drain virus (MDV) can cause equine encephalomyelitis (at least five cases reported). Experimental infection of pregnant ewes with MDV produced abortions and fetal malformation—CAHS (Edwards et al., 1997).

Human disease: unknown. BSL-2.

Geographic distribution: USA (California).

4.7. Akabane virus

Taxonomy: Simbu antigenic group, genus *Orthobunyavirus*. Prototype strain JaGAr-39 (Theiler & Downs, 1973).

History: first isolated by Oya, Okuno, Ogata, Kobayashi, and Matsuyama (1961) from *Ae. vexans nipponii* mosquitoes in Akabane town (Gumma prefecture, Honshu Island, Japan), 1959. Kurogi, Inaba, Goto, Miura, and Takahashi (1975) first ascribed teratogenic effects in pregnant cattle to Akabane virus (AKAV) infection.

Arthropod vectors: biting midges *Culicoides brevitarsis* and *C. wadai* (Australia); *C. oxystoma* (Japan), *C. imicola* and *C. milnei* (Africa); successful experimental transmission of AKAV by *C. variipennis* and *C. nubeculosus* (Jennings & Mellor, 1989). In Australia, long-distance wind-based dispersal of *C. brevitarsis* infected with AKAV was indicated in 1983, and, retrospectively, also in the 1974 and 1955 epizootics (Murray, 1987). However, AKAV has also been repeatedly isolated from mosquitoes (*Ae. vexans*, *Cx. tritaeniorhynchus*) in Japan.

Vertebrate hosts: domestic ruminants.

Animal disease: epizootic Akabane disease—usually, no overt clinical signs in nonpregnant ruminants, but abortions, stillbirth, and high incidence of severe teratogenic defects in newborn animals (CAHS, blindness) in cow, sheep, and goats (Doherty, 1977; Inaba, Kurogi, & Omori, 1975; Kurogi et al., 1976;

Narita, Inui, & Hashiguchi, 1979; Parsonson, Della-Porta, O'Halloran, et al., 1981a; Parsonson, Della-Porta, & Snowdon, 1977, 1981b; Parsonson, McPhee, Della-Porta, McClure, & McCullagh, 1988). However, Kamata et al. (2009) reported neurological signs (limb weakness, circling, astasia, torticollis, with relevant histological lesions in CNS) in several Japanese calves up to 15 months old, infected with AKAV.

An inactivated trivalent vaccine against AKAV, Aino virus (AINV), and Kasba virus (KASV) has been developed (Kim et al., 2011).

Human disease: unknown. BSL-2/3.

Geographic distribution: Japan, Korea, Australia (Queensland, New South Wales), Indonesia, Cyprus, Israel (major epizootics in 1969/70 and 2002/03), Oman, Africa (Kenya).

4.8. Aino virus

Taxonomy: Simbu antigenic group, genus *Orthobunyavirus*. Prototype strain JaNAr-28 (*Cx. tritaeniorhynchus*: Theiler & Downs, 1973). Closely related by CFT to AKAV, Simbu, Peaton, and Sathuperi viruses. Synonyms: **Samford virus** (Miura et al., 1978), **Kaikalur virus** (Kinney & Calisher, 1981).

History: first isolated from *Cx. tritaeniorhynchus* in Nagasaki prefecture of Japan, 1964 (Takahashi et al., 1968), and in Australia (as Samford virus) in 1968 (Parsonson, Della-Porta, & Snowdon, 1981c).

Arthropod vectors: mosquitoes *Culex* spp. (*Cx. tritaeniorhynchus*, *Cx. pipiens*, *Cx. pseudovishnui*), but the virus was also repeatedly isolated from biting midges *Culicoides brevitarsis*, *C. oxystoma*, and other *Culicoides* spp.

Vertebrate hosts: ruminants.

Animal disease: teratogenic virus—CAHS in calves (Noda et al., 1998; St George, 1989). An inactivated trivalent vaccine against AINV, AKAV, and KASV has been developed (Kim et al., 2011).

Human disease: unknown. BSL-2.

Geographic distribution: Japan, Korea, Indonesia, Australia (Queensland), India (Kaikalur strain).

4.9. Schmallenberg virus

Taxonomy: Simbu antigenic group, genus *Orthobunyavirus*. Nucleotide sequences obtained from Schmallenberg virus (SBV) were most closely

related to those of Shamonda (occurring in Nigeria and Japan, the vector is *C. imicola*, possibly other *Culicoides* spp.), Aino and Akabane viruses. A recent phylogenetic analysis of three genomic RNA segments revealed that SBV is a reassortant, with the M RNA segment from Sathuperi virus and the S RNA and L RNA segments from Shamonda virus (Yanase et al., 2012).

History: in summer 2011, a new illness of dairy cows was reported in Germany (North Rhine-Westphalia) and in the Netherlands that involved fever, anorexia, and reduced milk yield; the disease spread rapidly over western and central Europe (Conraths et al., 2013; Gibbens, 2012; Hoffmann et al., 2012).

Arthropod vectors: biting midges *Culicoides scoticus*, *C. obsoletus* s.s., *C. dewulfi*, *C. chiopterus* (De Regge et al., 2012; Elbers, Meiswinkel, van Weezep, van Oldruitenborgh-Oosterbaan, & Kooi, 2013; Rasmussen et al., 2012).

Vertebrate hosts: ruminants (sheep, goat, and cattle; Fig. 5.9)—but viremic period is short (3–5 days). Vertical transmission from female ruminants to their offspring is of particular importance. Virus RNA was detected in the semen of bulls with a history of SBV infection in German, Dutch, and French veterinary laboratories. In cattle inseminated with SBV RNA-positive semen, infection was detected by RT-PCR and antibody test in 2 of 6 animals (this finding has an international impact for semen trade). SBV has been detected by RT-PCR in bison, deer, moose, and alpacas; fallow deer, roe deer, red deer, and mouflon have been found to be seropositive.

Animal disease: an emerging disease of ruminants in Europe. Although the infection remains in adult animals usually asymptomatic, in pregnant sheep (and

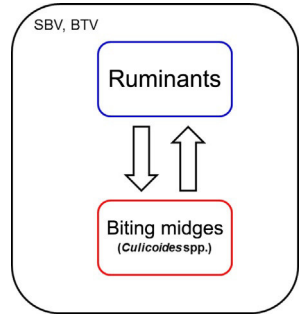

Figure 5.9 Schematic representation of the eco-epidemiological cycle of bluetongue and Schmallenberg viruses.

goat) transplacental transmission of the virus can lead to congenital disorders of the progeny: lambs (and kids) are sometimes born alive, but mostly not viable and malformed with CAHS—arthrogryposis, hydranencephaly, hydrocephalus, cerebellar hypoplasia or aplasia, ankylosis, torticollis, lordosis, scoliosis, brachygnatia, enlarged thymus, etc. Histologic lesions include lymphohistiocytic meningoencephalomyelitis, glial nodules in the mesencephalon and hippocampus, and neuronal degeneration and necrosis mainly in the brain stem; the skeletal muscles had myofibrillar hypoplasia. The lesions of SBV-associated abortion are similar to those caused by certain other viruses of the Simbu group bunyaviruses (AKAV, etc.). Clinical signs in dairy cattle involve fever, anorexia, diarrhea, reduced milk yield (up to 50%), but congenital disorders of calves are less often seen (Doceul et al., 2013; Herder, Wohlsein, Peters, Hansmann, & Baumgartner, 2012; Steukers, Bertels, Cay, & Nauwynck, 2012; van den Brom et al., 2012). As of June 2012, SBV affected a total of 2457 sheep, 79 goats, and 3040 cattle in Europe (OIE, 2012).

Human disease: unknown (and considered as "unlikely"). BSL-2.

Geographic distribution: Germany, Netherlands, Belgium, Denmark, Luxembourg, France, Great Britain, Ireland, Spain, Italy (including Sardinia), Switzerland, Austria, Czechland, Hungary, Slovenia, Croatia, Serbia, Poland, Latvia, Estonia, Finland, Sweden, Norway, and central European Russia (antibodies in cattle).

4.10. Shuni virus

Taxonomy: Simbu antigenic group, genus *Orthobunyavirus*. Prototype strain An 10107.

History: first isolated from the blood of a cattle at slaughter in Ibadan, Nigeria, in 1966 (Causey, Kemp, Causey, & Lee, 1972).

Arthropod vectors: *Culicoides* spp., but Shuni virus (SHUV) was also isolated from mosquitoes (*Cx. theileri*).

Vertebrate hosts: ruminants (sheep, cattle), equids.

Animal disease: SHUV caused neurologic disease in five horses (four were fatal) and a febrile illness in two additional horses in South Africa (van Eeden et al., 2012; Venter et al., 2010).

Human disease: unknown (a strain isolated from a man in Nigeria: Moore et al., 1975). BSL-2.

Geographic distribution: Africa (Nigeria, South Africa).

4.11. Rift Valley fever virus

Taxonomy: RVF antigenic group, genus *Phlebovirus*. Synonym: **Zinga virus**.

History: in 1912, an outbreak of RVF (diagnosed only clinically) occurred in Kenya (Peters & Meegan, 1981). The virus was first isolated during a severe sheep and cattle epizootic with a high mortality rate in lambs and abortion in pregnant ewes in the Great Rift Valley, Kenya, 1930 (Daubney, Hudson, & Garnham, 1931).

Arthropod vectors: mosquitoes of at least 30 species were found to be infected with Rift Valley fever virus (RVFV), belonging mostly to the genera *Aedes* and *Culex* (Chevalier, Pépin, Plée, & Lancelot, 2010), such as *Ae. mcintoshi* (TOT demonstrated, indicating reservoir potential of the mosquitoes), *Ae. vexans arabiensis* (TOT), *Ae. caballus*, *Ae. circumluteolus*, *Ae. tarsalis*, *Ae. lineatopennis*, *Ae. dentatus*, *Ae. palpalis*, *Ae. mcintoshi*, *Cx. pipiens*, *Cx. tritaeniorhynchus*, *Cx. theileri*, *Cx. poicilipes*, *Cx. antennatus*, and *Eretmapodites chrysogaster* complex (Karabatsos, 1985; Pepin, Bouloy, Bird, Kemp, & Paweska, 2010). The virus RNA has also been detected in (or the virus isolated from) *Anopheles gambiae* mosquitoes, sandflies, *Simulium* spp. blackflies and *Culicoides* spp. midges during RVF epizootics, and the role of biting midges remains to be determined. Increased precipitation in dry regions leads to enhanced hatching of mosquitoes and that caused enhanced risk of RVF. In experiment, stable flies (*Stomoxys calcitrans*), were able mechanically transmit RVFV to susceptible hamsters after probing on infected hamsters with high viral titers; therefore, stable flies, closely associated with domestic ruminants, may contribute to rapid spread of a RVF outbreak (Turell, Dohm, Geden, Hogsette, & Linthicum, 2010).

Vertebrate hosts: ruminants (domestic and wild, including African buffalo *Syncerus caffer* and antelopes; adult ruminants develop high viremia and thus serve as amplifying hosts); rodents (rats—genera *Arvicanthis* and *Rattus*) may play a reservoir role in the RVFV cycle in natural foci (Fig. 5.10); the virus was also isolated from bats (Olive, Goodman, & Reynes, 2012).

Animal disease: RVF is a severe disease of domestic ruminants, called first "enzootic hepatitis." Most susceptible to RVF, with a high mortality rate (more than 70%), are young animals (lamb, kid, calf); lethality is lower in adult sheep, while adult cattle, goat or buffaloes reveal severe disease though with a mortality rate below 10%. However, the disease can also be fatal for young dogs and cats, as well as for monkeys. Clinical manifestation in susceptible animals includes fever, weakness, depression, anorexia, vomiting, hemorrhagic diarrhea, blood-containing mucopurulent nasal discharge,

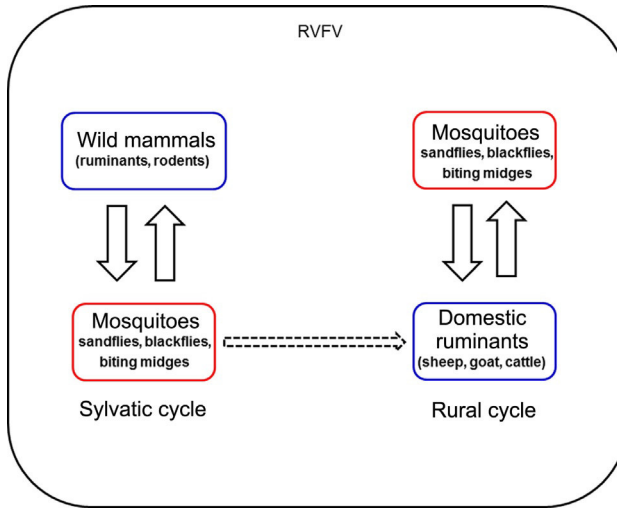

Figure 5.10 Schematic representation of the eco-epidemiological cycle of Rift Valley fever virus.

stiffness of gait, and necrotic hepatitis associated with icterus. Abortions in pregnant sheep, goats, and cows, sometimes accompanied with teratogenesis, are frequent (Bird, Ksiazek, Nichol, & MacLachlan, 2009; Chevalier et al., 2010; Coetzer & Barnard, 1977; McIntosh & Gear, 1981). No clinical symptoms have been observed in camel, horse, donkey, pig, rabbit, and birds.

Large outbreaks of RVF were reported from South Africa from 1950 to 1956, and then again from 1969 to 1976 (1950–1951: over 100,000 fatal cases in sheep and cattle: Alexander, 1951; Peters & Meegan, 1981; Weiss, 1957), Sudan (1976, 2007), Egypt (large outbreaks in 1977–1978, 1993–1994, 1997, 2003: Kamal, 2011), Senegal (1987), Kenya and Tanzania (1997–1998, 2006–2007), and Somalia (2007), often in association with livestock trade, and favorized by land use (irrigation) and increased rainfall (Chevalier et al., 2010). RVF is a notifiable disease (OIE, 2012). RVF epizootics cause extreme economic losses and big social impacts in pastoralist communities.

Both live (attenuated, e.g., MV P 12) and inactivated vaccines are available for livestock, the latter being less immunogenic but devoid of residual pathogenic effects that can sometimes occur when the attenuated live vaccine is applied (e.g., abortions and CAHS in pregnant animals: Coetzer & Barnard, 1977).

The virus has been classified as a potential bioterrorism (agroterrorism) agent.

Human disease: many cases, fatality rate comparatively low. BSL-3.

Geographic distribution: RVFV occurs in most countries of Africa (but until 1977, it only occurred in the sub-Saharan part of the continent): South Africa, Tanzania, Kenya, Uganda, Guinea, Nigeria, Mauritania, Somalia, Sudan, Egypt (1977), Senegal. However, RVFV recently spread (or has been introduced) outside African continent to Madagascar (2008), Mayotte (2007–2008), Yemen and Saudi Arabia (2000), where livestock trade plays an important role in the virus range expansion. Concerns therefore arise that "the introduction of RVF-infected animals on the eastern and southern shores of the Mediterranean Sea is a likely event" (Chevalier et al., 2010).

4.12. Bhanja virus

Taxonomy: Bhanja antigenic group, genus *Phlebovirus* (Matsuno et al., 2013). Prototype strain I 690 (*Haemaphysalis intermedia*, India: Theiler & Downs, 1973). Synonym or subtype: **Palma virus** (PoTi-4.92 strain, isolated from *Haemaphysalis punctata* in Portugal, 1992: Filipe et al., 1994); the mean cross-PRNT crosstiter differences among European, Indian and African strains of Bhanja virus (BHAV) have been found as great as 4–10-fold (Hubálek & Halouzka, 1985).

History: BHAV was first isolated from *H. intermedia* (syn. *H. parva*) ticks that had been collected from a paralyzed goat in Bhanjanagar (district Ganjam, Orissa State, India) in 1954 (prototype strain IG-690), but the record was published much later (Shah & Work, 1969).

Arthropod vectors: the virus is transmitted by metastriate ixodid ticks *H. intermedia*, *Boophilus decoloratus*, *B. annulatus*, *B. geigyi*, *Amblyomma variegatum*, *Hyalomma marginatum*, *H. detritum*, *H. dromedarii*, *H. truncatum*, *H. asiaticum* (TOT), *Rhipicephalus bursa*, and *R. appendiculatus*, in Europe *H. punctata*, *H. sulcata*, and *Dermacentor marginatus*.

Vertebrate hosts: probably sheep, goat, cattle; in Africa, BHAV was also isolated from the four-toed hedgehog (*Atelerix albiventris*) and striped ground squirrel (*Xerus erythropus*).

