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Autochthonous *Babesia canis*, *Hepatozoon canis* and imported *Babesia gibsoni* infection in dogs in the Czech Republic

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ABSTRACT: The epidemiology of tick-borne diseases as well as the ecology of the associated tick vectors is currently undergoing dynamic change. Canine babesiosis has, to date, only been reported in the Czech Republic as an imported disease, despite the presence of an established population of *D. reticulatus* in the South Moravian region. A recent report of autochthonous *H. canis* in dogs indicates that the spectrum of tick-borne diseases in dogs in the Czech Republic may be broader than previously expected and that a survey of a large set of dogs may be required. The aim of the present study was to survey the spectrum of tick-borne apicomplexan parasites infecting dogs in the Czech Republic and to assess the autochthonous character of detected infections. In the present study, we surveyed the spectrum of tick-borne protists in 418 dogs in the Czech Republic using PCR-based detection of the 18S rDNA gene of piroplasms and *H. canis*. We confirmed the presence of *H. canis* (4%), *B. canis* (1%, including the autochthonous cases) and a single imported case of *B. gibsoni* (0.2%) infection in sampled animals. Our results indicate that these pathogens deserve closer attention from veterinarian practitioners.

Keywords: tick-borne diseases; apicomplexan parasites; PCR detection; 18S rDNA

Apicomplexan tick-borne parasites are pathogens of great veterinary importance both in livestock and companion animals. In Europe, several species of piroplasms (*Theileria* and *Babesia* spp.) and *Hepatozoon canis* are known to infect dogs as well as free ranging carnivores. The distribution and epidemiology of these parasites is highly dynamic due to (I) the increasing movement of animals and (II) changes in the ecology and distribution of their tick vectors (Solano-Gallego et al. 2016). However, the increased application of DNA-based diagnostic techniques has also contributed to the increased rate of detection of these pathogens.

Several *Babesia* species, differing in geographical distribution as well as in their vectors, have been recognised to cause clinical disease in dogs in Europe. *B. canis* (sensu stricto) is the most common agent of canine babesiosis in Central Europe (Solano-Gallego and Baneth 2011; Matijatko et al. 2012; Solano-Galego et al. 2016). The local presence of this parasite largely depends on its vector *Dermacentor reticulatus* which has mosaic distri-

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bution throughout Europe in areas with mild and wet climates (Siroky et al. 2011; Foldvari et al. 2016; Rubel et al. 2016). In Mediterranean areas, two further species of *Babesia*, namely *B. vogeli* and *B. gibsoni,* are found (Cardoso et al. 2008; Solano-Galego et al. 2008; Beck et al. 2009; Trotta et al. 2009; Gabrielli et al. 2015; Rene-Martellet et al. 2015). *B. vogeli* is transmitted by *R. sanguineus* s.l. and the same vector is presumed to also transmit *B. gibsoni* (Solano-Gallego et al. 2016). However, *B. gibsoni* was reported also from Germany (Hartelt et al. 2007) and Slovakia (Vichova et al. 2016), countries which are considered *Rhipicephalus*-free (Estrada-Pena et al. 2013). An alternative route of transplacental transmission as well as infection via biting wounds was described for *B. gibsoni* (Birkenheuer et al. 2005; Fukumoto et al. 2005) and transplacental transmission was reported also for *B. canis* (Mierzejewska et al. 2014; Adaszek et al. 2016). The spectrum of piroplasms known to infect European dogs also includes *Theileria annae* (known also as *B. annae*/*B. vulpes*), an organism which causes severe clinical babesiosis in some parts of Europe (Zahler et al. 2000; Falkeno et al. 2013; Rene-Martellet et al. 2015). However, this parasite is found predominantly in red foxes which seem to be the natural (and reservoir) host (Karbowiak et al. 2010; Duscher et al. 2014; Najm et al. 2014).

