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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)