Research Article

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Variability of species of *Babesia* Starcovici, 1893 in three sympatric ticks (*Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna*) at the edge of Pannonia in the Czech Republic and Slovakia

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Abstract: The distribution, variability and host specificity of species of *Babesia* Starcovici, 1893 were studied in questing ticks collected on the northwestern edge of the Pannonian Basin in the south-easternmost part of the Czech Republic and in western Slovakia. The area is characterised by relatively natural floodplain habitats and the sympatric occurrence of three tick species possessing wide host spectra, namely *Ixodes ricinus* (Linnaeus), *Dermacentor reticulatus* (Fabricius) and *Haemaphysalis concinna* Koch. Analysis was carried out on 1,408 *I. ricinus*, 2,999 *D. reticulatus* and 150 *H. concinna* altogether, collected from 59 localities. We documented the presence of *Babesia* spp. not only in *I. ricinus* but also in *H. concinna* in the Czech Republic. Two isolates from *I. ricinus* were classified as *B. venatorum* Herwaldt, Cacciò, Gherlinzoni, Aspöck, Slemenda, Piccaluga, Martinelli, Edelhofer, Hollenstein, Poletti, Pampiglione, Löschenberger, Tura et Pieniazek, 2003 (formerly determined as *Babesia* sp. *EUI*), which is a zoonotic parasite and can cause human babesiosis. The rest of our amplicons were very similar to *B. canis* (Piana et Galli-Valerio, 1895), which is usually transmitted by *D. reticulatus*. Despite the huge amount of examined samples, all *D. reticulatus* ticks were *Babesia*-free. Due to this finding, we did not consider our obtained isolates to be *B. canis*, but other closely related species possessing a similar sequence of the studied portion of 18S rDNA. Although this genetic marker is most frequently used in PCR-based diagnostic methods of babesias, its low variability compromises its reliability in studies based only on this marker.

Keywords: host-specificity, piroplasms, species diagnosis, 18S rRNA

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The members of the genus *Babesia* Starcovici, 1893 are tick-transmitted apicomplexan haemoparasites. Their life cycle includes sexual reproduction within the tick vector, whereas merozoites are formed in the erythrocytes of their vertebrate host (Mehlhorn and Schein 1985). Babesiosis has a heterogeneous clinical picture corresponding to immune-mediated haemolytic anaemia. Some species of *Babesia* have zoonotic potential, three of them are present in Europe and they are transmitted by *Ixodes ricinus* (Linnaeus). *Babesia divergens* (M'Fadyean et Stockman, 1911) is the most common zoonotic species in Europe (Gray et al. 2010), with cattle as a natural host. Human infections with *B. divergens* have been reported from Great Britain, France, Ireland, Spain, Sweden and the former USSR (Zintl et al. 2003); so far more than forty clinical cases in Europe have been confirmed (Hildebrandt et al. 2013).

The most recently described zoonotic species is *B. venatorum* Herwaldt, Cacciò, Gherlinzoni, Aspöck, Slemenda, Piccaluga, Martinelli, Edelhofer, Hollenstein, Poletti, Pampiglione, Löschenberger, Tura et Pieniazek, 2003 (formerly described also as *Babesia* sp. *EUI*), infecting roe deers and reported in Europe from Belgium, France, Italy, the Netherlands, Poland, Slovenia and Switzerland (Duh et al. 2005, Casati et al. 2006, Cieniuch et al. 2009, Wielinga et al. 2009, Cassini et al. 2010, Gray et al. 2010, Lempereur et al. 2011, Reis et al. 2011).