Animal disease: BHAV is pathogenic for young ruminants (lamb, kid, calf), causing fever and CNS affection (meningoencephalitis), or leucopenia in cattle; experimental encephalitis was produced in lamb inoculated i.c., but not s.c. or i.v. (Camicas, Deubel, Heme, & Robin, 1981; Hubálek, 1987; Mádr et al., 1984; Semashko et al., 1976; Theiler & Downs, 1973) and rhesus monkey (Balducci, Verani, Lopes, & Nardi, 1970). Not fatal to adult goat (s.c.) or rabbit (i.c., i.n., s.c., i.v., p.o.; low viremia).

Human disease: about 10 cases reported. BSL-3.

Geographic distribution: India, Kirghizia, Kazakhstan, Azerbaijan, Armenia, Senegal, Guinea, Nigeria, Cameroon, Central Africa, Kenya, Somalia, Portugal, Italy, Croatia, Serbia, Bulgaria, Romania, and Slovakia. Antibodies were detected in Sri Lanka, Pakistan, Iran, Turkmenia, Uzbekistan, Tadjikistan, Uganda, Tanzania, Egypt, and Tunisia. Natural foci of BHAV are boskematic—pastoral steppe or forest-steppe ecosystems in xerothermic areas, or in karst habitats at more northern latitudes.

5. FAMILY *REOVIRIDAE*

5.1. African horse sickness virus

Taxonomy: AHS antigenic group, genus *Orbivirus*. There is a minor RNA homology with bluetongue virus (BTV). There are nine African horse sickness virus (AHSV) antigenic types, distinguishable by VNT and HIT while not by CFT. The antigenic heterogeneity of AHSV was revealed by A. Theiler as early as 1908 (Gorman, Taylor, & Walker, 1983; Theiler & Downs, 1973)—it results from antigenic drift and antigenic shift (reassortment) promoting evolution of AHSV (MacLachlan & Guthrie, 2010).

History: the disease AHS has been known in South Africa since the seventeenth century and associated with insect vectors (MacLachlan & Guthrie, 2010). AHS was studied microbiologically in South Africa since 1887, and the agent was first demonstrated to be a filterable virus by J. McFadyean and A. Theiler in blood samples collected from sick horses in South Africa, 1900–2001 and 1932 (Theiler & Downs, 1973).

Arthropod vectors: principal vectors are *Culicoides* spp. midges—confirmed experimentally by Du Toit (1944)—on *C. imicola*; experimental transmission of AHSV was also demonstrated in *C. sonorensis* and *C. obsoletus* (Mellor, Boned, Hamblin, & Graham, 1990; Mellor & Hamblin, 2004). Occasional vectors are mosquitoes of the genera *Aedes* and *Culex*—successful experimental transmission of AHSV by *Cx. pipiens*, *Ae. aegypti*, and *Anopheles stephensi* was described (Ozawa & Nakata, 1965).

Vertebrate hosts: equids (horse, mule, donkey, zebra) (Fig. 5.11). Zebra acts as a wildlife reservoir of the virus (viremia documented for up to 40 days) in African endemic foci (Gorman et al., 1983).

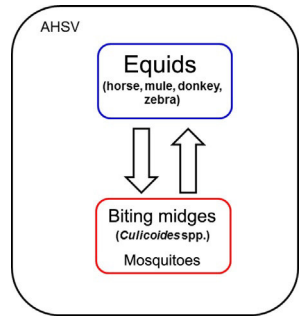

Figure 5.11 Schematic representation of the eco-epidemiological cycle of African horse sickness virus.

Animal disease: acute or subacute disease of equids (horse, zebra) that manifests in three ways—the lung ("dunkop") form, the heart ("dikkop") form, and the mixed form. The lung form is characterized by high fever, dyspnoea, nasal discharge, very high death rate (90%); the most common cause of death is pulmonary edema (hydrothorax). The heart form is with fever, edema of subcutis usually associated with head and throat edema (swelling of the head and eyes), loss of ability to swallow, internal hemorrhages, and hydropericarditis, mortality rate in equids is lower, usually about 50%. Some AHSV serotypes are neurotropic, others viscerotropic. The mixed form is characterized by signs of both the dunkop and dikkop forms of the disease. The horse is highly susceptible to inoculation with AHSV (s.c., i.v., i.n., i.p., p.o.), while clinical signs are infrequent in donkey, and only asymptomatic infection is produced in cattle, sheep, and goat (by any route); dogs die when fed with infected horse meat (Mellor & Hamblin, 2004). An AHS epidemic in India, starting in Jaipur, 1960, killed over 16,000 equids in 12 states (Theiler & Downs, 1973). AHS is a notifiable disease (OIE, 2012), and it regularly causes severe economic losses in affected areas.

Prevention: inactivated and cell-culture-derived attenuated live vaccines are available.

Human disease: unknown. BSL-2/3.

Geographic distribution: widely enzootic in sub-Saharan Africa, especially in South Africa. For instance, an outbreak of AHS in the Cape of Good Hope region killed about 70,000 horses in 1855 (MacLachlan & Dubovi, 2011). However, AHS has spread from Africa to the Middle East, Turkey, Pakistan, and India since 1959, and outbreaks occur periodically in North

Africa, India, Pakistan, Afghanistan, Iran, Iraq, Saudi Arabia, Yemen, Syria, Jordan, Israel, Turkey, and Cyprus. In Europe, the first AHS epizootic was diagnosed in southern Spain in 1966 (introduced by zebra *Equus burchellii* from Namibia) and then in 1987–1989, caused by serotype 4 (MacLachlan & Guthrie, 2010; Mellor et al., 1990; Sellers, Pedgley, & Tucker, 1977). AHSV was also introduced to south-eastern Portugal (outbreak in 1989). Infected vector midges may be carried by wind to remote areas and thus disseminate the virus—for example, to Spain from Morocco in 1966, Cyprus from Turkey in 1960, or Cape Verde Islands from Senegal in 1943 (Sellers, 1980; Sellers et al., 1977).

5.2. Kasba virus

Taxonomy: Palyam antigenic group, genus *Orbivirus*. Synonyms: **Abadina**, **Chuzan**, **Kagoshima viruses** (Jusa et al., 1994).

History: first isolated by C.N. Dandawate from *Culex vishnui* complex mosquitoes collected in Sathuperi, India in 1957 (Karabatsos, 1985).

Arthropod vectors: biting midges *Culicoides schultzei*, *C. oxystoma*, and other *Culicoides* spp. Occasional isolations from mosquitoes (*Culex vishnui*, *Aedes fowleri*).

Vertebrate hosts: cattle, goat.

Animal disease: Chuzan disease—CAHS in calves (Goto, Miura, & Kono, 1988; Kitano, Yamashita, & Makinoda, 1994; Oberst, 1993). An inactivated trivalent vaccine against KASV, AKAV, and AINV has been developed (Kim et al., 2011).

Human disease: unknown. BSL-2.

Geographic distribution: India, Africa (Abadina strain), Japan (Chuzan and Kagoshima strains).

5.3. Bluetongue virus

Taxonomy: Bluetongue antigenic group, genus *Orbivirus*; 26 distinct BTV serotypes with varying virulence are currently recognized (differentiated by VNT), including "Toggenburg virus," recently described from goats in Switzerland, proposed to be the 25th serotype (Hofmann et al., 2008). BTV genome consists of 10 linear segments; variation in VP2 and VP5 proteins determine the serotype (Schwartz-Cornil et al., 2008; Wilson & Mellor, 2009). Genetic drift and genetic shift (reassortments) have been described in BTV, causing its steady evolution (MacLachlan & Guthrie, 2010).

History: The ovine disease was first described in South Africa after European merino sheep were introduced in the region in the late eighteenth century (MacLachlan & Guthrie, 2010). In 1902, the disease was mentioned by Hutcheon as "malarial catarrhal fever of sheep," afterward named as "bluetongue" by Spreull in 1905 who first reported comprehensive clinical profile of the disease. The name of the disease originates from the Afrikaans name for the disease—"blaauwtong" (Spreull, 1905). BTV was first passaged by Dixon and Spreull from ovine spleen in South Africa, 1900, later by A. Theiler, who also demonstrated its filterability in 1906 (Du Toit, 1944; Karabatsos, 1985; Theiler & Downs, 1973).

Arthropod vectors: biting midges were confirmed experimentally as principal vector by Du Toit (1944). The most important vector is *C. imicola* (principal vector in Africa, the Middle East, much of south Asia, and parts of southern Europe), presumptive vectors in Europe are members of the *C. obsoletus* group (such as *C. obsoletus* s.s., *C. scoticus*, and *C. chiopterus*), *C. pulicaris*, *C. dewulfi*, *C. achrayi*; other vectors are *C. milnei* (Africa, Israel); *C. imicola* (South Africa); *C. variipennis*, *C. sonorensis*, and *C. cockerellii* (North America); *C. insignis* (Central and South America); *C. filarifer*, *C. trilineatus*, *C. furens*, and *C. pusillus* (Central America, USA), *C. brevitarsis* (principal vector) and *C. fulvus* (both in Australia), and *C. orientalis* (Indonesia) (Carpenter et al., 2012; Kampen & Werner, 2010; MacLachlan, 2011). Some mosquito species (e.g., *Ae. lineatopennis*) can play a role as secondary or mechanical vectors, as well as sheep ked *Melophagus ovinus*. TOT in biting midges has not been demonstrated but the virus could persist in long-lived adult *Culicoides* spp. that survive winter (MacLachlan, 2011). Importantly, *Culicoides* biting midges can be passively dispersed over long distances (>100 km) by prevailing winds leading to rapid spread of the viruses they carry (Ducheyne et al., 2011; Garcia-Lastra et al., 2012; Hendrickx, 2009; Mellor, 1993; Pedgley, 1983; Sellers, 1980; Sellers, Pedgley, & Tucker, 1978). Predictions made by "wind models" may contribute to forecast the spread of BTV outbreaks (Hendrickx, 2009).

Vertebrate hosts: sheep of certain breeds, non-African wild ungulates (Fig. 5.9) (Jessup, Osburn, & Heuschele, 1984), sporadically cattle and South-American camelids, and even carnivores. Viremia in certain ruminants may be high- and long term (2 weeks, but sometimes up to 50 days in cattle). In one case, BTV was said to persist in cattle for nearly 5 years, although vector-proof accommodation was unfortunately not provided (Luedke, Jones, & Walton, 1977). Possible persistence (overwintering) of BTV in other hosts (rodents, reptiles) is suspected (MacLachlan, 2011), as

well as that in infected T-cells of certain mammalian hosts (Takamatsu et al., 2003).

Animal disease: "bluetongue or ovine catarrhal fever"—a noncontagious disease of domestic and certain wild ruminants; the infection can often be subclinical or inapparent, but sometimes can lead to severe disease with high mortality in susceptible animals (sheep). BT has also been observed in American deer (*Odocoileus virginianus*, *O. hemionus*), elk (*Alces alces*), pronghorn antelope (*Antilocapra americana*), mouflon (*Ovis musimon*), bison, and camelids. The symptoms of bluetongue in sheep include fever, weakness, depression, diarrhea, vomiting, facial edema (involving lips, tongue, and head), oral erosions and ulcers, conjuctivitis, rhinitis with nasal exudate, excessive salivation, inflammation of the coronary band (above the hoof), lameness, hyperemia, and pain at mucocutaneous junctions as the gums and vulva, often (edematous) pneumonia; fetal death and CAHS can occur in lambs; mortality rate in sheep is 5–30% (MacLachlan, 2010). Postmortem lesions include hemorrhage and necrosis of the mucosal lining of the upper gastrointestinal tract from the oral cavity through the fore stomachs, edema, and hemorrhage of lymph nodes, s.c. hemorrhage and edema, pulmonary edema that is typically severe in fatal cases, pleural and pericardial effusion, edema of the facial planes of the abdominal muscles and those of the neck and head, subintimal hemorrhages in the pulmonary artery, and segmental necrosis of the myocardium and skeletal muscles (MacLachlan, 2011). In pregnant animals, abortion may occur. The blue tongue (cyanosis) after which the disease was named is seen only occasionally, in more serious clinical cases. Following recovery, animals may also exhibit a number of long-lasting secondary effects, such as reductions in milk yield and weight gain, severe wool break, and temporary infertility (Wilson & Mellor, 2009). The disease is noncontagious, but seminal shedding of BTV was demonstrated in viremic rams and bulls (Bowen, Howard, Entwistle, & Pickett, 1983). In general, sheep in endemic areas are naturally resistant to BT, but outbreaks of BT occur when susceptible sheep are introduced to these endemic areas. In cattle, BTV infection is largely asymptomatic but abortions (and CAHS) in cows have been described; interestingly, viremia is much longer (up to 4–5 months!) in cattle than in sheep. Cattle may thus serve as a source of BTV for several weeks while displaying little or no clinical signs of disease. Bluetongue is a notifiable disease (OIE, 2012). It causes marked economic problems in affected areas; for example, estimated direct (disease) and indirect (trade restrictions, costs of surveillance, and vaccination) losses attained over $3 billion per year according to a record from 1996 (Tabachnick, 1996).

Prevention: vaccines against BT currently available on market are either attenuated or inactivated (Bhanuprakash, Indrani, Hosamani, Balamurugan, & Singh, 2009; Savini, MacLachlan, Sanchez-Vizcaino, & Zientara, 2008). The attenuated vaccine has long been used to control BT in sheep in South Africa, Corsica, Balearic Islands, and Italy. It provides a robust protection for at least 1 year after one injection and it is relatively cheap, but some BTV vaccine attenuated virus(es) can be additionally spread by vectors with a potential to reversion of virulence and even reassortment with the genes of wild-type virus (Samal, Livingston, McConnell, & Ramig, 1987; Stott, Oberst, Channell, & Osburn, 1987). Some of the attenuated BTV vaccines caused abnormalities (including CAHS) in the fetuses of ewes vaccinated during the 5th and 6th week of pregnancy (Parsonson, Della-Porta, & Snowdon, 1981c). The inactivated vaccine produces after single vaccination only neutralizing antibodies, insufficient to provide long-term protection, and directed only to few serotypes. Several types of recombinant vaccines (using virus like particles or recombinant vectors) are in preparation (Savini, MacLachlan, et al., 2008). Different BTV serotypes and monovalent vaccines usually do not provide sufficient crossprotection to other serotypes. Moreover, no polyvalent vaccines are currently available in Europe (Hendrickx, 2009).

Human disease: exceptional cases. BSL-2.

Geographic distribution: BTV has been identified on all continents except Antarctis, in the latitude range between 35°S and 40°N with a potential expansion up to 50°N. It occurs in South and eastern Africa, Nigeria, and Egypt (since 1972); Israel and Palestine (since 1943); Cyprus and Syria (since 1943); Turkey and Iran (since 1944); Pakistan, India, Japan, Indonesia, Australia, and Central and North America (since 1948); and South America (since 1962). Prior to 1998, occasionally short-lived incursions of BTV occurred in southern Europe: Spain and Portugal (in 1956–1960, serotype BT-10 killed some 180,000 sheep: Manso-Ribeiro et al., 1957) and Greece (1979). However, since 1998, at least eight distinct BTV strains of six serotypes (1, 2, 4, 8, 9, 16) have invaded Europe, including Greece, Bulgaria, European Turkey, Balkan countries, France, and many northern countries (MacLachlan & Guthrie, 2010; Mellor, Carpenter, Harrup, Baylis, & Mertens, 2008). In 2006, African serotype BTV-8 was detected initially in the Netherlands, before spreading to Germany, Belgium, north-eastern France, and Luxemburg causing substantial losses among ruminants. Other European countries affected by this serotype (and/or some others) between 2006 and 2010 were Austria, Hungary (introduction with French cattle),

western Czechland (never observed here before), Denmark, Greece (BTV-4), Spain, Portugal, Italy (including Sardinia where almost 10,000 sheep died of BTV-2 infection up to 2012), Switzerland, United Kingdom, Norway (up to 60°N), and Sweden (Carpenter, Wilson, & Mellor, 2012; MacLachlan, 2010; Mellor et al., 2008; Wilson & Mellor, 2009). In 2008, serotypes 6 and 11 emerged in northern Europe (MacLachlan, 2010), and vaccine strain BTV-14 was detected in cattle in Russia, Latvia, Lithuania, and Poland during 2011–2012 (OIE, 2012).

5.4. Epizootic hemorrhagic disease virus

Taxonomy: EHD antigenic group, genus *Orbivirus*. Prototype strain: New Jersey (isolated from white-tailed deer). There are at least seven antigenic types.

History: Epizootic hemorrhagic disease virus (EHDV) was first recovered by R. E. Shope from internal organs of a dead white-tailed deer in New Jersey, 1955 (Karabatsos, 1985).

Arthropod vectors: principal vectors are biting midges *C. variipennis* in North America, *C. brevitarsis* in Australia, *C. kingi*, *C. schultzei*, and other spp. in Africa. The virus has also been occasionally isolated from mosquitoes. Winds are a contributory factor for a distant spread of *Culicoides*-borne EHDV (Kedmi et al., 2010).