Imported cases of clinical babesiosis caused by *B. canis* have also been repeatedly reported from the Czech Republic (CR) (Kucera 1992; Svobodova and Svobodova 2004), but no autochthonous case has been confirmed to date. The permanent presence of *D. reticulatus* in southern Moravia (Siroky et al. 2011), together with a number of imported cases raised concerns that *B. canis* may become established as an endemic pathogen in this part of the CR. In a study performed by Konvalinova et al. (2012) on a group of 41 non-travelling dogs and 340 adult unfed *D. reticulatus* from the South Moravian region, the authors did not detect the parasite by PCR, but found 12% of dogs to be serologically positive.

H. canis is common in dogs in Mediterranean areas and is transmitted through the ingestion of its vector, the brown dog tick *Rhipicephalus sanguineus* s.l. Although the pathogenicity of this parasite is generally low, some infected animals can develop mild or severe clinical disease (Baneth 2011). Reports of *H. canis* in domestic dogs from localities outside the *Rhipicephalus* range are rare (Hamel et al. 2011; Maguire et al. 2011; Hornok et al. 2013), commonly attributable to travel. Conversely, the parasite is commonly reported in red foxes (*Vulpes vulpes*) and Eurasian golden jackals (*Canis aureus*) also in *Rhipicephalus*-free geographic areas (Majlathova et al. 2007; Karbowiak et al. 2010; Duscher et al. 2013; Duscher et al. 2014; Farkas et al. 2014; Mitkova et al. 2014; Najm et al. 2014). In the CR, *H. canis* was reported for the first time in red foxes and in hunting dogs only recently (Mitkova et al. 2016), indicating the possibility of occasional transmission of the parasite between dogs and foxes in areas with an apparent absence of *R. sanguineus* s.l. as the typical vector.

Dynamic changes in the epidemiology of canine tick-borne diseases (TBDs) in Europe, suitable conditions for the establishment of *B. canis* foci in Moravian region together with a recent report of *H. canis* infection, prompted us to investigate a larger set of dogs from different parts of the CR for the presence of *H. canis, Babesia* spp. and *T. annae*.

MATERIAL AND METHODS

A total of 418 dogs including pet dogs, dogs from animal shelters and hunting dogs from the Czech Republic were sampled as part of an epidemiological survey on canine dirofilariosis in the summer of 2015. The majority of sampled dogs (345) originated from south and central parts of the South Moravian region, 32 dogs were from South Bohemia, 29 from Prague and the remaining 12 dogs came from different localities of Central Bohemia. The same set of samples was subjected to examination for *Hepatozoon* parasites and piroplasmids.

DNA was isolated from 200 μl of EDTA blood using a commercial kit (Genomic DNA Mini Kit, Geneaid Biotech, Taiwan). Two PCRs targeting 18S rDNA were performed. Nested PCR using the primers BTH-1F, BTH-1R in the first run and GF2, GR2 in the second run was employed (Zintl et al. 2011; Hodzic et al. 2015), to obtain approximately 560 bp amplicon of *Babesia*/*Theileria* 18S rDNA. For detection of *Hepatozoon* DNA, a conventional PCR in which approximately 625 bp long fragment was amplified was performed using the HEP F and HEP R primers (Inokuma et al. 2002) with modified conditions (Mitkova et al. 2016).

PCR products were visualised using 1% agarose gel electrophoresis with GoodView DNA stain

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(Beijing SBS Genetech, China). PCR products from the second run of nested piroplasmid PCR and from *Hepatozoon* PCR were purified (Gel/PCR DNA Fragment Extraction Kit, Geneaid Biotech, Taiwan) and subjected to sequencing using the amplification primers (Macrogen Europe, the Netherlands). All obtained sequences were edited and analysed using Geneious® 9.1.2 (Kearse et al. 2012) and compared to the GenBank database using the BLAST algorithm.