Babesia microti (Franca, 1912) is prevalent in ticks across the whole Europe, but the only autochthonous case in Europe was confirmed in 2006 (Hildebrandt et al. 2007). Surveys in Germany, Poland and Switzerland have proved the presence of antibodies against *B. microti* in, 5.4% (25 of 467), 5% (2 of 39) and 1.5% (5 of 366) humans exposed

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to tick bites, respectively (Foppa et al. 2002, Hunfeld et al. 2002, Chmielewska-Badora et al. 2012). These studies suggest the possibility of more undiagnosed babesiosis cases in Europe. Infection rates in ticks are usually low and range from 0.9 to 20% (Hildebrandt et al. 2013).

Babesia venatorum represents a nomenclatural problem since the original description by Herwaldt et al. (2003), who provided a molecular genetic characterisation, computer-generated images of a new *Babesia* species on a Giemsa-stained smear, a catalogue accession number (2002/9) of three smears deposited in Upper Austrian Museum Linz, and also a complete etymological '*derivatio nominis*'. Remarkably, after such a quite useful description, the authors stated that 'this description is not a description' and suggested a candidatus status instead (Herwaldt et al. 2003). We think that the paper has properties of valid description of the new species in spite of the authors' statement of its provisional status, and we consider *B. venatorum* to be a valid species.

Regarding the Czech Republic, *B. venatorum* was first documented from *I. ricinus* collected near Ostrava in 2010 (Venclíková et al. 2015) and *B. microti* was confirmed by Rudolf et al. (2005) in the nymphs of *I. ricinus* collected in South Moravia at the southeast of the Czech Republic. Despite the presence of zoonotic species of *Babesia*, no autochthonous case of human babesiosis has been reported in the Czech Republic so far. A *B. microti* infection imported from the USA was the only case of human babesiosis reported in the Czech Republic (Nohýnková et al. 2002).

Babesiosis also occurs in domestic animals such as cattle namely *B. bovis* (Babès, 1888), *B. bigemina* (Smith et Kilborne, 1893) and *B. divergens*; the last species causes serious economic losses (Zintl et al. 2003), and dogs – *B. canis* (Piana et Galli-Valerio, 1895) and *B. gibsoni* (Patton, 1910) (see Uilenberg et al. 1989, Boozer and Macintire 2003). Canine babesiosis caused by *B. canis* is considered an emerging disease spreading with its vector *Dermacentor reticulatus* (Fabricius) (Zygner et al. 2009). Clinical cases of babesiosis caused by *B. canis* are milder and have been reported from countries neighbouring with the Czech Republic, such as Austria, Germany, Poland and Slovakia (Zahler et al. 2000, Chandoga et al. 2002, Adaszek and Winiarczyk 2008, Majláthová et al. 2011, Silaghi et al. 2011). The more pathogenic *B. gibsoni* has been recently reported from Slovakia (Víchová et al. 2016). The distribution of species of *Babesia* infecting dogs was reviewed by Solano-Gallego et al. (2016).

The present study was realised in the western part of the Pannonian Basin along the lower courses and confluence of the rivers Dyje and Morava in the Czech Republic and to the lowlands along the River Morava in western Slovakia. It is an area with the sympatric occurrence of three tick species – *Ixodes ricinus*, the vector of zoonotic babesias, *Dermacentor reticulatus*, the vector of *B. canis*, and the less studied *Haemaphysalis concinna* Koch. This makes this area an ideal model for the study of vector capabilities of various tick species. Despite this situation, *B. microti* is the only babesia so far reported from this region (Rudolf et al. 2005). This study aims to fill the gaps in our knowledge

of the variability, host specificity and distribution of babesias in this area.

MATERIAL AND METHODS

Tick sampling

The ticks were collected by flagging (Sonenshine 1993) with a 1 m × 1 m white denim flag in southern Moravia and western Slovakia, during the spring and autumn season of 2009–2011. The collected ticks were placed in plastic tubes with 96% pure ethanol and stored at room temperature. The sex and species determination of the ticks was performed using the identification key by Nosek and Sixl (1972). We used 150 *H. concinna*, 1,408 *I. ricinus* and 2,999 *D. reticulatus* ticks for further analysis, altogether collected from 59 localities (35 in the Czech Republic and 24 in Slovakia – Table S1).