Vertebrate hosts: white-tailed deer (*Odocoileus virginianus*), antelopes.

Animal disease: acute disease of wild ruminants (deer) with fever, rapid difficult breathing, excessive salivation, nasal exsudate, swollen tongue, and generalized hemorrhagic symptoms—hemorrhages are observed in many organs at the time of death of the animals (Karstad, Winter, & Trainer, 1961). EHD occurs in epidemics among the white-tailed deer in several U.S. states. Mortality rate of white-tailed deer might be high (up to 90% during the New Jersey outbreak in 1955). Other epizootics of deer were recorded in Alberta 1962, North Dakota 1970, and south-eastern USA 1971. EHDV has been found to be also pathogenic for cattle in the Mediterranean (for instance causing big economical losses in Israel 2006: Kedmi et al., 2010), but it is not pathogenic for sheep, goats, horse, dog, pig, and rabbit. EHD is a notifiable disease (OIE, 2012).

Human disease: unknown. BSL-2.

Geographic distribution: United States (New Jersey, Michigan, Washington, North Dakota, South Dakota), Canada (Alberta), Israel, Turkey, North Africa, Nigeria, Australia.

5.5. Ibaraki virus

Taxonomy: EHD antigenic group, genus *Orbivirus*. No antigenic relationships with BTV. Ibaraki virus (IBAV) is sometimes regarded as a subtype 2 of EHDV.

History: first isolated from blood of a sick cow in the Ibaraki prefecture of Japan in 1959 (Karabatsos, 1985).

Arthropod vectors: biting midges (*Culicoides* spp.).

Vertebrate hosts: ruminants.

Animal disease: Ibaraki disease—bluetongue-like symptoms in cattle with fever, ulcerative stomatitis, dysphagia, leucopenia, degeneration of striated muscles, abortion, and stillbirths (Inaba, 1975; MacLachlan & Dubovi, 2011; Omori et al., 1969). Low pathogenicity for sheep.

Human disease: unknown. BSL-2.

Geographic distribution: Japan, Indonesia, Taiwan.

5.6. Equine encephalosis virus

Taxonomy: genus *Orbivirus*; seven serotypes have been differentiated.

History: prior to the recent outbreak of equine encephalosis in Israel (2008–2009: Mildenberg et al., 2009), Equine encephalosis virus (EEV) had only been isolated from equids in South Africa.

Arthropod vectors: biting midges (*C. imicola*, *C. bolitinos*).

Vertebrate hosts: equids (horse, donkey, and zebra).

Animal disease: similar to AHS—fever, unrest, anorexia, edema of the neck, legs, lips and eyelids, accelerated pulse and breathing rates, congested mucosae, and encephalitis in horses, sporadically fatal. However, in most cases, EEV infection results in a mild disease (MacLachlan & Guthrie, 2010; Mildenberg et al., 2009).

Human disease: unknown. BSL-2.

Geographic distribution: South Africa, East Africa (Gambia, Ethiopia, Ghana), Israel (Mildenberg et al., 2009; Oura et al., 2012).

5.7. Peruvian horse sickness virus

Taxonomy: genus *Orbivirus*. Synonym: **Elsey virus** (Attoui et al., 2009).

History: in 1997, a new virus was isolated during a disease outbreak in horses, donkeys, cattle, and sheep in Peru. Peruvian horse sickness virus (PHSV) was subsequently also isolated during 1999, from diseased horses in the Northern Territory of Australia (Elsey virus).

Arthropod vectors: mosquitoes.

Vertebrate hosts: equids, ruminants.

Animal disease: fever with neurological disorders and up to 78% mortality in horses.

Human disease: unknown. BSL-2.

Geographic distribution: South America (Peru), Australia (MacLachlan & Guthrie, 2010).

5.8. Yunnan virus

Taxonomy: genus *Orbivirus*. Synonyms or subtypes: **Middle Point virus** (MPOV: Cowled et al., 2007) and **Rioja virus** (RIOV: Attoui et al., 2009).

History: Yunnan virus (YUOV) was originally isolated from mosquitoes in China (Attoui et al., 2005: but in the paper there is no mention when it was isolated), MPOV from asymptomatic sentinel cows in North Australia, 1994, and RIOV during a disease outbreak among domestic animals in Peru, 1997.

Arthropod vectors: mosquitoes (e.g., *Cx. tritaeniorhynchus*).

Vertebrate hosts: equids, ruminants.

Animal disease: fever, with neurological disorders in donkey, cattle, sheep, and dog. In MPOV, usually asymptomatic infection in cattle, with a very long-term viremia (exceptionally up to 35 weeks: Cowled et al., 2012).

Human disease: unknown. BSL-2.

Geographic distribution: China, North Australia (MPOV), South America (Peru: RIOV).

6. FAMILY *RHABDOVIRIDAE*

6.1. Bovine ephemeral fever virus

Taxonomy: genus *Ephemerovirus*.

History: isolated by R.L. Doherty, H.A. Standfast, and I.A. Clark from the blood of a febrile calf after experimental inoculation with BEF cattle-passage material obtained in North Queensland in 1968. However, BEF has been recognized in Africa since 1867, and the agent was adapted to mice and cell cultures in South Africa and Japan (1951) prior to the studies of Doherty et al. (Karabatsos, 1985).

Arthropod vectors: principal vectors are probably mosquitoes, for example, *Cx. annulirostris*, *Anopheles annulipes* (St George, 2008). However, biting midges could be additional vectors: *Culicoides schultzei*, *C. coarctatus*, and

C. imicola in Africa; *C. algecirensis* in central Asia; and *C. brevitarsis* in Australia (Karabatsos, 1985). Winds are a contributory factor for distance spread of *Culicoides*-borne Bovine ephemeral fever virus (BEFV) (in addition to animal transport).

Vertebrate host: cattle.

Animal disease: usually benign but high fever in cattle and water buffaloes, called "three-day sickness" (Inaba, 1973), with a sudden and severe drop in milk production in milking cows, respiratory symptoms, s.c. emphysema, anorexia, salivation, nasal discharge, arthralgia, muscle tremor, lameness (affected animals are reluctant to move), and sometimes limb paralyses. The case fatality rate is low, about 1%. At histopathology, the disease is associated with endothelial hyperplasia and perivascular infiltrates, especially in synovial membranes, tendon sheaths, muscles, and skin. BEFV is not pathogenic for sheep, goat, and pig. Major epizootics of BEF occurred in Australia 1936–1937, 1955–1956, and 1967–1968, with significant economical impact (Doherty, 1977).

Prevention: an attenuated vaccine has been produced in Australia and Japan.

Human disease: unknown. BSL-2.

Geographic distribution: South Africa, Nigeria, Kenya, Egypt, Arabian Peninsula, Israel, Jordan, Turkey, Iran, Turkmenistan, Korea, Japan, China, Taiwan, Australia, Indonesia.

6.2. Kotonkan virus

Taxonomy: genus *Ephemerovirus* (Blasdell et al., 2012).

History: originally isolated (IbAr 23380) by V. Lee from biting midges in cattle barns, Nigeria, 1967 (Karabatsos, 1985; Kemp et al., 1973).

Arthropod vectors: biting midges *Culicoides* spp.

Vertebrate hosts: ruminants (cattle).

Animal disease: an ephemeral fever-like illness in cattle (Tomori, Fagbami, & Kemp, 1974).

Human disease: unknown. BSL-2.

Geographic distribution: Africa (Nigeria).

6.3. Vesicular stomatitis—New Jersey virus

Taxonomy: genus *Vesiculovirus*.

History: first isolated by L. Mott from snout epithelium of an ill domestic pig in Jeff City, Georgia (USA) in 1952 (Karabatsos, 1985).

Arthropod vectors: insects—possibly sandflies, mosquitoes, simuliid flies (one isolation in Colombia, but it was not demonstrated whether the blackflies were biological vectors); occasionally isolated also from biting midges *C. variipennis* and *C. stellifer* in North America.

Vertebrate hosts: cattle, horse, pig, deer, raccoon (Yuill, 1981).

Animal disease: disease of cattle, horse, pig (including feral swine)—vesicular stomatitis: salivation, vesicle formation on snout, fever. Vesicular stomatitis is notifiable disease (OIE, 2012).

Human disease: exceptional cases. BSL-2.

Geographic distribution: United States, Canada (Manitoba 1937 and 1939), Mexico, Panama, Colombia, Venezuela, Ecuador.

6.4. Vesicular stomatitis—Indiana virus

Taxonomy: genus *Vesiculovirus*.

History: vesicular stomatitis was recognized in horses already during the Civil War in the United States, and in cattle in 1904 (Yuill, 1981). Vesicular stomatitis—Indiana virus (VSIV) was first isolated by W.E. Cotton from tongue epithelium of a cattle with vesicles on lips and tongue in Indiana but shipped from Kansas, 1925 (Karabatsos, 1985).

Arthropod vectors: sandflies (*Lutzomyia trapidoi*—TOT demonstrated), probably also mosquitoes.

Vertebrate hosts: ruminants.

Animal disease: disease of cattle and horse—vesicular stomatitis, with marked salivation, fever, inappetence, vesicles in the mouth, on lips and tongue, mastitis in cows, and lameness. Vesicular lesions after rupture and erosions heal quickly—within about 2 weeks. Vesicular stomatitis is a notifiable disease (OIE, 2012).

Human disease: exceptional cases. BSL-2.

Geographic distribution: United States (mainly south-western states), Mexico, Panama, northern South America.

6.5. Vesicular stomatitis—Alagoas virus

Taxonomy: genus *Vesiculovirus*. Sometimes regarded as VSIV subtype 3.

History: isolated by C. Moraes Andrade from tongue epithelium of a mule with vesicular lesions on tongue and feet in Alagoas (Brazil), 1964 (Karabatsos, 1985).

Arthropod vectors: sandflies and mosquitoes.

Vertebrate hosts: antibodies were found in horses, humans, monkeys, and bats in Brazil.

Animal disease: tongue vesicles and fever in cattle and equids.

Human disease: exceptional cases. BSL-2.

Geographic distribution: Brazil.

6.6. Cocal virus

Taxonomy: genus *Vesiculovirus*. Related to VSIV, sometimes regarded as VSIV subtype 2.

History: first isolated from *Gigantolaelaps* sp. mites collected from a rodent *Oryzomys laticeps* in Trinidad (Theiler & Downs, 1973), and later from horses with vesicular disease in Argentina (Yuill, 1981).

Arthropod vectors: probably mites, sandflies, possibly mosquitoes (experimental transmission demonstrated).

Vertebrate hosts: rodents (e.g., *Heteromys anomalus*, *Zygodontomys*, and *Oryzomys* spp.); possibly bats (long-term experimental viremia demonstrated in *Myotis lucifugus*).

Animal disease: vesicular lesions in cattle.

Human disease: unknown. BSL-2.

Geographic distribution: Trinidad, Panama, Brazil, Argentina.

7. FAMILY *ORTHOMYXOVIRIDAE*

7.1. Thogoto virus

Taxonomy: genus *Thogotovirus*. Prototype: Ken-IIA (mixed metastriate ticks, Kenya, 1960). African topotype: IbAr-2012 (*Boophilus* spp., Nigeria, 1964); European topotype: SiAr-126 (*Rhipicephalus bursa*, Sicily, 1969). Thogoto virus (THOV) shares only 15–20% nucleotide identity with influenza orthomyxoviruses. Virions are spherical, 80–120 nm, enveloped, contain ss(−)RNA arranged in six segments with a total size of 10 kbp, and one surface glycoprotein.

History: first isolated from a pool of *B. decoloratus* and *Rhipicephalus* spp. ticks collected on cattle in Thogoto Forest near Nairobi, Kenya in 1960 (Haig, Woodall, & Danskin, 1965). In Europe, it was first isolated from ticks collected on ruminants in Sicily, 1969 (Albanese, Bruno-Smiraglia, Di Cuonzo, Lavagnino, & Srihongse, 1972) and then in Portugal in 1978 (Filipe & Calisher, 1984).

Arthropod vectors: Arthropod vectors are metastriate ticks only—*B. decoloratus*, *B. annulatus*, *Amblyomma variegatum*, *R. appendiculatus*, *R. sanguineus* (Portugal), *R. bursa* (Sicily), *R. evertsi*, other *Rhipicephalus* spp., *Hyalomma truncatum*, and *H. a. anatolicum*.

Vertebrate hosts: cattle, camel, and man (isolations in Africa). Antibodies were also detected in sheep and goat.

Animal disease: afebrile leucopenia in cattle, and fever and abortion in sheep (Davies, Soi, & Wariru, 1984). Fatal to, and highly hepatotropic or pantropic in, adult mouse (Filipe, Peleteiro, Monath, & Calisher, 1986) and adult Syrian hamster (i.p.).

Human disease: sporadic but severe cases (usually laboratory infections). BSL-3.

Geographic distribution: THOV occurs in Nigeria, Kenya, Uganda, Ethiopia, Cameroon, Central African Rep., Egypt, Iran, Sicily, Portugal. Tick-infested domestic animals (e.g., camels) and migratory birds could disseminate the virus over a wide geographic range (Calisher, Karabatsos, & Filipe, 1987). Natural foci are boskematic—pastoral xerothermic ecosystems.

8. FAMILY *ASFARVIRIDAE*

8.1. African swine fever virus

Taxonomy: genus *Asfivirus*. The only DNA arbovirus pathogenic for animals. There are four antigenic types and 22 genotypes of African swine fever virus (ASFV), while no recognized prototype strain.

History: the virus was first isolated by R.E. Montgomery from a sick pig at Kabete (Kenya) in 1910 (Karabatsos, 1985), and the first extensive outbreak of ASFV with 100% mortality resulted when ASFV was transmitted from wild-African pigs to domestic pigs in 1921.

Arthropod vectors: soft ticks *Ornithodoros moubata* and *O. porcinus* (reservoir: TOT demonstrated—Plowright, Perry, & Peirce, 1970), while *O. erraticus* in North and West Africa and south-western Europe. However, contact infections among pigs are also very common.

Vertebrate hosts: common warthog *Phacochoerus africanus* (main host in the African sylvatic cycle), bushpigs *Potamochoerus porcus* and *P. larvatus*, giant forest hog *Hylochoerus meinertzhageni* (Jori & Bastos, 2009), and *Sus scrofa* (domestic and wild pigs) (Fig. 5.12). Usually, asymptomatically infected wild suids (except for *S. scrofa*) are amplifying hosts or even the reservoir of ASFV (Hess, 1971). Transportation of living pigs and infected pork meat play an

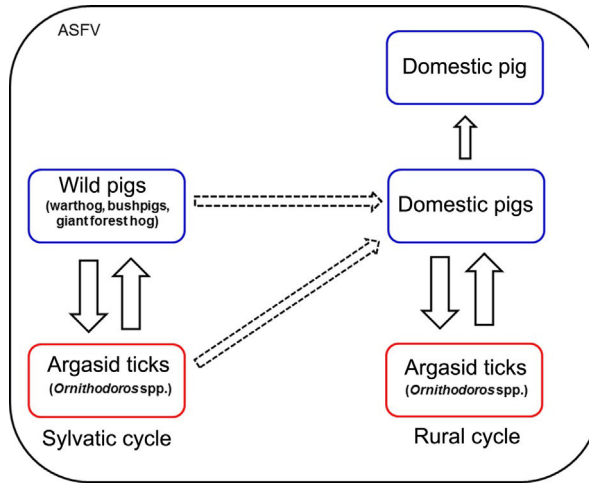

Figure 5.12 Schematic representation of the eco-epidemiological cycle of African swine fever virus.

important role in the epidemiology of ASF. In addition, ASFV is markedly resistant to inactivation under ambient temperature.

Animal disease: ASF is an acute or peracute, highly contagious and fatal pantropic disease of pigs, clinically resembling classical swine fever (hog cholera) of Europe and North America, with fever, cough, anorexia, lethargy, skin cyanosis, movement incoordination, diarrhea, and abortion; destruction of lymphoreticular elements, vasculitis, widespread hemorrhages (skin and visceral organs), thromboses, infarction, necrosis areas, hydropericardium, and hydrothorax (Schlafer & Mebus, 1984). Mortality rate (death within 10 days) is up to 100% with virulent strains in naive commercial pig populations, while some ASFV strains may produce mild disease and carriership. The virus can persist in the flesh of infected pigs for 5 months, when cooled. Interestingly, neutralizing antibodies do not appear in vertebrates after infection (while CF, IF, hemadsorbing, and precipitating antibodies do). African swine fever can have devastating impact: for example, during an epizootic in Central African Republic over 8000 pigs on two farms died in 2012. ASF is a major cause of loss in Africa and has an impact on the economic health of particular regions. ASF is a notifiable disease of pigs (OIE, 2012). Cattle, sheep, goat, dog, and rabbit are insusceptible (at s.c. or i.v. inoculation) though virus recovery was reported from rabbit and goat.