All sequences of piroplasmids and selected representatives of *Hepatozoon* species were deposited in GenBank and used in phylogenetic analyses. A set of chosen nucleotide sequences of corresponding fragments from GenBank, covering a broad geographical and host origin, was aligned with the sequences described here, using the ClustalW algorithm (Larkin et al. 2007). Appropriate evolution models were determined by a likelihood ratio test using R software (R Core Team 2012). Phylogenetic analyses were performed using the maximum likelihood method in PhyML 3.0 software (Guindon and Gascuel 2003). Phylogenetic trees were visualised and edited in FigTree v. 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

PCR followed by amplicon sequencing confirmed *H. canis* positivity in 16 dogs (4%), *B. canis* positivity in five dogs (1%) and *B. gibsoni* positivity for one dog (0.2%); coinfections were not observed. The geographic origin of positive animals is summarised in Table 1. Importantly, the owners of 10 *H. canis*-positive dogs and three *B. canis*-positive dogs from the South Moravian region confirmed that their dogs had no history of travel and had never crossed the borders of the Czech Republic.

Obtained sequences of the *Hepatozoon* 18S fragment showed only limited variability. In BLAST analysis, all sequences revealed 99–100% identity to sequences from red foxes and a golden jackal from Hungary (KC886726, KJ572978, KJ572979). Our sequences clustered together with *H. canis* sequences from wild canids and dogs from central and southern Europe (Czech Republic, Austria, Hungary, Croatia, Italy; Figure 1). The sequences from our study are available in GenBank under the numbers KY021177–KY021184.

Table 1. Numbers of sampled dogs and prevalence of positive findings (%) in different regions of the Czech Republic

Region	Dogs	*H. canis*	*B. canis*	*B. gibsoni*
South Moravia	345	13 (4)	4 (1)	1 (0.3)
South Bohemia	32	1 (3)	1 (3)	0
Prague	29	2 (7)	0	0
Central Bohemia	12	0	0	0

Sequences of piroplasms were 99–100% identical to *B. canis* from dogs from Estonia and Poland (KT008057, EU622793) and from *Ixodes ricinus* from Slovakia and Poland (KU362904, KR003829). One of our sequences (KY021187) showed 100% identity to *B. gibsoni* from a dog from China (KP666168). Our *B. canis* (KY021185, KY021186, KY021188–KY021190) clustered together with other *B. canis* sequences deposited in GenBank. The only sequence differences of *B. canis* isolates were in AG/GA nucleotides responsible for their clustering to genotype A (two sequences) or genotype B (three sequences). A single *B. gibsoni* sequence is placed within the *B. gibsoni* clade (Figure 2).

DISCUSSION

Changes in the epidemiology of TBDs have recently been reported from all around Europe (Otranto et al. 2015; Solano-Gallego et al. 2016). The most commonly discussed reasons include the changing ecology of ticks (Medlock et al. 2013; Pfaffle et al. 2013; Foldvari et al. 2016; Rubel et al. 2016) and international travel or transport of infected animals (Hamel et al. 2011). Both of these situations apply also for the Czech Republic (Kucera 1992; Svobodova and Svobodova 2004; Materna et al. 2008). The common use of highly sensitive PCR-based diagnostic tools, often combined with phylogenetic analyses, contributes not only to a growing rate of detection of these pathogens, but also to a better understanding of their diversity (Schnittger et al. 2012; Carcy et al. 2015).

B. canis is an emerging pathogen in Europe (Solano-Gallego et al. 2016) and, in the last decades, has become locally endemic in all countries bordering the Czech Republic. Canine babesiosis is present in localities with established populations of *D. reticulatus* in Germany (Naucke 2008), Austria (Halos et al. 2014; Pantchev et al. 2015), Poland (Mierzejewska et al. 2015) and Slovakia (Kubelova

