

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

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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 eppgradient (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 H₂O) 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 MgCl₂·6H₂O, 0.6 M NaAc.3H₂O). 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.

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
<i>Babesia</i> 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.

KJ465869). Another 4 positive *Babesia* spp. specimens were assigned only to the genus *Babesia*.

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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