Prevention: there is no effective commercial vaccine against ASFV available at present—inactivated virus does not induce sufficient immunity and

attenuated ASFV causes chronic infection and carriership in pigs. Control measures for domestic cycle of ASF have been proposed (Penrith, Vosloo, Jori, & Bastos, 2013).

Human disease: unknown. BSL-2.

Geographic distribution: Africa (mainly sub-Saharan: East and South, but also West and North Africa), Madagascar, Mauritius (ASF genotype II, 2007), Portugal (an epizootic in 1957 caused by introduction of genotype I from Angola, and 1960, eradicated in 1993: Filipe, 1980), Spain (1960–1994, eradicated 1995: Oleaga-Perez, Perez-Sanchez, & Encinas-Grandes, 1990; Mur et al., 2012), Italy including Sardinia (1967, 1983: Swaney, Lyburt, & Mebus, 1987; ASF is still endemic in Sardinia), Malta; recently (since 2007), the Caucasian region (genotype II: Georgia, Armenia, Azerbaijan) and southern Russia (regions Chechnya, North Ossetia, Krasnodar, Saratov, Ingushetia, 2008–2011), central European Russia and Ukraine (2012–2013), Belarus (2013); temporarily also France (1964), Belgium (1985), the Netherlands (1986). In the early 1970s, ASF genotype I causing severe epidemics appeared in Brazil (1978–1981), and on some Caribbean islands (Cuba, Dominican Republic, Haiti: 1978–1984).

Natural foci of ASF (sylvatic cycle) are situated mainly in tropical and subtropical pastoral ecosystems and consist principally of a wild hog/pig-*Ornithodoros* cycle. Moreover, circulation in pig pens occurs in rural habitats. ASF has a complex epidemiology (Costard, Mur, Lubroth, Sanchez-Vizcaino, & Pfeiffer, 2013).

9. CONCLUSIONS

The 50 arboviruses reported here and known to cause disease in endogenous (homeotherm) vertebrate animals (those affecting exclusively man have been omitted) belong to seven families: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, and *Asfarviridae*.

They are transmitted to animals by hematophagous arthropods belonging to five groups of the subphyllum *Chelicerata* (order Acarina, families *Ixodidae* and *Argasidae*—ticks) or the class *Insecta*: mosquitoes (family *Culicidae*); biting midges (family *Ceratopogonidae*); sandflies (family *Psychodidae*, subfamily *Phlebotominae*); and cimicid bugs (family *Cimicidae*).

Arboviral diseases in endotherm animals may thus be classified as:

- tick-borne: louping ill and TBE, Omsk hemorrhagic fever, Kyasanur Forest disease, Tyuleniy fever, Nairobi sheep disease, Soldado fever, Bhanja fever, Thogoto fever, African swine fever;

- mosquito-borne: Eastern, Western, and Venezuelan equine encephalomyelitides, Highlands J disease, Sindbis disease, Middelburg disease, Getah disease, Semliki Forest disease, yellow fever, Japanese encephalitis, Murray Valley encephalitis, West Nile encephalitis, Usutu disease, Israel turkey meningoencephalitis, Tembusu disease (duck egg-drop syndrome), Wesselsbron disease, La Crosse encephalitis, Snowshoe hare encephalitis, Cache Valley disease, Main Drain disease, Rift Valley fever, Peruvian horse sickness, Yunnan disease;
- sandfly-borne: vesicular stomatitis—Indiana, New Jersey, and Alagoas, Cocal disease;
- midge-borne: Akabane disease, Aino disease, Schmallenberg disease, Shuni disease, African horse sickness, Kasba disease, bluetongue, epizootic hemorrhagic disease of deer, Ibaraki disease, equine encephalosis, bovine ephemeral fever, Kotonkan disease;
- cimicid-borne: Buggy Creek disease.

In addition to fever and various nonspecific signs, main clinical syndromes (groups of clinical symptoms) that occur in animals infected with particular pathogenic arboviruses are:

1. neurological (meningitis, encephalitis, encephalomyelitis): EEEV, WEEV, VEEV, BCRV, SINV, MIDV, JEV, MVEV, WNV, USUV, ITMV, TMUV, LIV, TBEV, OHFV, KDFV, TYUV, BHAV, LACV, SSHV, MDV, SHUV, EEV, PHSV, YUOV;
2. hemorrhagic: TMUV, OHFV, NSDV, RVFV, AHSV, EHDV, ASFV;
3. abortion and congenital disorders (CAHS): WSLV, NSDV, CVV, MDV, AKAV, AINV, SBV, RVFV, KASV;
4. vesicular stomatitis: VSIV, VSNJV, VSAV, COCV.

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PRÁCE 42

Rizzoli A., Silaghi C., Obiegala A., **Rudolf I.**, Hubálek Z., Foldvari G., Plantard O., Vayssier-Taussat M., Bonnet S., Špitálská E., Kazimírová M. 2014. *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health. *Frontiers in Public Health.* 2, 251.

Stručná charakteristika: minireview shrnující údaje o patogenech přenášených klíštaty *I. ricinus* v urbánním a periurbánním ekosystému a naznačující možné trendy v oblasti 'public health'. V review jsou shrnuty prevalence jednotlivých patogenů např. *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Rickettsia* spp. a '*Candidatus* Neoehrlichia mikurensis' v klíštěti *I. ricinus* v rámci Evropy včetně definování jednotlivých obratlovců jako hostitelů, případně rezervoárů onemocnění a podílících se na cirkulaci jednotlivých agens v urbánním či periurbánním ekosystému.

Hlavní přínos práce: jde o ucelený přehled mapující data o riziku přenosu patogenů klíštětem *I. ricinus* v urbánním a periurbáním biotopu, který může sloužit především expertům v oblasti 'public health' při nastavení preventivních a kontrolních opatření v oblasti klíšťaty přenášených nákaz.

Příspěvek autora k dané práci: autor se podílel zejména na podkapitole týkající se klíšťat *I. ricinus* jako přenašečů *B. burgdorferi* v urbánním ekosystému a také celkové revizi rukopisu.

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Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health

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Tick-borne diseases represent major public and animal health issues worldwide. *Ixodes ricinus*, primarily associated with deciduous and mixed forests, is the principal vector of causative agents of viral, bacterial, and protozoan zoonotic diseases in Europe. Recently, abundant tick populations have been observed in European urban green areas, which are of public health relevance due to the exposure of humans and domesticated animals to potentially infected ticks. In urban habitats, small and medium-sized mammals, birds, companion animals (dogs and cats), and larger mammals (roe deer and wild boar) play a role in maintenance of tick populations and as reservoirs of tick-borne pathogens. Presence of ticks infected with tick-borne encephalitis virus and high prevalence of ticks infected with *Borrelia burgdorferi* s.l., causing Lyme borreliosis, have been reported from urbanized areas in Europe. Emerging pathogens, including bacteria of the order Rickettsiales (*Anaplasma phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis," *Rickettsia helvetica*, and *R. monacensis*), *Borrelia miyamotoi*, and protozoans (*Babesia divergens*, *B. venatorum*, and *B. microti*) have also been detected in urban tick populations. Understanding the ecology of ticks and their associations with hosts in a European urbanized environment is crucial to quantify parameters necessary for risk pre-assessment and identification of public health strategies for control and prevention of tick-borne diseases.

Keywords: ticks, *Ixodes ricinus*, tick-borne pathogens, urban habitats, Europe

INTRODUCTION

Tick-borne infections are arthropod-borne diseases frequently reported worldwide. Ticks are known to transmit a great variety of pathogenic agents producing the highest number of human disease cases compared to other vector-borne diseases in Europe (1, 2). In general, the eco-epidemiology of zoonotic vector-borne diseases is very complex. It depends on the interactions of the vectors with the reservoir hosts and the pathogenic agents, which are modulated by several abiotic and biotic factors that vary in space and time. Certain tick-borne infections have recently been emerging in new regions or re-emerging within endemic sites and create an increasing concern for public health, food security, and biodiversity conservation (3–5). Global warming obviously affects the spread of tick-borne diseases, but climate alone does not determine the geographical distribution of tick species, their population densities and dynamics, the likelihood of their infection with microorganisms pathogenic for humans and animals, nor the frequency of contacts of humans and domestic animals with infected ticks (4, 6, 7). Socio-demographic factors, agricultural and wildlife management, deforestation and reforestation, are known to exert a big impact on the transformation of biotopes, thus affecting tick host assemblages as well as tick infection rates (8–10).

Urbanization as one of the socio-demographic factors has increased worldwide in recent decades (11, 12). Currently, more than half of the world's population lives in urban areas, and it is expected that 70% will live in urban areas by 2050 (13). Nowadays, more than 75% of Earth's ice-free lands show evidence of alteration as a result of human residence and land use, with less than a quarter remaining as wildlands. Europe shows the highest level of urbanization worldwide (14). Urbanization, due to restriction of natural areas, is known to dramatically change the composition of wildlife communities and affect the associated tick populations. In European cities, public parks, gardens, peri-urban

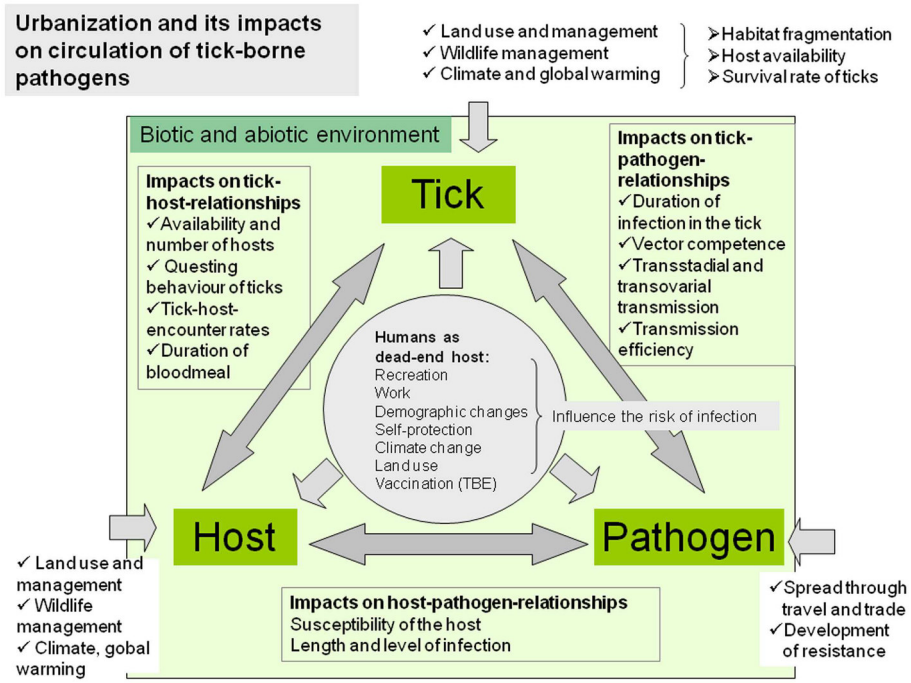

FIGURE 1 | Complex factors of the biotic and abiotic environment influence the tick–host–pathogen interaction and consequently the occurrence of tick-borne diseases in urban and peri-urban environments.

leisure-time areas, and cemeteries became particularly important places where humans and domesticated animals can encounter potentially infected questing ticks (2).

Urban areas are highly fragmented environments composed of a mosaic of patches of various sizes, vegetation, and land-use types. Urban and peri-urban habitats are generally characterized by lower biodiversity of wildlife species compared to natural ecosystems. Urbanization often produces a certain gradient of homogenization in densely built-up areas, where synanthropic species adapted to urban habitats can be found and where species richness is reduced (15). On the other hand, suburban habitats are also occupied by native species comprising medium-sized mammalian predators and ground-foraging, omnivorous, and frugivorous birds that produce abundant populations there. But urbanization can also result in variation of animal species composition, e.g., by introduction of non-native species that replace native ones (16, 17).

Majority of the wildlife species commonly found in urban and peri-urban sites can serve as tick-maintenance hosts and also as reservoirs of tick-borne pathogens (18, 19). Furthermore, the majority of these species are generalists and are able to adapt to the urban and peri-urban environment and reach higher population densities than in natural sites (12, 20, 21). In urban habitats of Europe, rodents (mice, voles, dormice, squirrels, and rats), hedgehogs, shrews, birds, lizards, and companion animals (dogs and cats), but in peri-urban areas also medium-sized and larger mammals like foxes, roe deer, and wild boars, play the major role as tick-maintenance hosts and reservoirs of tick-borne pathogens (19, 22). Adaptation of wild animals to urban environment can also lead to increased contacts with humans and to increased risk of exposure to zoonotic agents. In addition, animal populations in urban areas can show genetic differentiation from wild populations of the same species. Thus urbanization can alter the biology and population densities of ticks and hosts and may lead to increased transmission of pathogens between vectors and urban-adapted hosts (11, 23). Moreover, urbanization is followed by increased mobility of humans, intensive long-distance trade, and new contacts of humans and companion animals with nature, which may contribute to changing of epidemiological and epizootiological conditions in urban and peri-urban areas (12) (**Figure 1**).

Understanding the ecology of ticks and their association with various hosts in a changing urban and peri-urban European environment is therefore crucial to quantify various parameters necessary for the risk pre-assessment and for the identification of the best public health strategies for tick-borne disease management and prevention. The cascade of events including fluctuations in wildlife community composition and abundance, tick density and emergence, and spread of tick-borne pathogens in various habitat types in Europe are now being modeled as part of the EU FP7 project EDENext[1]. In this review, we focus on *Ixodes ricinus*, one of the principal vectors of pathogens causing arthropod-borne infections in Europe, its associations with hosts and pathogens and risk of infection of humans in urbanized areas.

[1]http://www.edenext.eu

IXODES RICINUS – VECTOR OF MULTIPLE PATHOGENS

Ixodes ricinus (Acari: Ixodidae) is the most widespread tick species in Europe and transmits several viral, bacterial, and protozoan agents of medical and veterinary importance (8, 24–28).

The distribution area of *I. ricinus* has significantly expanded over the past decades. Recently, the species can be found in more northern areas and habitats at higher altitudes than a few decades ago (29–31). Increase in abundance, habitat expansion, including urbanized areas, and prolongation of the questing activity periods of *I. ricinus*, reported in recent years, are attributed to multiple and interacting factors (19, 26, 32). They include changes in land cover and land use due to alterations in agriculture and forestry management, changes in climate, changes in abundance, and distribution of wildlife due to altering wildlife management, and shifts in socioeconomic factors affecting the rate of exposure of humans to infected ticks (25, 26).

Risk factors associated with *I. ricinus* transmissible diseases can be divided generally into: (i) those directly related to climate change (acting on the tick, the host, or their habitats), (ii) those related to changes in the distribution of tick hosts (which may be a direct or indirect effect of human intervention), and (iii) other ecological changes (also commonly influenced by human intervention) (26).

Ixodes ricinus is primarily associated with shrubs and deciduous and mixed forests, with a high abundance of small, medium, and large wild vertebrate hosts. However, as a consequence of changing land use and wildlife management, persistent tick populations and high prevalences of infections with tick-borne pathogens have also been observed in urban and peri-urban sites in many European countries (33–41). *Ixodes ricinus* is a generalist exophilic tick species that is able to feed on over 300 different vertebrate species (42). It has a long-lasting life cycle, involving three active life stages (larvae, nymphs, and adults) that quest and attach to a host and feed on blood for a few days before detachment (parasitic life period) and subsequent molting or laying eggs (females). Each developmental stage requires its specific microhabitat comprising various biotic and abiotic factors. The parasitic on-host life of *I. ricinus* is limited to 3–5 days (larvae), 4–7 days (nymphs), and 7–11 days (females) of feeding on vertebrate hosts, whereas, the non-parasitic off-host life period of all developmental stages can last for several months or years (43). This extremely complex life cycle makes the tick vulnerable to alterations in habitat structure and availability of host animals.

In urban and peri-urban areas, the requirement for high relative humidity (above 80%) for extended periods of time by the off-host stages restricts the occurrence of *I. ricinus* to city parks with litter layers, forest patches, gardens, and cemeteries (22) where the continuous use of water to maintain the vegetation also increases the relative humidity. The other limiting biotic factor for *I. ricinus* in urban environments is the availability of medium-sized and large mammals as hosts of the adults, maintaining persistent and independent tick populations. Shifts in the tick–host associations to, e.g., hedgehogs, foxes, hares, domestic dogs, or cats, due to lack of large mammalian hosts can evoke changes in *I. ricinus*-borne pathogen spectrum, prevalence, and distribution. On the other hand, populations of large animals like deer and wild boar have become more abundant in large city parks and peri-urban areas around European cities, leading to the establishment of tick populations, shift of natural transmission cycles of some pathogens, and increase of the disease risk for humans and domestic animals (19).