DNA extraction

The total genomic DNA from *I. ricinus* and *D. reticulatus* was extracted by alkaline hydrolysis, using the method of Rijpkema et al. (1996), modified by Kubelová et al. (2011). The ticks were air-dried and placed in 1.5 ml test tubes with 500 µl of 0.36 M ammonium hydroxide. The ticks were crushed with sterile single-use plastic pipette tips and boiled at 100 °C for 30 min in closed tubes and another *ca* 30 min in opened tubes, to evaporate any remaining ammonia until 100 µl of lysate left. Tubes containing only ammonium hydroxide were included as negative controls in each of the isolation runs. Negative controls were checked for presence of DNA to detect contamination during extraction process.

DNA from *H. concinna* (originally used in a different study) was isolated by the modified phenol-chloroform method using guanidine isothiocyanate (GT) (Chomczynski and Sacchi 2006). The ticks were also air-dried, placed into 1.5 ml test tubes and crushed in 150 µl of Tris-EDTA buffer. After homogenisation, 450 µl of GT was added. Precipitation of the proteins was achieved by adding 100 µl of chloroform and centrifugation. Supernatant was placed into a new test tube with 600 µl of 2-propanol, samples were placed into freezers at -20 °C over night. The next day samples were centrifuged in 4 °C and the liquid was spilled. Then 800 µl of 70% ethanol was added and after centrifuging the liquid was displaced again. In the last step the pellet was resuspended in 50 µl of PBS buffer and the samples were incubated at 55 °C for 15 min. All obtained samples were stored at -20 °C.

PCR analysis

The PCR diagnosis consisted of a nested PCR reaction. An *ca* 790 bp long portion of 18S rDNA was amplified by nested PCR according to the protocol established by Jefferies et al. (2007) and modified by Kubelová et al. (2011), while the annealing temperature was lowered. We used primers BTF1 (5'-GGCT-CATTACAACAGTTATAG-3') and BTR1 (5'-CCCAAA-GACTTTGATTTCTCTC-3') for the first step, followed by BTF2 (5'-CCGTGCTAATTGTAGGGCTAATAC-3') and BTR2 (5'-GGACTACGACGGTATCTGATCG-3') for the second step. The mixture for PCR reaction (volume 25 µl) was composed of 12.5 µl of Combi PPP MasterMix (TopBio, Vestec, Czech Republic), 9.5 µl of PCR H₂O (TopBio), 1 µl of each primer (KRD, Praha, Czech Republic) and 1 µl of template DNA. The initial denaturation at 94 °C for 3 min was followed by 35 cycles com-

Fig. 1. Phylogenetic tree of *Babesia* spp. as revealed by Bayesian analysis based on 773 bp long alignment of 18S rDNA. Branch lengths indicate expected numbers of substitutions per nucleotide site. Maximum Likelihood (ML) tree provided identical topology. Numbers along branches indicate Bayesian posterior probabilities/percent bootstrap values as obtained by ML analysis (posterior probabilities <0.8 and bootstrap values <50 not shown). Branches with posterior probabilities <0.5 and/or bootstrap values <50 were collapsed. Isolates sequenced in this study are highlighted in bold.

posed of 1 min of denaturation at 94 °C, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min. The final extension at 72 °C lasted 7 min. Positive (*B. canis* DNA isolated from *D. reticulatus*) and negative (Ultra Pure PCR H₂O) controls were included in each reaction.

The acquired positive PCR products were purified using a Gel/PCR Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan) according to the manufacturer's protocol and sent for sequencing to Macrogen (Amsterdam, the Netherlands).