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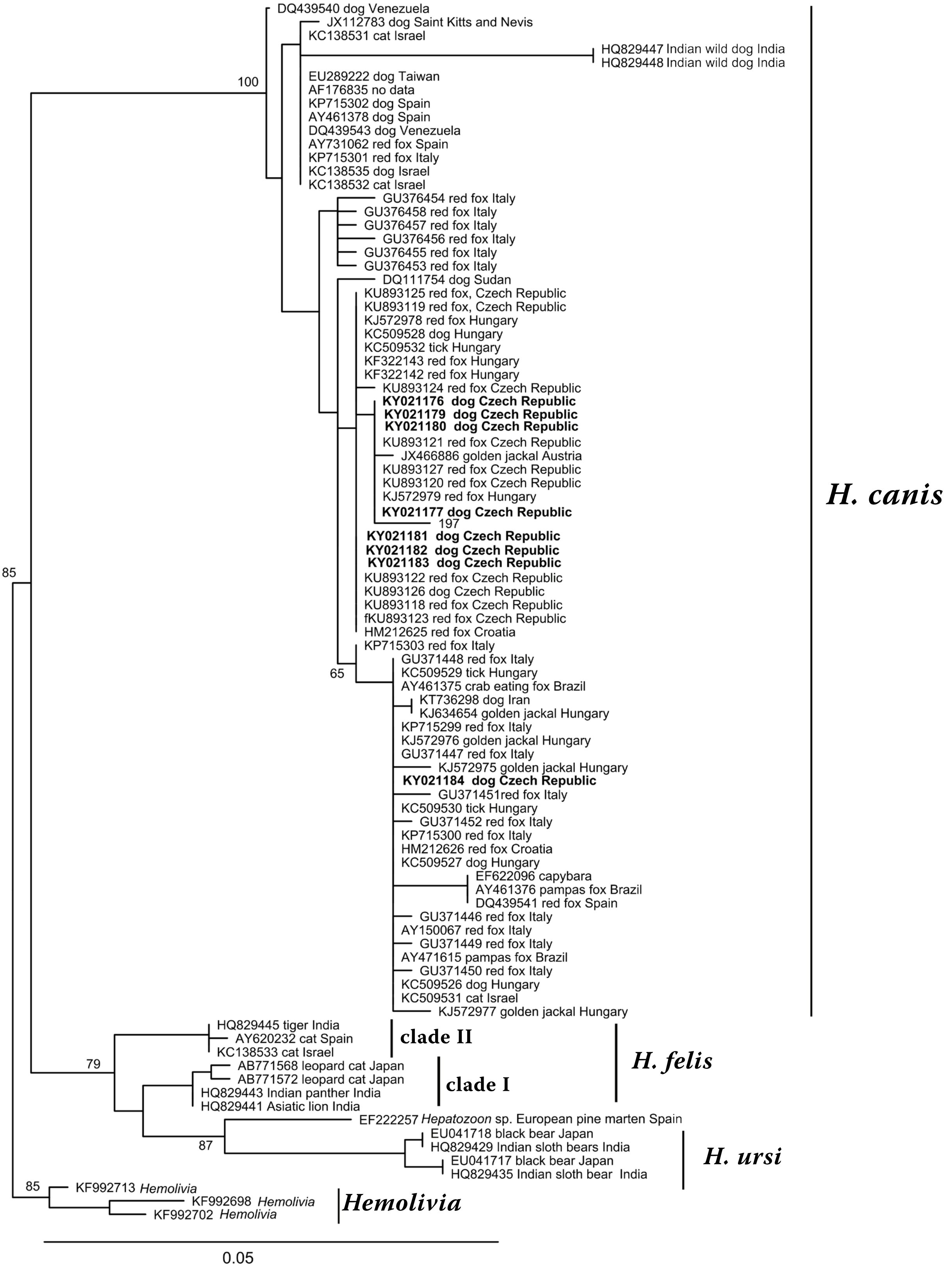

Figure 1. A maximum likelihood tree based on a 505 nt long alignment of 18S rDNA sequences of *Hepatozoon* spp. Sequences from this study are in bold; sequences of *Hemolivia* spp. are used as an outgroup; bootstrap values from 1000 replicates above 60% are shown; scale bar represents the probability of nucleotide change per site