VERTEBRATE HOSTS OF TICKS AND TICK-BORNE PATHOGENS IN URBAN AREAS

Terrestrial vertebrate hosts are key players in the epidemiology of tick-borne diseases for at least two reasons. Firstly, they serve as maintenance hosts for ticks as a food resource and secondly, as reservoir hosts they are often responsible for the long-term maintenance of pathogens in both natural and urban habitats. Although many reports exist about the presence of pathogens in various hosts or ticks removed from them, the reservoir capacity for each of the pathogens in many cases remains to be experimentally defined. A reservoir host of tick-borne pathogens must fulfill certain criteria: (i) it must feed infected vector ticks, at least occasionally; (ii) it must take up a critical number of infectious agents during an infectious tick bite; (iii) it must allow the pathogen to multiply and to survive in at least certain parts of its body; and last but not least (iv) the pathogen has to find its way into other feeding ticks (44, 45). For this reason, the simple recording of pathogens (or nucleic acid of them) in a vertebrate host is not sufficient for classifying that host as a reservoir, but only a candidate reservoir when physiological and behavioral features may theoretically support pathogen amplification and transmission to the vector, or a simple carrier host, or a dead end host. Similarly, a higher prevalence in ticks removed from the vertebrate host compared to prevalence in questing ticks is only a good indication that the host is a candidate reservoir. However, to unambiguously prove the reservoir status of a host, xenodiagnostic experiments have to be carried out. They involve feeding of specific pathogen-free tick larvae from a laboratory colony on the tested host and the subsequent analysis of them for pathogens after their molt into the next stage. Unfortunately, for most pathogens and hosts, xenodiagnostic experiments have not been performed so far and the key hosts in the natural (and urban) cycle of tick-borne pathogens remain to be tested. The few exceptions are some species of mice, voles, rats, dormice, squirrels, and shrews (see details in **Table 1**) that had already been proven reservoirs of some tick-borne pathogens.

Urban environments represent many special ecological characters in the complex communities of pathogens, ticks, and hosts. From a public and veterinary health perspective, city parks and peri-urban recreational areas are typical meeting places for humans (their pets) and ticks. Ticks in this respect serve as a bridge for pathogens, connecting reservoir hosts with humans. In addition to the frequent and likely encounter of humans with ticks, vertebrate host communities also differ substantially in many urban habitats compared to natural settings. Some important tick-maintenance and pathogen reservoir hosts (e.g., hedgehogs, squirrels, and songbirds) have no or very few natural enemies within urban environments, thus their populations might reach significantly higher densities compared to natural ones (21, 74). Besides the lack of predators, these urbanized vertebrates can also make use of man-made structures and anthropogenic food resources, like waste and pet food. Hedgehogs are one of the most successful urban adapters reaching up to nine times higher densities in urban areas than in rural areas (74). In Great Britain, red fox density was

Table 1 | Most important mammal hosts of *I. ricinus* and pathogens transmitted by this tick species with urban or peri-urban occurrence.

Order	Species	Associated *I. ricinus* stage	Associated pathogens	Reference
Rodentia	*Apodemus flavicollis*	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 46–50)
	Apodemus sylvaticus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 46, 48–52)
	Apodemus agrarius	L, N	***Borrelia afzelii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* ***Babesia microti***	(42, 50, 53)
	Myodes glareolus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 48–50, 54, 55)
	Microtus agrestris	L, N	**TBEV** *Borrelia afzelii* *Babesia microti* *Cand.* N. mikurensis *Anaplasma phagocytophilum*	(42, 49–51, 56)
	Microtus arvalis	L, N	*Cand.* N. mikurensis *Anaplasma phagocytophilum* *Babesia microti*	(53, 55, 56)
	Rattus norvegicus	L, N	***Borrelia afzelii*** ***Borrelia spielmanii***	(46, 57)
	Rattus rattus	L, N	*Borrelia afzelii* *Anaplasma phagocytophilum*	(46, 50, 57)
	Eliomys quercinus	L, N	***Borrelia spielmanii***	(46)
	Muscardinus avellanarius	L, N	***Borrelia spielmanii***	(58)
	Glis glis	L, N	**TBEV** *Borrelia afzelii*	(42, 51)
	Sciurus carolinensis	L, N	***Borrelia afzelii*** *Borrelia burgdorferi* s.s.	(42, 59)
	Sciurus vulgaris	L, N	**TBEV** *Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*	(51, 60, 61)
	Eutamias sibiricus	L, N	*Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*	(62)

(Continued)

Table 1 | Continued

Order	Species	Associated *I. ricinus* stage	Associated pathogens	Reference
Lagomorpha	*Lepus europaeus*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*	(50, 63)
	Lepus timidus	L, N, A	*Borrelia burgdorferi* s.l.	(63)
Soricomorpha	*Sorex araneus*	L, N	**TBEV** *Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum* *Babesia microti*	(49–51, 63)
	Sorex minutus	L, N	*Borrelia burgdorferi* s.l.	(63)
Erinaceomorpha	*Erinaceus europaeus*	L, N, A	*Borrelia afzelii* *Borrelia spielmanii* *Borrelia bavariensis* *Anaplasma phagocytophilum*	(64–68)
	Erinaceus roumanicus	L, N, A	**TBEV** *Borrelia afzelii* *Borrelia bavariensis* *Anaplasma phagocytophilum* *Cand.* N. mikurensis	(47, 64, 69)
Artiodactyla	*Capreolus capreolus*	L, N, A	*Anaplasma phagocytophilum* *Babesia venatorum*	(70)
	Cervus elaphus	L, N, A	*Anaplasma phagocytophilum*	(71)
	Dama dama	L, N, A	*Anaplasma phagocytophilum*	(71)
Carnivora	*Vulpes vulpes*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*	(42, 72)
	Meles meles	L, N, A	*Borrelia afzelii* *Borrelia valaisiana*	(73)

Mammal species that are experimentally proven reservoirs for pathogens are in ***bold****. Borrelia burgdorferi s.l. refers to studies with no species identification (genotyping) of the spirochetes. L, larva; N, nymph; A, adult.*

found at least 10-fold higher in cities than in rural areas (75, 76). The tendency to preserve green spaces inside cities is not only a positive aspect to humans but also for many tick-maintenance and reservoir hosts (12). For these urban dwellers, well established and dense shrubbery in parks offers shelter and nest sites. Furthermore, higher temperatures, especially during winter (heat island effect), are highly beneficial (74). All these factors can lead to an unbalanced vertebrate community that easily provides favorable ecological conditions for tick and pathogen cycles.

MAMMALS

Rodents are among the most important maintenance hosts for the subadult stages of *I. ricinus* (77). Furthermore, as pointed out by a recent analysis (78), ecologically widespread, synanthropic species with high density and fast life history such as rodents are often the most competent reservoirs for multi-host pathogens. As a consequence, mice and voles are also known to be important reservoirs for several pathogenic agents like tick-borne encephalitis virus (TBEV), *Borrelia afzelii*, and "*Candidatus* Neoehrlichia mikurensis" (**Table 1**). In addition to well-established rodent populations in cities, the frequent migration of these animals between human dwellings and natural environments can easily bring infected larvae and nymphs of *I. ricinus* into gardens and houses (79). Fluctuations in rodent densities are very important factors of disease risk (24, 80) with different ecological factors affecting rodent population dynamics in different parts of Europe. However, rodent population dynamics are less studied in urban and peri-urban parks than in natural areas. Rodents can harbor different endophilic (nidicolous) tick species (e.g., *Ixodes trianguliceps* and *I. acuminatus*). These do not pose a direct human hazard since they do not feed on humans. Their co-occurrence with *I. ricinus* on the same rodent, however, can lead to an exchange of pathogens among different tick species.

Little is known about the role of rats (*Rattus rattus* and *R. norvegicus*) in the urban maintenance of ticks and tick-borne pathogens. As one of the most efficient urban adapters, despite the control actions usually undertaken, they might be involved in the urban maintenance of Lyme borreliosis (LB) spirochetes (46, 57, 77). Other urbanized rodents, like garden dormice (*Eliomys quercinus*), hazel dormice (*Muscardinus avellanarius*) (46, 58) and hedgehogs (*Erinaceus europaeus* in Western Europe and *E. roumanicus* in Central and Eastern Europe) are also involved in the urban ecology of LB (**Table 1**). Hedgehogs have not only a longer life span compared to rodents but they also have the great

advantage for ticks being able to feed not only larvae and nymphs but also a considerable number of adults (21, 81). Thus, they can easily maintain stable *I. ricinus* populations in urban areas in the long run (64).

In some cases, anthropogenic introduction of mammals into a new area can lead to the emergence of tick-borne pathogens even previously unknown for that region (12). The gray squirrel (*Sciurus carolinensis*) is native to North America, but an invasive species in the UK that has spread across the country and has largely displaced the native red squirrel (*S. vulgaris*). This species is a frequent urban dweller and can be an indirect source of human tick-borne infections since it has been experimentally shown to be reservoir for *B. afzelii* (59). Siberian chipmunks (*Eutamias sibiricus*) appeared as pets in many European countries but soon these rodents were recorded in urban parks of Rome (82, 83), Geneva (84), Brussels, and in and around many other towns (12). Chipmunks seem to be perfect hosts for subadult *I. ricinus* (85). Pisanu et al. (86) showed that these introduced rodents are more heavily infested by *I. ricinus* than native rodents such as the wood mouse (*Apodemus sylvaticus*) and the bank vole (*Myodes glareolus*). It was also found that the introduced rodent is associated with three species of *B. burgdorferi* sensu lato (s.l.), whereas, the native rodents are associated with only one species (62).

Lagomorphs (hares and rabbits) also inhabit anthropogenic landscapes and serve as blood sources for ticks (79). The European hare (*Lepus europaeus*) and the mountain hare (*L. timidus*) were shown to be not only effective tick-maintenance hosts but also reservoirs for *B. burgdorferi* s.l. (63). The European rabbit (*Oryctolagus cuniculus*) belongs to the most invasive mammalian species and its urban populations can harbor a variety of endo- and ectoparasites including *I. ricinus* (87). These lagomorphs can reach high densities and due to their ability to host adult *I. ricinus* as well, they are able to maintain an infective tick population even in urban and suburban areas where large mammals are not necessarily present. This double epidemiological function (tick-maintenance and reservoir host), which makes them key players in urban cycles of tick-borne pathogens is unique for lagomorphs and hedgehogs.

Bats can also carry different stages of *I. ricinus* ticks, thus they can also transport ticks to urban areas (88). Species especially adaptive in human dwellings, e.g., the lesser horseshoe bat (*Rhinolophus hipposideros*), can serve as tick-maintenance hosts but the role of these flying mammals in the pathogen life cycles remains to be clarified (54). Experimental TBEV viremia was shown in the greater mouse-eared bat (*Myotis myotis*), which is also a common urban inhabitant (51).

Among larger mammalian hosts, which can affect the circulation of tick-borne pathogens in peri-urban areas, roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), and red foxes (*Vulpes vulpes*) are particularly important, because they can host all three active life stages of *I. ricinus*, and they increasingly live in urbanized areas (89, 90). Studies on roe deer abundance and movements can provide critical information for predicting tick dispersal and TBEV hazard (91, 92). Deer density is also suggested to be related to the LB incidence (31).

Tick density can be influenced by abundance and distribution of roe deer and red deer (*Cervus elaphus*) (93–95). Roe deer and red deer can inhabit a great variety of tick-infested habitats. Roe deer can even occur in some city parks, e.g., in Munich, Germany (70). Furthermore, the ability of deer to migrate more than 100 km carrying a high number of ticks is also known. This may facilitate the spreading of ticks to other areas (95, 96) and therefore potentially also of tick-borne pathogens from one area to another, although for some pathogen such as *Borrelia* spp., these species dilute the infection rate (97).

Wild boar populations have increased in Europe in recent decades and these animals are well adapted to live in urban and suburban forest areas (98). This species can provide a significant contribution to maintaining tick populations, although its role of reservoir of various tick-borne pathogens is only partially known (98, 99).

Foxes inhabit most urban areas across Europe and population increases have been seen in many European countries, e.g., in Great Britain and Switzerland (100, 101). In a recent study, *I. ricinus* was the most frequently detected tick on foxes in Germany, and all stages of this tick species were found on the animals (90). In Romania, *I. ricinus* infested almost 30% of foxes, indicating that these animals may play a significant role in the epidemiology of tick-borne diseases (102).

Urbanization largely concentrates humans in an area as well as a high number of pets (12). Among these, stray dogs represent an especially effective host for ticks, many of which are *I. ricinus* adults (103). They not only roam in large areas connecting natural and urban habitats, but they also get minimal or no treatment against ticks. Although we have limited knowledge on dogs' role in the maintenance of tick-borne human pathogens (104–106), as effective hosts for *I. ricinus* adults they certainly contribute to the size of tick populations within gardens, parks, and sub-urban areas. The estimated 100 million free roaming dogs (owned and stray) living in Europe (107) certainly need to be taken into consideration during urban surveillance and control of ticks and tick-borne diseases.

BIRDS

Birds play an important role in the introduction of ticks and associated pathogens into urban areas (108, 109). Birds, especially ground-feeding song birds, are important maintenance hosts for larval and nymphal stages of *I. ricinus*. Common urban bird species foraging mostly on the ground and low shrub vegetation, such as common blackbird (*Turdus merula*), song thrush (*Turdus philomelos*), and European robin (*Erythacus rubecula*) were shown to be frequently infested with *I. ricinus* (110–112). More specifically, migratory birds have been shown to carry ticks and pathogens to large distances (113). However, the knowledge on the role of migratory birds in favoring the introduction of ticks and pathogens within new sites is so far very limited (114). Furthermore, earlier onset of spring migration and reproduction with more active ground-feeding activity of birds in the period of questing activities of *I. ricinus* larvae and nymphs may represent an additional risk factor for TBEV spread (115, 116). A recent study highlighted that migratory bird species were infested by more ticks than residents, with urbanized birds being the most parasitized (117). Thus in case of cities being close to bird resting or breeding sites (like cities and towns located on river banks) there is a

realistic chance for the introduction and the maintenance of tick-borne pathogens (12). Birds as carriers of infected ticks probably play a role in the geographical spread of pathogens, such as *Rickettsia helvetica*, *Anaplasma phagocytophilum*, *Babesia microti*, and *B. venatorum* (118–120).

LIZARDS

Lizards have long been known as important hosts for ticks capable of feeding large amounts of immature *I. ricinus* (121) and they can often find suitable habitats in cities. In areas inhabited by lizards they can be as important tick-maintenance hosts as rodents (122, 123). Compared to rodents, however, lizards are more suitable hosts for nymphal *I. ricinus* (as shown by a lower larva/nymph ratio) (124–126). Sand lizards (*Lacerta agilis*), common wall lizards (*Podarcis muralis*), and green lizards (*Lacerta viridis*) are the most common species that can contribute to the urban maintenance of *I. ricinus* populations (122, 123, 125).

The role of lizards in the circulation of tick-borne pathogens has been underestimated compared to that of mammals and birds, but they have been proved to be reservoirs of LB spirochetes (122) and might also be involved in the life cycle of other tick-borne pathogens (124). However, experimental and field studies are needed to shed light on this epidemiological issue.

PATHOGENS TRANSMITTED BY *IXODES RICINUS*

Among the pathogens transmitted by *I. ricinus*, the western European TBEV subtype (TBEV-Eur), causing tick-borne encephalitis (TBE) (127) and spirochetes of the *B. burgdorferi* s.l. complex, the causative agents of human LB (128) have the greatest impact on human health. *I. ricinus* can also harbor bacteria of the order Rickettsiales that are of rising medical and veterinary importance. Among them, *Anaplasma phagocytophilum* can lead to granulocytic anaplasmosis in both humans and animals (50); the emerging pathogen "*Candidatus* Neoehrlichia mikurensis" can cause severe febrile illness in immunocompromised patients (129) and fever in humans without any primary disease (130); rickettsiae of the spotted fever group (SFG) (*Rickettsia helvetica*, *R. monacenis*) cause rickettsioses in humans (131). Protozoans of the genus *Babesia*, mainly *B. divergens* and *B. microti*, cause babesiosis in humans, and for *B. venatorum* pathogenicity to humans is suspected (132). The role of *I. ricinus* in transmission of *Bartonella* species (e.g., *B. quintana* and *B. henselae*) causing bartonellosis in humans is suspected (28, 133). *Francisella tularensis*, causing tularemia, and the Q fever agent *Coxiella burnetii* have also been detected in *I. ricinus*, but the role of this tick species in the epidemiology of these diseases is probably not significant (28, 133).

TICK-BORNE ENCEPHALITIS VIRUS

Tick-borne encephalitis is the most important tick-borne arboviral infection of humans in Europe and eastern and central Asia and is caused by the TBEV (Flaviviridae) (134–136). *Ixodes ricinus* is the principal vector for the western European (TBEV-Eur) subtype of the virus (127, 137). TBE is now endemic in 27 European countries (138) and its expansion northward and into higher altitudes has been observed in recent years (137, 139). There is a considerable lack of knowledge in the current fine scale spatial distribution of TBE, including urban areas, thus the risk of infection is still underestimated, especially considering that about two-thirds of human TBE infections are asymptomatic (135).