Sequence analysis

The obtained sequences were aligned in MEGA6 (Tamura et al. 2013) and compared to the GenBank database using the BLAST algorithm (Altschul et al. 1990). The final alignment with additional sequences from GenBank (see Fig. 1 for Acc. Nos.) consisted of 773 bp. Akaike information criterion was used to identify the most appropriate model of nucleotide substitution. Bayesian inference analysis was carried out in the Mr.Bayes 3.2.6 plugin in Geneious 9.1.5 (Ronquist and Huelsenbeck 2003, Kearse et al. 2012) with the GTR+G+I model for 10⁷ generations, with phylogenies sampled every 1,000 generations. The trees were summarised after removing burn-in (1,000 trees). Maximum Likelihood (ML) phylogenetic analysis was conducted by PhyML 2.2.0 plugin in Geneious 9.1.5 (Guindon and Gascuel 2003, Kearse et al. 2012) with the GTR+G+I model and parameters estimated from the data; nodal supports were generated with 1,000 bootstrap replicates. The resulting trees including *Theileria equi* (Laveran, 1901) as an outgroup were visualised using Tree-Graph 2.12.0 (Stöver and Müller 2010).

RESULTS AND DISCUSSION

The DNA of *Babesia* spp. was detected in 22 (1.6%) of analysed *Ixodes ricinus* and in six (4%) of *Haemaphysalis concinna*. We found no DNA of any piroplasm in *Derma-centor reticulatus*. The BLAST analysis of the obtained sequences showed that two of our products from *I. ricinus* were 100% identical to the *B. venatorum* sequence from GenBank (Acc. No. AY046575), whereas the remaining sequences either from *I. ricinus* or *H. concinna* were most similar to the GenBank records of *B. canis*. Those of our sequences reported hereafter as *B. canis*-like correspond with 99% similarity to nucleotides 162 to 953 of the published *B. canis* 18S rDNA (GenBank Acc. No. AY072926). *Babesia venatorum*-positive samples (GenBank Acc. Nos. KX857479 and KX857480) were collected at two localities near the Nové Mlýny Dam Lake on the Dyje River in the Czech Republic. The DNA of *B. canis*-like (GenBank Acc. Nos. for each unique sequence in Table 1) was found at nine locations in the Czech Republic and two locations in Slovakia (Table S1).

A comparison of our sequences with the GenBank record (Acc. No. AY072926) of *B. canis* showed five single nucleotide polymorphisms (Table 1). Sequences from isolates from *I. ricinus* can be clearly distinguished from isolates from *H. concinna* as they contain T, A, and G on positions 399, 717 and 888, respectively, whereas isolates from *H. concinna* contain C, G and A on these positions. Further variability is located on positions 606–607, which is used to differentiate between two genotypes 'A' (nucle-

Table 1. Sequence variability and GenBank accession numbers of *Babesia canis*-like isolates.

GenBank Acc. Nos.	Isolate	Host	Nucleotides on selected positions				
			399	606	607	717	888
AY072926	<i>Babesia canis</i> (Piana et Gal- li-Valerio, 1895)	dog	C	G	A	G	A
KX857481	5375	<i>Ixodes ricinus</i> (Linnaeus)	T	A	G	A	G
KX857482	5424	<i>I. ricinus</i>	T	A	G	A	G
KX857483	5488	<i>I. ricinus</i>	T	A	G	A	-
KX857484	5583	<i>I. ricinus</i>	T	A	G	A	-
KX857472	6959	<i>Haemaphysalis concinna</i> Koch	C	G	A	G	A
KX857473	6927	<i>H. concinna</i>	C	G	A	G	A
KX857474	6906	<i>H. concinna</i>	C	A	G	G	A
KX857475	6961	<i>H. concinna</i>	C	G	A	G	A
KX857476	6962	<i>H. concinna</i>	C	G	A	G	A
KX857477	6958	<i>H. concinna</i>	C	A	G	G	A

otides A-G) and 'B' (nucleotides G-A) of *B. canis*. Four of our isolates from *H. concinna* correspond to genotype 'B', which is described as being the more pathogenic of the two (Adaszek and Winiarczyk 2008). All isolates from *I. ricinus* and two isolates from *H. concinna* have nucleotides A-G on these positions, so they belong to genotype 'A'.