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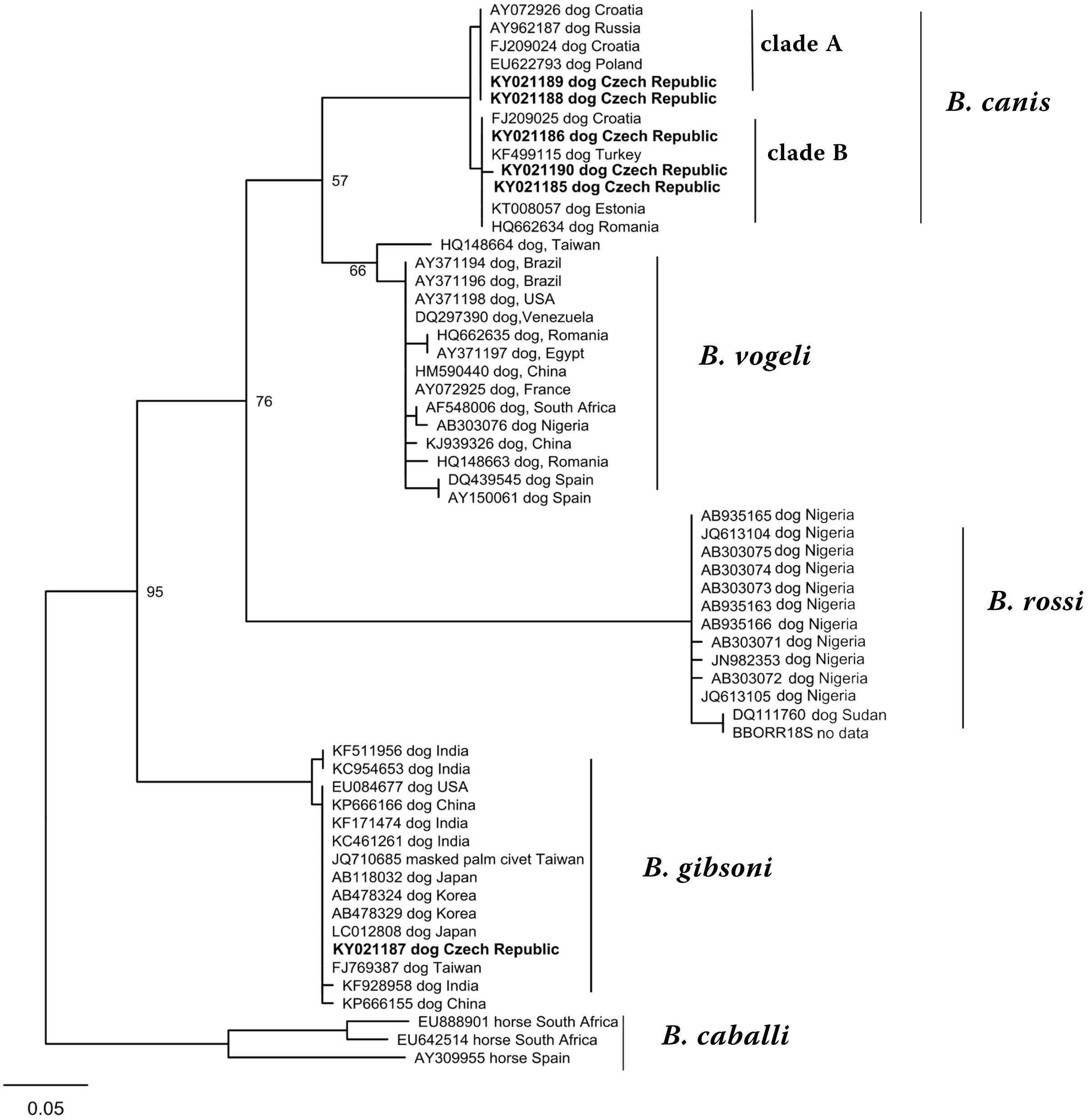