Incidence of TBE in Europe has been changing in a heterogeneous manner during the last decades, with spatial expansion in some areas and decrease in others (140–142). TBE ecology and epidemiology is expected to be affected considerably by climate change (143) and other drivers like changing in land-use patterns, expansion of forest coverage, increase of abandoned areas, and the creation of new suitable and fragmented landscapes for ticks and hosts within urban areas. Exposure to infected ticks is dependent on several and regionally variable socio-economical factors such as recreational and occupational human activities, public awareness, vaccination coverage, and tourism (26, 94, 144).

The majority of human TBE infections are acquired through bites of infected ticks, more rarely by the alimentary route through consumption of raw milk of infected goats, sheep, or cattle, or unpasteurized dairy products (145–147). As organic markets become more popular, city dwellers also have to be aware of the TBEV infection risk associated with unpasteurized cow and goat milk and milk products.

Tick-borne encephalitis incidence appears to be increasing, including urban areas, partially as a result of improvements in the diagnosis and reporting of TBE cases, but also due to increased exposure of humans to TBE due to outdoor activities. The risk of exposure to TBE was found to be relatively high even in the immediate surroundings of patients' homes, e.g., in the Czech Republic (148), and an enhanced surveillance of TBE cases in Poland revealed that more than 50% of patients resided in urban areas (149).

Tick-borne encephalitis virus circulates mainly in natural sylvatic cycles involving vector ticks and reservoir hosts. However, due to expansion of urban sites to previously natural habitats and penetration of small and large wild animals into urban areas, reservoir hosts for TBEV as well as large tick-maintenance hosts can be present also in urban and peri-urban sites and thus ensure circulation of the virus there (150). Ticks remain infected throughout their life and it is suggested that they are not only vectors, but also long-term reservoirs of the virus (151). Rodents (*A. flavicollis*, *A. sylvaticus*, *M. glareolus*, and *M. arvalis*, see **Table 1**) are important reservoir hosts for TBEV-Eur (152, 153) and probably may maintain the virus in nature through latent persistent infection (154, 155). Co-feeding tick to tick transmission of TBEV, even in the absence of detectable viremia in these rodent species (156), is crucial to explain the focal distribution of the TBE foci and their potential variation over time (157). Experimental TBEV viremia has been demonstrated also in two lizard species (*L. viridis* and *L. agilis*) often occurring in urban areas (51), but field data on their reservoir competence for TBEV are missing. Migratory birds may play an important role in the geographic dispersal of TBEV-infected ticks, which can contribute to the emergence of new foci of disease, including gardens and urban parks, in case abiotic conditions and the vertebrate host spectrum are favorable for the maintenance of the pathogen (158). Among birds, thrushes (*Turdus* spp.) are the most frequently infested with *I. ricinus* ticks and also carry the most frequently infected ticks (159), however, the prevalence of TBEV-infected bird-feeding ticks is relatively low.

Wild and domestic ungulates, carnivores (foxes and dogs), and hares frequently occurring in peri-urban parks and forest patches within urbanized areas, are important actors in the dynamics of TBE, mainly as tick-maintenance hosts and carriers of infected ticks (160–162). Variation in abundance of roe deer was found to considerably affect TBE risk, depending on the threshold densities of tick, rodent, and large vertebrate populations in the area (31, 91, 92, 163). Ungulates probably do not contribute to the amplification of the virus, but may serve as sentinels to identify TBE foci (163, 164).

Accompanying dogs also represent an important risk factor for humans to acquire TBE. They are accidental hosts, but can become ill with TBE. In addition, during walking in natural forest or hunting activities, dogs come in contact with infected ticks and can carry them home or to urban parks, where they may later infest humans (165).

In general, data on TBEV prevalence in tick populations and seroprevalence in reservoir and sentinel hosts in urban areas and on the circulation of various virus strains in Europe are scarce (166–169). Furthermore, our knowledge on the mechanism favoring TBEV persistence and amplification in urban sites is very limited. TBEV infection rate in ticks is usually very low (<1%) (170–172), but can amount up to 15% in microfoci (173). TBEV-positive *I. ricinus* ticks have recently been detected, e.g., in a highly urbanized region in Southern Poland (estimated pool prevalence ranging from 0.19 to 1.11% for positive locations), suggesting the presence of active foci (174). TBEV-infected *Dermacentor reticulatus* adults were also detected in an urban area (Warsaw) in Poland, with higher prevalence (3.12%) than in natural areas. But our knowledge about the importance of this tick species in TBE epidemiology is still limited (175).

Generally, screening of ticks by PCR cannot be recommended for assessment of human TBE risk and alternative methods of environmental TBEV monitoring should be considered, such as serological long-term monitoring of rodents and other wild and domestic animals, which would serve as sentinel species (169).

BORRELIA BURGDORFERI SENSU LATO

In little more than 30 years, Lyme borreliosis (LB), which is caused by the spirochete *B. burgdorferi* s.l., has risen from relative obscurity to become a global public health problem and a prototype of an emerging pathogen (176). During this period, we have accumulated enormous progress in knowledge of its phylogenetic diversity, molecular biology, genetics, host interactions, pathogenicity for humans as well as other vertebrate species, and preventive measures including vaccine development. But relatively little is known about public health consequences of LB in terms of eco-epidemiology issues and risk of acquiring infection in suburban and urban habitats.

Lyme borreliosis is the most abundant tick-borne disease of humans worldwide, though it only occurs in the northern hemisphere. LB occurs in North America (from the Mexican border in the south to the southern Canadian provinces in the north), the whole Europe, parts of North Africa (Maghreb), and northern Asia (Russian Siberia and the Far East, Sakhalin, Japan, China, and Korea). The geographical distribution of LB correlates closely with the range of the principal vectors, ticks of the *I. ricinus* complex (177). LB occurs between approximately 35° and 60°N in Europe, and between 30° and 55°N in North America. In countries at the southern limits of the LB range, its incidence decreases rapidly along the north-to-south gradient (178).

The *B. burgdorferi* s.l. complex now comprises up to 19 *Borrelia* species. Of these, only *B. afzelii*, *B. burgdorferi*, and *B. garinii* are proven agents of localized, disseminated, and chronic manifestations of LB in Europe, whereas, *B. spielmanii* has been detected in early skin disease, and *B. bissettii* and *B. valaisiana* have been detected in samples from single cases of LB (179, 180). The clinical role of *B. lusitaniae* remains to be substantiated.

Principal vectors of *B. burgdorferi* s.l. in Europe, including urban and suburban ecosystems, are two tick species: *I. ricinus* and *I. persulcatus*, the latter only occurring in eastern and north-eastern Europe. Moreover, the occurrence of *I. hexagonus* in the urban environment, due to the presence of suitable hosts, such as hedgehogs, cats, dogs, and foxes in gardens and public parks, could contribute to transmission of LB (65).

The risk of infection is particularly high in deciduous or mixed forest ecosystems or woodlands, along with city parks and urban gardens, especially gardens close to forests (181). The higher risk of contracting LB in the ecotones between forests and arable fields (178) or meadows, although higher densities of infected vector ticks are within forests, is an effect of frequent human presence along the edges of these habitats (182). Also forest fragmentation in suburban areas theoretically poses a greater risk due to enhanced proportion of ecotones (183). Other risks include reforestation (with increased population of forest rodents, but also deer, the principal host of adult vector ticks). For example, in the Czech Republic Zeman and Januska (184) found that LB risk correlated with overall population density of game (red deer, roe deer, mouflon, and wild boar) regardless of rodent abundance. Nevertheless, increased populations of reservoir hosts (forest rodents) usually stimulate the LB incidence.

All activities that increase human contact with ticks present risk for contracting LB, especially recreational (leisure time) activities in forested and urban areas (jogging, berry/mushroom picking, walking, and hiking), seasonal and occasional living by urban residents in country cottages, mowing and clearing of brush around the home in forested areas and gardening. Ownership of pet dogs and cats could also present a relative risk for humans when the pets are frequently parasitized by ticks and the owner tries to remove the ticks (178, 181). Moreover, outdoor employment and work (forestry workers, military personnel in the field, farmers, gardeners, gamekeepers, hunters, and rangers) are at risk. However, in most European countries, occupational exposure generally constitutes only 2% of LB cases (185), whereas, permanent residence in endemic areas with a high prevalence of infectious ticks (e.g., forested peri-urban areas) is a serious risk factor for LB.

Small rodents (*A. sylvaticus*, *A. flavicollis*, and *M. glareolus*) are regarded as the main reservoir hosts of LB pathogens in urban and suburban habitats across Europe (**Table 1**). Garden dormice (*E. quercinus*) (186) and hazel dormice (*M. avellanarius*) are especially competent reservoirs of the human pathogenic *B. spielmanii* (46, 58). Important role in the urban maintenance of *B. spielmanii* and *B. afzelii* could also be played by rats (*R. norvegicus* and *R. rattus*) (46, 57, 187). Other key urban players in the maintenance

of LB spirochetes are hedgehogs (*E. europaeus* and *E. roumanicus*) (64, 65, 188). Red squirrels (*S. vulgaris*) were found to be heavily infested by ticks and feeding ticks showed high prevalence of infection in enzootic areas in Switzerland (60) and might consequently contribute to maintenance of spirochetes also in urban foci.

Dogs and cats are heavily infested with ticks and might act as hosts (probably not reservoirs) or sentinels for LB. The risk of exposure of dogs to numerous vector-borne pathogens has increased, and close relationship with humans in urban areas poses new concerns for human public health (106).

Ground-foraging bird species such as blackbird (*T. merula*), song thrush (*T. philomelos*), robin (*E. rubecula*), and pheasant (*Phasianus colchicus*) play a unique role in the epidemiology of LB and also contribute to the transmission cycle of *B. burgdorferi* s.l. in urban and suburban areas (189–192). Due to their specific immunity (complement system), certain bird species are resistant to some LB spirochetes but susceptible to others (193). They usually carry *B. valaisiana* and *B. garinii* and transmit these spirochetes to ticks. In 1998, two xenodiagnostic studies clearly defined the reservoir role of birds in the epidemiology of LB, one on a passerine bird, the blackbird (190), the other on a gallinaceous species, and the pheasant (194). However, the reservoir competence of other bird species needs to be clarified. A recent study showed that circulation of LB spirochetes is partly maintained by bird-specific tick species, and bridged by *I. ricinus* to other host types (195).

The role of lizards in the maintenance of *B. burgdorferi* s.l. is still controversial, since several lizard species have been shown to possess a complement with borreliacidal activity (196). However, in some areas LB spirochetes are more prevalent in sand lizards (*L. agilis*) and common wall lizards (*P. muralis*) than in rodents (122). The lizard-associated LB spirochete is *B. lusitaniae*, a genospecies previously thought to occur only in Mediterranean and Central Europe (197), but it was shown that it has a far more widespread geographical distribution involving the green lizard (*L. viridis*), the Balkan wall lizard (*Podarcis taurica*), and the sand lizard (*L. agilis*) (123, 125, 126).

We have reviewed the occurrence of *B. burgdorferi* s.l. in host-seeking urban *I. ricinus* ticks across Europe according to the literature (**Table 2**). There are also several additional papers demonstrating the presence of borreliae in ixodid ticks collected in (sub)urban areas (198–202). All accessible data show that borreliae in *I. ricinus* ticks collected in urban parks, gardens, or suburban habitats are prevalent approximately at the same rate as in *I. ricinus* ticks living in forests (203). In urban areas, therefore the risk of contacting LB could be as high as in natural environment.

We should consider that most studies dealing with eco-epidemiology of LB in patients living in urban areas may have limitation, because not always the exact location (or area) where they acquired the vector tick is known. While popular opinion is that outdoor occupations and hiking are risk activities, several studies have implied that infection is often acquired near the home, during gardening and dog walking associated with increased risk (148, 226–228).

ANAPLASMA PHAGOCYTOPHILUM

Anaplasma phagocytophilum is a small, gram-negative obligate intracellular alpha-Proteobacterium and infects neutrophilic, eosinophilic granulocytes, and monocytes of mammals. There, it replicates within a cytoplasmatic, cell-membrane derived vacuole. *A. phagocytophilum* is transmitted by ticks of the *I. ricinus* complex in the Northern hemisphere and in European countries mainly by *I. ricinus* (50).

The bacterium has been known since the last century to cause diseases in domestic ruminants (229) and since the 1960s in horses (230). The first human case was described in the USA in 1994 (231). The causative agents of the diseases were at the time classified into the granulocytic group of the genus *Ehrlichia*, which contained *E. phagocytophila* as agent of tick-borne fever of ruminants, *E. equi* as agent of equine granulocytic ehrlichiosis and the human granulocytic ehrlichiosis (HGE)-agent. In 2001, a reorganization of the order Rickettsiales, based on homologies in the *16S rRNA* gene, reclassified the granulocytic *Ehrlichia*-group as the new bacterial species *A. phagocytophilum* and the respective diseases were then called granulocytic anaplasmosis (232). Clinical cases are also occurring in dogs and cats, then known as canine and feline granulocytic anaplasmosis (233, 234).

After the first cases appeared in the US in the 1990s, human granulocytic anaplasmosis (HGA) has become one of the most important tick-borne diseases in the US, with an incidence in 2010 of 6.1 cases per 1 million inhabitants[2]. The first human case in Europe was described in the 1990s (235), and around 100 cases have been described since then in several European countries, e.g., in Slovenia, Croatia, Czech Republic, Slovakia, Austria, Latvia, the Netherlands, Norway, Poland, Spain, France, and Sweden (236–252). Seroprevalence rates in humans in Europe are around 1–20% and they fluctuate depending on anamnesis, tick exposure, and age of the patients (253).

Mammalian host species (**Table 1**) such as wild ruminants (e.g., roe deer, red deer, fallow deer, but also mountain ungulates), small mammals such as rodents and insectivores, but also foxes, bears, wild boars, birds, and reptiles are infected with *A. phagocytophilum* (50). Prevalence rates in wild ruminant species in Europe are generally high, e.g., ranging in roe deer and red deer from around 12% to over 85% (70, 254–256). On the other hand, prevalence rates in small mammals are from 0% to about 20% (50).

Anaplasma phagocytophilum is detected with varying prevalences in questing *I. ricinus* ticks, and has been found in Europe in nearly 30 countries. The prevalence ranged, for example, in Norway from 0.4 to 17.1%, in Estonia from 3 to 6.5%, in Slovakia from 1.1 to 8.3%, and in Germany from 1.0 to 17.4% [reviewed in Ref. (50)]. So far, transovarial transmission has not been shown in *Ixodes* ticks. As such, for the current state of knowledge, a reservoir host is necessary to keep up the endemic life cycle of *A. phagocytophilum* in nature.

The discrepancy of a high occurrence of *A. phagocytophilum* in ticks and mammals as well as high seroprevalence rates in Europe in contrast to few clinical cases has been explained by the potential underdiagnosing of the disease, or the potential occurrence of less virulent strains in Europe in comparison to the USA. The discrepancy could also be explained by a higher awareness of US physicians to the disease because in the USA it is a notifiable

[2]www.cdc.gov/anaplasmosis

Table 2 | Occurrence of *Borrelia burgdorferi* sensu lato in questing *Ixodes ricinus* ticks in urban and suburban areas in Europe.