Detection of isolates with 18S rDNA very similar to *B. canis* is surprising, since no case of autochthonous canine babesiosis caused by this species has been described in the Czech Republic so far. Canine babesiosis is widespread in neighbouring countries. The first clinical case in Slovakia was described almost 15 years ago (Chandoga et al. 2002). Since then many clinical cases have been reported (Majláthová et al. 2011, Cook and Swann 2016). A prevalence of up to 50% in questing ticks was confirmed in Hungary (Hornok et al. 2016), and up to 11% in Poland (Zygner et al. 2008). A molecular study showed a more than 25% prevalence of *B. canis* in the blood of asymptomatic dogs in Poland (Welc-Falęciak et al. 2009). Kubelová et al. (2011) studied the distribution of *B. canis* in *D. reticulatus* in the Danubian Lowland and documented the westernmost *B. canis*-positive *D. reticulatus* near Gabčíkovo Waterworks close to Bratislava.

Despite the number of examined *D. reticulatus*, the definitive host of *B. canis*, originating from the studied area, we did not find any of them to contain *Babesia* DNA. The absence of *B. canis* in examined samples could be caused by lower concentration of *Babesia* DNA in our samples. Nevertheless, *B. canis*-positive samples isolated from *D. reticulatus* collected in year 2009 and extracted with the same method are still used in our laboratory as *Babesia*-positive control with very high concentration, so we do not expect low yield due to this extraction method. In an earlier study, Konvalinová et al. (2012) described

a 12% prevalence of anti-*B. canis* antibodies among 41 asymptomatic PCR-negative dogs from district Břeclav and Lanžhot with no history of travelling abroad. The geographic occurrence of these serologically positive dogs corresponds with our findings of *Babesia*-positive ticks. It is notable that *B. canis*-like infection has been discovered in other than the known definitive tick host species. The phenomenon of the presence of *B. canis* in other tick species than *D. reticulatus* has previously been reported. Cieniuch et al. (2009) described the presence of *B. canis* from *I. ricinus* in Poland and Tomanović et al. (2012) proved the presence of *B. canis* in *H. concinna* in Serbia. However, the authors amplified a sequence (146 bp) of 18S rDNA, which was too short for reliable species diagnosis and provided no details about homology with GenBank records.

Infection by *B. canis* in a dog with clinical signs of babesiosis and the presence of large babesias has also been reported in Norway, out of the range of *D. reticulatus* (see Øines et al. 2010). Possible explanations are as follows: (1) the introduction of an infected vector, (2) the undisclosed travelling history of the dog, or (3) infection by another, nearly identical species of *Babesia*, transmitted by another tick vector. The resolution of 18S rDNA between various species of *Babesia* is generally very low; nevertheless, fragments of 18S rDNA are clearly most available for sequence comparison. From a species diagnostics point of view, a multi-gene approach would be more appropriate. Accordingly, we tried to obtain complete 18S sequences and sequences of the *cox1* gene from our positive samples, but we were not successful, most probably due to the age and inappropriate storage of the isolated DNA.

The species determination of babesias is still complicated in spite of the growing number of available sequence data and an improvement in PCR-based methods. Taking into account the absence of *Babesia*-positive *D. reticulatus*, the absence of clinical cases of canine babesiosis caused by *B. canis*, and the lower reliability of 18S rDNA in the species diagnosis of babesias, we might suggest that our isolates are not *B. canis*, but another very closely related species possessing a similar studied portion of sequence of 18S rDNA. Due to the presence of seropositive dogs in the same geographic area as *Babesia*-positive ticks, we cannot exclude the possibility of a cross-immunity reaction between *B. canis* and *Babesia* spp. from other tick species.

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