Figure 2. A maximum likelihood tree based on a 391 nt long alignment of 18S rDNA sequences of *Babesia* sp. infecting dogs. Sequences from this study are in bold; sequences of *B. caballi* used as an outgroup; bootstrap values from 1000 replicates above 60% are shown; scale bar represents probability of nucleotide change per site

et al. 2011; Majlathova et al. 2011; Kubelova et al. 2013). *B. canis* was previously reported from the CR; however, all cases were in dogs with a history of travel to endemic areas (Kucera 1992; Svobodova and Svobodova 2004). The most recent study failed to detect parasite DNA in non-travelling dogs or in ticks from the Czech Republic, but did report serologically positive individuals (Konvalinova et al. 2012). Our study provides PCR confirmation of *B. canis* in non-traveling dogs from the South Moravian region, which was suspected as a potentially endemic area with well-established populations of *D. reticulatus* (Siroky et al. 2011), having spread from the Panonian lowlands of north-east Austria and western Slovakia. Based on this, one can assume that the spread of *B. canis* follows the natural distribution of its vector. However, the data on *B. canis* infection in dogs or ticks in areas of Slovakia and Austria adjacent to the borders of the Czech Republic are either absent, or infection was not confirmed at the molecular level despite the presence of clinical cases (Kubelova et al. 2011; Majlathova et al. 2011; Kubelova et al. 2013; Halos et al. 2014; Pantchev et al. 2015). Recent phylogenetic studies suggested intraspecies diversity of *B. canis*. Three strains were described (A, B and 34) based on 18S rDNA and *Bc28.1* gene analyses, differing in geographical distribution and severity of clinical signs (Adaszek and Winiarczyk 2008; Beck et al. 2009; Carcy et al. 2015). Based on analyses of 18S sequences, our sequences clustered within A and B clades, which corresponds well with published evidence that both genotypes are present in Central Europe (Carcy et al. 2015). Published reports raised the possibility of alternative transmission routes of *B. canis,* e.g. the vectorial role of *Ixodes ricinus* and transplacental transmission (Cieniuch et al. 2009; Iori et al. 2010; Mierzejewska et al. 2014; Adaszek et al. 2016; Hamsikova et al. 2016). Four out of five dogs found positive in our study originated from an area where *D. reticulatus* is known to be present. The remaining single positive dog was sampled in an area without any known presence of *Dermacentor*; however, data on the travel history of this dog are missing. So far, we do not have enough evidence for the presence of *B. canis* infection outside the range of *D. reticulatus* in the Czech Republic.

Our study provides the first report of *B. gibsoni* in the CR. The infected dog was an American pit bull terrier with clinical signs of acute babesiosis. In fact, the animal originated from Slovakia, where *B. gibsoni* was already reported (Vichova et al. 2016). *T. annae* was not detected in our sample set, although clinical disease in dogs caused by this parasite was reported from several European countries (Zahler et al. 2000; Falkeno et al. 2013; Rene-Martellet et al. 2015) and the parasite is apparently common in red foxes in Central Europe (Karbowiak et al. 2010; Duscher et al. 2014; Najm et al. 2014).

H. canis was recently reported in red foxes and in hunting dogs from the CR (Mitkova et al. 2016). The present study confirms its presence in the studied dog population, however, at a relatively low prevalence (4%). We also provide further evidence that *H. canis* infection is endemic in dogs in *Rhipicephalus*-free areas, although the infection route remains unknown. It is probable that red foxes represent a reservoir of the infection. Their role in the epidemiology of *H. canis* infection in Central Europe, as well as the involvement of alternative tick vectors, deserves future attention, (Najm et al. 2014). None of the examined dogs positive for *H. canis* DNA showed clinical signs of canine hepatozoonosis; however, the presence of infection in clinically healthy dogs is common throughout the range of this canine pathogen, and is related to the intensity of parasitaemia (Baneth and Weigler 1997; Baneth 2011).

Our study provides the first evidence for autochthonous infections of *B. canis* in dogs in the Czech Republic from localities with the presence of its tick vector. This infection deserves more attention in small animal practice and babesiosis should be suspected also in dogs with no travel history. Moreover, we report a case of *B. gibsoni* infection imported from Slovakia, affirming the necessity of proper determination of piroplasm species. We confirm the presence of *H. canis* in domestic dogs in the CR; however, the real distribution of this pathogen in Central Europe remains unclear and further research is necessary to explain the discrepancies between the epidemiology of this infection inside and outside the *R. sangineus* s.l. range.

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