Country	City/region (habitat), year	No. of examined ticks	Prevalence[a]	Method	Genomic spp.	Reference
Czech Republic	Prague (U, S)	2,490 N, 143 F, 184 M	2–22%	IFA		(204)
	Prague (U, S) 1994–1997	12,287	3.3–13.3%	IFA		(205)
	Prague 1995–1997	462 N, 173 A	1.9% N, 12.7% A	PCR	Bg 18, Ba 13	(206)
	Brno – outskirts 1988	1,005	3.8% N, 16.4% F, 12.7% M	IFA		(207)
	Brno (U parks) 1992	34 N, 64 F, 65 M	14.7% N, 29.7% F, 30.8% M	DFM		(208)
	Brno-Pisárky (S) 1996–1998	643 N, 123 F, 107 M	10.0% N, 13.8% F, 18.7% M	DFM (and PCR)		(209)
	Brno-Pisárky (S) 2002	243 N, 19 F, 22 M	15.8% N + F + M	DFM (PCR)	Bg 15, Ba 14, Bb 2, Bv 2	(210)
Finland	Helsinki (U, S)	303 N, 189 F, 234 M	32.2% N + F + M	DFM, PCR, BSK	Ba 70%, Bg 25%	(35)
France	Paris (U, S)	360 N, 69 F, 129 M	32% F, 10% N, 20% M	PCR	Ba/Bv 36%, Bg/Bl 60%, Bm 4%*	(211)
Germany	Berlin – West (U, S)	1,414 N, 132 F, 165 M	2.4% N, 9.1% F, 6.1% M (MIR)	BSK		(212)
	Bonn (U, S) 2003	865 N, 241 F, 288 M	17.3% N, 26.6% F, 12.5% M	PCR	Ba 39%, Bg 28%, Bb 16%, Bv 9%	(36)
Hungary	Budapest (parks, forests, and cemeteries) 2013	240 F	40.8%	PCR		(213)
Italy	Imola (U parks) 2006		10.4% N + A	PCR		(214)
Lithuania	Vilnius (city park) 2005	39 A	25%	DFM, PCR	Ba, Bg, Ba + Bg	(215)
The Netherlands	Bijlmerweide (city park) 2000–2002	384 N + F + M	6.8%	PCR	Ba 10, Bb 1, Bv 1	(38)
Poland	Gdansk, Sopot, Gdynia (U, S)	701 N + F + M (164 F, 139 M)	12.4%, 11.6% F, 10.1% M	PCR		(216)
	Szczecin (U, S)	193 N, 22 A	17.7%	DFM		(217)
	Warsaw (U, S), 1996		19.2–31.0%	IFA (PCR)	Bg, Ba, Bv	(218)
	Warsaw (city parks)		6.1%	PCR		(219)
Serbia	Belgrade (U, S) 1996–2005	10,158 N + A	21.9% N + A	DFM (BSK, PCR)	Ba 75%, Bb 22%, Bg 3%	(220)
Slovakia	Bratislava (U, S) 1986–1988	77	7.8%	DFM		(221)
	Košice (U, S) 1991–1995	660 N, 2,904 A	9.2% N, 14.8% A	DFM and IFA		(222)
	Košice, Bardejov (U, S) 2008–2010	670	10.1%	PCR	Ba, Bg, Bv, Bb	(223)
Switzerland	Basel (U, S) 2003	172 N, 35 A	16.4% N + A	PCR		(224)
United Kingdom	London (U parks)	65 F	7.7% F	PCR		(225)

U, urban; S, suburban; Ixodes ricinus: N, nymph; F, female; M, male; A, adult; DFM, dark-field microscopy; IFA, indirect immunofluorescence assay; BSK, cultivation in BSK II medium; Ba, Borrelia afzelii; Bb, B. burgdorferi s.s.; Bg, B. garinii; Bv, B. valaisiana; Bl, B. lusitaniae; Bm, B. miyamotoi; MIR, minimum infection rate.

[a] *Different PCR methods were used that differ in their sensitivity.*

* *No sufficient discrimination between Bg and Bl and between Ba and Bv.*

disease. However, *A. phagocytophilum* shows also genetic heterogeneity and potential differences concerning the potential host tropisms and pathogenicity (118). A potential human pathogenic strain of *A. phagocytophilum* in Europe has been especially suspected to be connected with wild boars. This was confirmed in recent studies (257, 258).

Several studies have investigated the genetic heterogeneity on the basis of several genes such as *16S rRNA*, *groEL* heat-shock protein, major surface protein coding genes, and the *ankA* gene (255, 259–261). Several distinct clusters were found where, in general, strains derived from domestic animals or ruminants clustered together. Roe deer strains often clustered separately from

Table 3 | Occurrence of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks in urban and suburban areas in Europe[a].

Country	City/region (habitat)	No. of ticks posit./examined	Prevalence[b] (%)	Reference
Austria	Graz (RA)	5/518	1	(264)
Czech Republic	Dvur Kralove (U forest)	8/138	5.8	(265)
	Ostrava (U park)	276 (tested in pools)	9.4	(266)
France	Paris (S forests)	2/558	0.7	(211)
Germany	Hamburg (U RA)	51/1,400	3.6	(267)
	Hannover (U RA)	94/2,100	4.5	(268)
	Bavaria (U parks)	500/5,569	9.0	(269)
	Bavaria (U parks)	103/2,862	2.9	(270)
	Bavaria (U parks)	172/2,800	6.1	(271)
	Leipzig (U, S RA)	47/539	8.7	(55)
	Hannover (U RA)	52/1,646	3.2	(272)
Hungary	Budapest (30 sites: U parks, forests, and cemeteries)	21/240	8.8	(213)
Poland	S forests	18/124; 6/46	14.5; 13.0	(273)
Slovakia	Bratislava (U, S forests)	10/248	4	(265)
	Malacky (U park)	4/101	4	(265)
	Košice (U forest)	10/224	4.5	(265)
	Bardejov Poštárka (S forest)	2/75	2.7	(40)
	Košice Adlerova (S forest)	10/261	3.8	(40)
	Jazero (U forest)	5/91	5.5	(40)
	Košice (S forests)	1,075	1.4–5.5	(274)

U, urban; S, suburban; RA, recreational area.

[a] *Negative results not shown,*

[b] *different PCR and real-time PCR methods were used that differ in their sensitivity.*

strains derived from other animals. No evidence was found that wild ruminants are involved in the transmission cycles of potentially pathogenic strains. This was shown again by a recent multi locus sequence typing study (262). However, another study found pathogenic strains associated mostly to ungulates (118).

Furthermore, in a recent large-scale analysis, four *A. phagocytophilum* ecotypes with significantly different host ranges were identified based on *groEL* heat-shock protein gene sequences of various European vertebrate and tick samples (99). So far, all human cases clustered in ecotype I with the broadest host range (including domesticated animals, red deer, wild boar, and urban hedgehogs). Ecotype II was associated with roe deer and some rodents, ecotype III included only rodents. Birds seem to have a different enzootic cycle from all these (ecotype IV). Based on population genetic parameters, ecotype I showed significant expansion, which might have occurred through an increase in either the population of *I. ricinus* ticks, or in the (often urban) vertebrate host species, or in both (99).

Only recently, a HGA case of a German patient has been published having acquired the infection whilst on holidays hiking in Scotland (263). This shows that the risk of contracting this infectious agent can also be in leisure time whilst hiking, or even in the cities whilst being in urban or peri-urban park areas.

In about the last 5 years, considerable research effort has been undertaken in Europe to investigate the epidemiology of *A. phagocytophilum*, especially in urban areas and high prevalences of this pathogen have been found with seasonal and geographic variability. An overview of recent studies investigating questing *I. ricinus* in urban and suburban areas is shown in **Table 3**. However, when considering *A. phagocytophilum* prevalence rates in ticks, the genetic variability has to be taken into account as not all strains may be pathogenic to humans.

CANDIDATUS NEOEHRLICHIA MIKURENSIS

"*Candidatus* Neoehrlichia mikurensis" (*Candidatus* N. mikurensis) is a tick-borne pathogen, which is probably transmitted by *I. ricinus* ticks (24). However, transovarial transmission in this tick species has not been reported yet.

Currently, the genera *Wolbachia*, *Ehrlichia*, *Neorickettsia*, *Aegyptianella*, and *Anaplasma* belong to the rickettsial family Anaplasmataceae (232). Most certainly, the new genus "*Neoehrlichia*" will be included in this family in future. The pathogens of this family are intracellular bacteria transmitted by arthropods and may cause severe diseases in humans and animals. For at least three of the five existing genera within this family (*Anaplasma*, *Ehrlichia*, and *Neorickettsia*) serological cross reactions are not known so far (275). *Candidatus* N. mikurensis is an obligate intracellular gram-negative bacterium, which is characterized by an endothelial cell tropism but it could not be cultivated *in vitro* thus far. Therefore, the status "Candidatus" is still preserved.

A previous study published data on not taxonomically grouped *Ehrlichia* DNA in engorged *I. ricinus* ticks from roe deer in the

Table 4 | Occurrence of *Candidatus* N. mikurensis in questing *Ixodes ricinus* ticks in various habitats in Europe.

Country	No. of sites, habitat	No. of ticks examined	Prevalence[a]	Reference
Austria	U, S, 2002–2003	518	4.2%	(264)
Czech Republic	U, 2010	69	0.4%	(265)
Denmark	Three sites, S, sylvatic, 2011(+tick DNA from archive)	79[a]	3.8%	(285)
France	Two sites, sylvatic	60	1.7%	(282)
Germany	Ten sites, U, S	542	8.1%	(282)
	U, S, 2008–2009	782	24.2–26.6%	(52)
Hungary	Nine sites, 2007	2,004	n.a. 9 of 35 sites positive	(286)
Italy	U, S, 2006–2008	138	10.5%	(287)
The Netherlands	Three sites, sylvatic	180	8.6%	(288)
	Twenty-one sites, U, S, sylvatic, 2006–2010	5,343	5.6%	(289)
The Netherlands/Belgium	n. a., 2006–2010	2,375	7%	(281)
Russia	S, sylvatic, 1997–1998	295	7.1%	(277)
Slovakia	S, sylvatic, 2006	68	2.9%	(290)
	Ten sites, U, S, sylvatic, 2008, 2010	670	2.4%	(40)
	U, S		1.1–4.5%	(265)
Spain	S, 2013	100	2%	(291)
Sweden	Four sites, sylvatic, 2010–2011	949	4.5–11%	(292)
Switzerland	Eleven sites, U, S, 2009–2010	818	6.4%	(293)
	Four sites, U, S, 2009	1,916	3.5–8%	(294)

U, urban; S, suburban.

[a]*Different PCR and real-time PCR methods were used that differ in their sensitivity. n.a., not available.*

Netherlands (276). This pathogen was then named after the senior author as "Schotti-Variant" (276). Similar sequencing results were published for *I. ricinus* and *I. persulcatus* ticks from the Baltics in 2001 (277). Between 1998 and 2001, DNA of a pathogen, suggested to be called *Cand. Ehrlichia walkerii* spp. nov., was found in engorged *I. ricinus* ticks that fed on asymptomatic patients from Italy (278). In 2003, DNA sequences of this new pathogen were detected in DNA extracted from *I. ricinus* ticks from Germany, followed by first investigations on possible reservoir hosts (279). In 2003, a pathogen was found via examination by conventional PCR in three wild rats (*R. norvegicus*) in China. This examination was followed by DNA sequencing of this pathogen, which was then called the "Rattus Variant" (280). In 2004, DNA of this "new" pathogen was found in 7 out of 15 brown rats from a Japanese isle called Mikura (275). The pathogen was passaged in Wistar rats and first investigations on the ultrastructure and the phylogenetic analysis were done, which lead to the currently valid taxonomic denomination "*Candidatus* Neoehrlichia mikurensis." The close genetic similarity of the *16S rRNA* and the *groEL* gene puts *Candidatus* N. mikurensis in the family of Anaplasmataceae.

Candidatus N. mikurensis was found widespread in *I. ricinus* throughout Europe (281, 282). It could be detected in Italy, France, Sweden, Russia, and other European countries (**Table 4**). The prevalences ranged between 1 and 11% but focal areas were found with prevalence rates up to 26.6% (49) (**Table 4**). Furthermore, *Candidatus* N. mikurensis was detected in one out of 126 *I. ricinus* ticks that were collected in Moldavia back in the year 1969 (283) and it was only detected in the genus of *Ixodes* ticks so far (284). Positive ticks were not only found in sylvatic and non-anthropogenic sites but also in urban and peri-urban sites with human influence in Europe (**Table 4**).

Previous studies on potential reservoir hosts revealed that rodents, especially bank voles and yellow-necked mice, but also common voles (*M. arvalis*) were infected at high rates, suggesting a role as reservoir hosts (52, 281, 295, 296), but insectivores were found to be negative for *Candidatus* N. mikurensis thus far (52). Recently, the reservoir role of *Apodemus* mice (*A. flavicollis* and *A. sylvaticus*) and bank voles (*M. glareolus*) has unambiguously been proven in a xenodiagnostic study [(48); **Table 1**]. Urban hedgehogs (*E. roumanicus*) with high density in a Budapest city park were found to be carriers of *Candidatus* N. mikurensis, indicating that non-rodent reservoirs might be also involved in the maintenance of this pathogen, especially in human dwellings (69). Additionally, *Candidatus* N. mikurensis was detected in dogs from Germany and Nigeria (297, 298).

In the past, the detection of *Candidatus* N. mikurensis in rodents and ixodid ticks was an interesting but only incidental

finding without any medical importance (299). In contrast to this assumption, it was recently found in humans (50) with immune deficiency but without being in an occupation group at risk for tick bites over the last decade. *Candidatus* N. mikurensis caused unspecific symptoms such as fever, septicemia, malaise, and weight loss in these patients (300–302). Until October 2012, the first six clinical cases of neoehrlichiosis were the only human cases confirmed by laboratory diagnostic methods. All of these patients suffered from a primary disease, were immunocompromised and came from European countries, such as Germany (301), the Czech Republic (303), Sweden (302), and Switzerland (300). Nevertheless a primary disease is not a necessary precondition to develop neoehrlichiosis as *Candidatus* N. mikurensis could be detected in blood of 7 out of 622 patients from China suffering from fever (130). The authors of these clinical reports emphasize that these seven patients were otherwise healthy and did not suffer from a chronic or immunosuppressive disease. The most recent two human cases were reported in Switzerland, where both patients recovered quickly after a treatment with Doxycycline (294). The data, gained in the last decade, lead to the assumption that *Candidatus* N. mikurensis is an emerging pathogen that might be found by increasing numbers in ticks from sylvatic and urban sites, in small mammals and humans in future (281, 304). Further investigations are needed on the spread, maintenance, and potential reservoir hosts to assess the risk potential of *Candidatus* N. mikurensis.

RICKETTSIAE

Rickettsiae are Gram-negative, obligate, aerobic, intracellular bacterial parasites of eukaryotes that survive freely within the cytosol of the host cell, and belong to the family Rickettsiaceae and order Rickettsiales. Rickettsiae are traditionally subdivided into the typhus and the spotted fever group (SFG). SFG rickettsiae are associated with hard ticks (Ixodidae), with the exception of *Rickettsia akari* (mite-borne) and *R. felis* (flea-borne). Hard ticks can transmit them transstadially and transovarially and serve both as vectors and reservoirs of these pathogens. Vertebrates are suspected to serve as reservoirs of rickettsiae, but they may also be accidental hosts and acquire infection by a tick bite (305). However, in a recent xenodiagnostic experiment infected rodents were not able to transmit *R. helvetica* or *R. monacensis* to *I. ricinus* larvae (48).

In Europe, *R. felis*, *R. typhi*, *R. prowazekii*, *R. akari*, *R. conorii*, *R. slovaca*, *R. sibirica mongolotimonae*, *R. raoultii*, *R. massiliae, R. aeschlimanni*, *R. helvetica*, and *R. monacensis* have been implicated in human diseases or reported as emerging pathogens or isolated from vectors or humans (131, 306–308). Furthermore, the candidate species "*Candidatus* Rickettsia kotlanii," "*Candidatus* Rickettsia barbariae," or "*Candidatus* Rickettsia vini" have been found in ticks in Europe (309–311). Numerous rickettsiae are regularly associated with ticks and have been called symbionts, microsymbionts, or endosymbionts (living in endocellular symbiosis). However, their potential for pathogenicity is still unknown (312).

The presence of tick-borne rickettsiae has been reported from almost all European countries. The current view on geographic distribution of *Rickettsia* species in the world is summarized by Parola et al. (131).

In Europe, *I. ricinus* ticks are known to carry mainly *R. helvetica* and *R. monacensis*. However, *R. massiliae* was also detected in *I. ricinus* ticks (313). The following rickettsial genotypes were detected only by molecular tools in *I. ricinus* ticks collected in Europe: "*Candidatus* R. vini" was proposed as a new *Rickettsia* spp. detected in *I. arboricola* and *I. ricinus* collected from three different bird species in Spain (311), *Rickettsia* spp. strain Davousti, previously found in *Amblyomma tholloni* ticks in Africa, was detected in *Ixodes* spp. collected from migratory birds in Sweden (314),"*Candidatus* Rickettsia moreli" (GenBank accession numbers Y08784 and Y08785) was detected in *I. ricinus* from Spain, and *Rickettsia* spp. clone KVH-02-3H7 (GenBank accession number GQ849216) was detected in *I. ricinus* in the Netherlands (131).

Rickettsia helvetica was first isolated from *I. ricinus* in Switzerland and it was confirmed to be a new member of the SFG rickettsiae in 1993 (315, 316). It has been generally accepted that *I. ricinus* is the main vector and natural reservoir of *R. helvetica.* However, *D. reticulatus* ticks were found to be infected with *R. helvetica* in Croatia (317). *R. helvetica* has been detected in questing and bird-feeding *I. ricinus* ticks in at least 24 European countries (131). The prevalence rates vary from 0.5% in a bird conservation island named Greifswalder Oie in the Baltic Sea to 66% in the Netherlands (318, 319). For example, the highest infection rate of *R. helvetica* in *I. ricinus* from Denmark was found in May, followed by July, August, and October (320). The presence of *R. helvetica* was also confirmed in *I. ricinus* in some urban and peri-urban sites in Slovakia, the Czech Republic, Germany, Portugal, Serbia, and Poland (**Table 5**).

In 1999, *R. helvetica* was associated with chronic perimyocarditis in sudden cardiac death in Sweden (328). This species has been cultivated from a patient with subacute meningitis (329). The hypothetical role of *R. helvetica* as an etiological agent of sarcoidosis could not be confirmed (330). The illness is associated with fever, headache, arthralgia, and myalgias and less frequently with rash and/or an eschar (331, 332).

Rickettsia monacensis was originally isolated as new species from *I. ricinus* collected in a city park in Germany (333). Phylogenetic analyses of the *16S rRNA*, *gltA*, and *rompA* gene sequences demonstrated its close relationship with *Candidatus* Rickettsia spp. IRS3 and *Cand. Rickettsia* sp. IRS4 isolated from *I. ricinus* in north-eastern and south-western Slovakia (334, 335). The prevalence rates of *R. monacensis* in *I. ricinus* ticks vary from 0.5% in Germany to 34.6% in Turkey (322, 336). *R. monacensis* has been detected in *I. ricinus* ticks in at least 18 European countries (131). The presence of *R. monacensis* was also confirmed in *I. ricinus* ticks in some urban and peri-urban sites in Slovakia, the Czech Republic, Germany, Portugal, Serbia, and Poland (**Table 5**). In 2005, *R. monacensis* was identified as a human pathogen in two patients in Spain (in June and September) and latter in one patient in Sardinia, Italy (in April) (337, 338). In addition to fever and flu-like symptoms, the inoculation eschar was identified in an Italian patient, and a generalized rash including the palms and soles was identified in a Spanish patient.

Rickettsia massiliae was originally isolated from *Rhipicephalus sanguineus* ticks collected near Marseille, France, in 1992 and then detected in *R. sanguineus*, *R. turanicus*, *R. pusillus*, *R. bursa*, and *I. ricinus* ticks in France, Greece, Portugal, Switzerland, Spain,

Table 5 | Occurrence of *Rickettsia* spp. in questing *Ixodes ricinus* ticks in various habitats in Europe.

Country	City/region (habitat)	No. examined ticks	Prevalence of *Rickettsia* spp.	Identified species (*n*)	Reference
Czech Republic	Ostrava (U park), 2010	180 N	2.2% (MIR)	14 *Rh,* 6 *Rm*	(266)
		96 A	4.2% (MIR)		
	Proskovice (mixed forest), 2010	1,114 N	3.5% (MIR)		
		83 A	2.5% (MIR)		
France	Paris (S)	360 N, 69 F, 129 M	5.8%	*Rh*	(211)
Germany	Munich, 2006	961 N	1.0%	138 *Rh,* 13 *Rm*	(321)
		1,900 A	7.3%		
	Saarland (RA), 2008–2009	36 N	16.7–47.2%	8 *Rh*	(322)
	Bavaria/Munich (natural alluvial forest), 2008–2009	79 A	21.5%		
	Leipzig/Saxony (coal surface-mining area), 2008–2009	28 N	21.4%		
		100 A	19.0%		
		98 N	8.2–27.6%		
		431 A	9.7%		
	Munich, Regensburg, Ingolstadt, Augsburg, Berg (U parks), 2009–2010	774 L	2.1–9.8%	15 *Rh*, 1 *Rm*	(37)
		1,190 N	6.8%		
		2,495 A	7.5%	77 *Rh,* 4 *Rm*	
		244 L	–		
		742 N	–	180 *Rh,* 8 *Rm*	
		1,142 A	–		
	Munich, Regensburg, Lake Starnberg (U, S)	24 L	2.2–7.5%	29 *Rh,*1 *Rm*	(323)
	Lake Starnberg and Lake Ammersee, pastures	500 N	5.0%		
	Augsburg, forest, 2011	889 A	8.7%		
		140 N	15.7%	9 *Rh*	
		225A	13.3%		
		139 L	2.2–10.1%		
		120 N	17.5%	9 *Rh*	
		79 A	13.9%		
	Hanover (U park), 2010	31 L	16.0%	268 *Rh*	(268)
		1,697 N	25.5%		
		372 A	30.4%		
Poland	Warsaw, national parks and natural areas, 2011	1,147 N 442 A	3.7% (MIR)	38 *Rh*, *Rm*	(41)
			5.9% (MIR)		
Portugal	Alentejo (safari park), 2006–2009	35 A	82.9%	14 *Rh*, 15 *Rm*	(324)
Serbia	Four natural sites, 2 sites (RA), 2007, 2009	26	23.1%	2 *Rh*, 4 *Rm*	(325)
Slovakia	Bratislava (S forest, cemeteries), 2006–2011	445 N	8.3%	61 *Rh*, 3 *Rm*	(326)
		471 A	10.2%		
	Malacky (U park), 2006–2011	59 N	6.8%	10 *Rh,* 3 *Rm*	
		62 A	14.5%		
	Martin (U park), 2006–2011	3 N	0		
		12 A	16.7%		
	Martinské hole Mts (mountain forest), 2006–2011	276 N	5.4%	6 *Rh,* 2 *Rm*	
		482 A	10.0%		
	Vojka nad Dunajom (RA), 2011–2012	2 N	0	30 *Rh,* 3 *Rm*	(327)
		280 A	11.7%		

U, urban; S, suburban; RA, recreational area; Ixodes ricinus: L, larva; N, nymph; A, adult; MIR, minimum infection rate; Rh, R. helvetica; Rm, R. monacensis.

including islands: Sardinia and Sicily (Italy), the Canary Islands (Spain), Cephalonia (Greece), and Cyprus (131). *R. massiliae* was identified in four *I. ricinus* ticks removed from humans at hospitals in Castilla y León, Spain (313). However, to our knowledge, there are no other studies of this species in urban areas.

BABESIA

Ixodes ricinus is the vector of three intraerythrocytic protozoan parasites circulating in Europe and involved in human babesiosis: *B. divergens*, *B. venatorum* (originally designated *Babesia* spp. EU1), and *B. microti*. To date, no other Piroplasmida affecting humans have been reported to be transmitted by this tick species, even though it feeds on a very large spectrum of hosts, which are potentially infected by several parasite species including numerous other *Babesia* species associated to wildlife or domestic animal diseases. However, the list of potential or known tick-borne pathogens is constantly evolving, either due to: (i) the description of *Babesia* species new for science, (ii) the spread of parasite species previously unknown in Europe, or (iii) the discovery of a *Babesia* species previously restricted to animals but now known to be associated with humans. Thus, emergence or re-emergence of tick-borne diseases leads to the development of unknown health risks (339). Therefore, there is a real concern that tick-borne diseases due to parasites will appear in areas previously free of such diseases, and there is a real necessity of an epidemiological surveillance of the parasitic communities hosted, and potentially transmitted by ticks (340).

Although best known as an animal disease, babesiosis is a zoonotic disease, classified as emerging by some authors. Approximately 50 human cases of babesiosis have been reported in Europe, which is probably underestimated because of a large proportion of asymptomatic infections, as suggested by seroprevalence studies (341). Among the *Babesia* species pathogenic for humans, the bovine parasite *B. divergens* is thought to be responsible for most European cases of human babesiosis (342). However, since 2003, cases of human babesiosis have also been attributed to *B. venatorum* in Austria, Italy, and Germany (343, 344) as well as to *B. microti* in a single case in Germany (341). Whilst the clinical signs of human babesiosis are usually limited to splenectomized patients, two human cases (one attributed to *B. divergens*, the other to an unknown origin) have been detected in immunocompetent patients in eastern France (345). It is also noticeable that, as an example, 0.38% of the French population is splenectomized (346). Moreover, the rising number of HIV-positive individuals and the increasing population of immunocompromised humans, especially in urban areas, may therefore lead to boost the number of human babesiosis cases (341). The proportion of the population at risk of *Babesia* infection is thus higher than previously suspected and *Babesia* spp. likely represents real potential agents of an emerging zoonotic disease and needs increased attention and vigilance.

Besides transstadial transmission, transovarial transmission within ticks is characteristic for most *Babesia* spp. (differentiating them from *Theileria* species), which implies that ticks constitute a real parasite reservoir in the field, facilitating the long-term persistence of *Babesia* species in the ecosystem (sometimes over several tick generations) (347). In Europe, infection rates of *Babesia* spp. in ticks are usually rather low, but published values range from 0.9 to 20% (341).

Babesia divergens is a bovine parasite transmitted by *I. ricinus*, and is thought to be responsible for most cases of human babesiosis in Europe (342). This parasite is the most widespread and pathogenic *Babesia* species infecting cattle in northern temperate areas (342). Thus, any urban or peri-urban area where cattle and *I. ricinus* are found is potentially at risk. For example, *B. divergens* has been found in an *I. ricinus* tick collected in an urban park in Germany (37). Recently, the discovery of this parasite in questing *I. ricinus* from a forest area in Eastern France (340), as well as in *I. ricinus* collected from wild cervids in Belgium (348), may suggest that its geographical distribution is increasing, even within forested areas without cattle farms, which would require the existence of reservoir hosts other than cattle. Indeed, it was reported that *B. divergens* is also able to infect ungulates (roe deer, fallow deer, red deer, mouflon, and sheep), splenectomized rats, as well as non-splenectomized reindeer, sheep, and gerbil [see review in Ref. (347)]. Thus, this parasite has been shown to have a wider vertebrate host range than previously thought, leading to a potential risk not only in rural areas but also in peri-urban ones.

Babesia venatorum, implicated in human cases of babesiosis in Europe (343, 344), seems to phylogenetically lie in a sister group with *B. divergens* (343), and some serological cross-reactivity between *B. divergens* and *B. venatorum* has been reported (349). Roe deer were strongly suspected to be the wildlife reservoir of this parasite (350, 351) and its transmission by *I. ricinus* was validated both *in vivo* (351, 352) and *in vitro* (353). In addition, *B. venatorum* has been identified in *I. ricinus* in several European countries including Slovenia (354), Switzerland (355), the Netherlands (356), Poland (357), Italy (358), Belgium (359), Germany (37), and France (211, 351), with prevalence varying from 0.4 to 1.3%, demonstrating a wide geographical spread across the continent. Increasing reports of *B. venatorum* in ticks and wild ruminants make this parasite an excellent candidate for the emergence of a new zoonotic tick-borne disease, in particular in the current context of a growing number of wild hosts such as deer. As roe deer is often found even in suburban or peri-urban parks (if they are connected to more natural or semi-natural areas such as forests or rural areas), *I. ricinus* sampled in such places have already been reported as infected by *B. venatorum* (55, 323). This parasite has been detected in 1.3% of questing *I. ricinus* collected in France in a forest located in the South of Paris metropolitan area in the middle of an urban zone (211). Because of its location and the recreational activities available, this forest is visited by over 3 million people every year, emphasizing the public health risk. Similarly, the first detection of *B. venatorum* in Poland has been reported from ticks collected in an urban area (357), and a later study performed in recreational areas, corresponding to peri-urban forest near Warsaw city, showed also the presence of *B. venatorum* in questing *I. ricinus* (360).

Recent molecular phylogenetic investigations have convincingly established *B. microti* as forming a distinct and early diverging clade relative to other *Babesia* species (including the clade containing *B. divergens* and *B. venatorum*) as well as to *Theileria* species (361–363). *B. microti* is responsible for several hundred cases reported yearly in the USA in both spleen-intact and asplenic patient (132). This rodent parasite is known to be transmitted by *I. ricinus*, and now seems to be widely established in Europe, although only one human case has been reported to date (341). The substantial difference in the human pathogenicity of the North-American and European *B. microti* strains need further studies. It has been identified in *I. ricinus* in several European countries

such as Switzerland (364), Poland (365), Hungary (366) Slovenia (367), Germany (368), the Netherlands (356, 369), Belgium (359), and France (340). Microtine rodents and probably shrews are the reservoirs of *B. microti* (**Table 1**). Infectious tick bites are most likely to occur in deciduous woodland and peri-domestic settings (37, 55, 323). Indeed, this parasite was recovered in questing ticks from a forest in Poland that was qualified as "one of the most popular tourist destinations in Poland," highlighting the risk for humans during recreational activities (360).

NEW OR NEGLECTED TICK-BORNE PATHOGENS: STILL UNKNOWN BACTERIAL, PARASITIC, AND VIRAL SPECIES TO BE DISCOVERED?

Due to advances in molecular biology, new species, strains, or genetic variants of microorganisms are being detected in ticks, resulting in an ever-increasing list of pathogens capable of infecting domesticated animals and humans. Some of them have been linked to human or animal diseases only many years after their first discovery in ticks or animal reservoirs (299). An emblematic example is that of *B. henselae*, the agent of Cat Scratch Disease, known to be transmitted from cat to human by cat scratch (or by fleas). For years, cases of *B. henselae* infection had been described in patients without history of contact with cat without any idea how these people could be infected. By screening pathogens in ticks, *B. henselae* DNA, and RNA were identified in *I. ricinus* (370–373). After many years of debate to know whether *B. henselae* was or was not a tick-borne pathogen, the direct link between tick bites, *B. henselae*, and disease in humans was finally demonstrated (374). Another striking example is the one of *Borrelia miyamotoi.* This *Borrelia* species has been isolated for the first time in Japan in 1995 from *Ixodes* ticks and has been considered as non-pathogenic endogenous tick bacteria until the first human cases of *B. miyamotoi* infection were reported in Russia in 2011 (375). Since then, human infections have been described in the USA and in 2013 in the Netherlands (376–379). In France, *B. miyamotoi* was found to circulate in *I. ricinus* as well as in the bank vole *M. glareolus* (380), and this French genotype was identical to the genotype isolated from a sick person in the Netherlands. These findings have important implications for public health, especially considering that *B. miyamotoi*-positive ticks and rodents were collected from different sites in close proximity to human dwellings. Up to now, no human cases of *B. miyamotoi* infections have been reported in most European countries, however, symptoms caused by *B. miyamotoi* could easily be confused with symptoms caused by other pathogens, which are better known by practitioners, suggesting that surveillance urgently needs to be improved.

A more recent example of neglected pathogens is a new phlebovirus that has been described in humans from northwestern Missouri, USA independently presented to a medical facility with fever, fatigue, diarrhea, thrombocytopenia, and leukopenia, and all had been bitten by ticks 5–7 days before the onset of illness. Electron microscopy revealed viruses consistent with members of the Bunyaviridae family. Next-generation sequencing and phylogenetic analysis identified the viruses as novel members of the phlebovirus genus (381). All these examples demonstrate that new or unexpected tick-borne pathogens are characterized, as soon as they are looked for, in patients bitten by ticks.

CONCLUSION

Tick-borne diseases in urban and peri-urban areas represent a rising hazard for public and animal health in Europe. The rapid global changes that planet Earth is facing, especially due to the human ecological footprint, are also affecting the ecology and epidemiology of infectious diseases, including tick-borne diseases. The *I. ricinus* tick being the principal vector of a plethora of viral, bacterial, and protozoan pathogenic microorganisms is showing adaptations to new habitats and ecological conditions. Persistent and potentially increasing populations of this tick species are present in green areas within European cities. Public parks, small forest patches, gardens, and cemeteries are of increasing interest as they represent places where humans, companion, and domestic animals can encounter ticks and be exposed to infected tick bites. The presence of large vertebrates, that serve as tick-maintenance hosts and find conditions to survive and reproduce in the peri-urban environment, reduces the extinction risk of tick populations. Furthermore, majority of tick-maintenance hosts are ecologically classified as generalist species and in many cases serve as reservoirs of a number of emerging zoonotic pathogens, including those transmitted by *I. ricinus.* The combination of urbanization, climate change, and alterations in land-use patterns along with socio-economic factors (outdoor sports and leisure-time activities, gardening, an increased density of pets, and companion animals near human settlements) act in creating favorable conditions for increasing the exposure of humans to ticks, thus favoring the transmission of tick-borne pathogens in urban and peri-urban areas.

Risk communication campaigns aimed at implementing preventive measures against infectious tick bites in urban and peri-urban habitats therefore deserve particular public health efforts. However, several knowledge gaps and lack of quantitative ecological, epidemiological, and socioecological data limit our ability to provide precise quantitative risk pre-assessment. Therefore, more eco-epidemiological research and surveillance specifically focused on the occurrence of ticks, their infection with pathogenic microorganisms as well as on the presence of tick-maintenance and reservoir vertebrate hosts in urbanized areas is urgently needed. Only a multidisciplinary "One-Health" approach integrating research outputs of specialists from different disciplines (veterinarians, zoologists, ecologists, molecular biologists, epidemiologists, physicians, sociologists, and public health experts, etc.), combined with appropriate outreach and dissemination campaigns, can bring success in making urban and peri-urban areas safer from infection by tick-borne pathogens.

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