Occurrence and diversity of arthropod-transmitted pathogens in red foxes (*Vulpes vulpes*) in western Austria, and possible vertical (transplacental) transmission of *Hepatozoon canis*

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(*Received 7 April 2017; revised 23 May 2017; accepted 31 July 2017; first published online 24 August 2017*)

SUMMARY

Red fox (*Vulpes vulpes*) is the most abundant wild canid species in Austria, and it is a well-known carrier of many pathogens of medical and veterinary concern. The main aim of the present study was to investigate the occurrence and diversity of protozoan, bacterial and filarial parasites transmitted by blood-feeding arthropods in a red fox population in western Austria. Blood ($n = 351$) and spleen ($n = 506$) samples from foxes were examined by PCR and sequencing and the following pathogens were identified: *Babesia canis*, *Babesia* cf. *microti* (syn. *Theileria annae*), *Hepatozoon canis*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia sp. and *Bartonella rochalimae*. Blood was shown to be more suitable for detection of *Babesia* cf. *microti*, whilst the spleen tissue was better for detection of *H. canis* than blood. Moreover, extremely low genetic variability of *H. canis* and its relatively low prevalence rate observed in this study may suggest that the parasite has only recently been introduced in the sampled area. Furthermore, the data presented here demonstrates, for the first time, the possible vertical transmission of *H. canis* from an infected vixen to the offspring, and this could explain the very high prevalence in areas considered free of its main tick vector(s).

Key words: vector-borne pathogens, *Hepatozoon canis*, transplacental transmission, fox, *Vulpes vulpes*, Austria.

INTRODUCTION

Red foxes (*Vulpes vulpes*) play an important role in the transmission of a broad spectrum of pathogens of medical and veterinary importance, and this is mostly due to their increasing population density, wide distribution, high susceptibility to relevant pathogens and their good adaptation to urban and periurban environments (Duscher *et al*. 2015). Increasing number and urbanization of foxes consequently increase the risk of pathogen transmission to humans and domestic animals (Barandika *et al*. 2016). In the last few years, foxes have been intensively investigated worldwide for the presence of protozoan (e.g. *Babesia*, *Hepatozoon*, *Leishmania*), bacterial (e.g. Anaplasmataceae, *Bartonella*, *Borrelia*) and filarial (e.g. *Dirofilaria*) infections transmitted by haematophagous arthropod vectors, as they seem to play an important role in the maintenance and transmission of the agents (Henn *et al*. 2009; Torina *et al*. 2013; Millán *et al*. 2014; Penezić *et al*. 2014; Cardoso *et al*. 2015; Dumitrache *et al*. 2015; Hodžić *et al*. 2015). The incidence, diversity and geographic range of the pathogens harboured by red foxes and other wild carnivore species have increased in the recent past due to several factors, and these mainly include climate changes, destruction of wildlife habitats, urbanization, but also increased awareness of the scientific community and establishment of more reliable and accurate diagnostic tools (Aguirre, 2009; Alvarado-Rybak *et al*. 2016).

However, many aspects of the epidemiology of vector-borne diseases such as host range, vector host use, conservation threats, possible non-vectorial routes of transmission and zoonotic potential are still poorly understood. Therefore, investigation of the pathogen distribution, vector-host–pathogen associations and dynamics of infections among wild and domestic carnivores are of a great importance for better understanding the epidemiology and also for filling the existing gaps required for establishing efficient control strategies (Otranto *et al*. 2015; Alvarado-Rybak *et al*. 2016).

The data on the occurrence and diversity of vector-borne pathogens in foxes in Austria are fragmentary

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doi:10.1017/S0031182017001536

and limited to a very small area located in the north-eastern part of the country (Gänserndorf, Lower Austria) (Duscher *et al*. 2014). Therefore, this comprehensive molecular study focused on the following aspects: (i) prevalence and genetic diversity of pathogens transmitted by hematophagous arthropods in a red fox population in western Austria; (ii) genetic variations and haplotype composition of the two most common apicomplexan haemoparasites in foxes namely *Babesia* cf. *microti* and *Hepatozoon canis*; (iii) comparative suitability of blood and spleen tissues for molecular detection of haematozoa, and (iv) possible vertical transmission of *H. canis* from an infected vixen to its offspring, and importance of the non-vectorial transmission pathway in the circulation of this parasite.

MATERIAL AND METHODS

Study area and sample collection

The study was conducted in the two westernmost Austrian provinces, Tyrol and Vorarlberg between November 2013 and March 2015. Carcasses of 506 red foxes (245 males and 261 females) were collected as a part of *Trichinella* and *Echinococcus multilocularis* control programs, and delivered to the Institute for Veterinary Disease Control in Innsbruck, Austria. A complete necropsy was performed and spleen tissue was sampled from all animals and stored at −20 °C until processed for molecular analysis. In addition, blood samples from 351 foxes were taken from body cavities (primarily thoracic cavity) and spotted (100 μL) onto filter paper. Animals were sexed and divided into two age groups based on tooth wear: juveniles (<1 year; 337 individuals) and adults (⩾1 year; 169 individuals).

DNA extraction, PCR amplification and sequencing

Genomic DNA was isolated from the spleen tissue with DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA extraction from the blood spots was performed using the protocol previously described (Fuehrer *et al*. 2010). Briefly, disks of spotted and air dried blood, 4 × 4 mm^2 in size, were cut out of the filter paper and soaked in 100 μL phosphate-buffered saline (PBS) at 4 °C overnight. After the isolation step, DNA was purified with InstaGene™ Matrix (Bio-Rad, Austria). DNA extracts from spleen and blood samples were used as templates for polymerase chain reaction (PCR) amplification and molecular detection of pathogens. For subtyping of genetic variants, all samples positive for *Anaplasma phagocytophilum* in the 16S rRNA gene were additionally tested by PCR targeting the *groEL* heat shock operon segment. Primer sequences, targeted genes and protocols used in the study are listed in Table 1. All PCR reactions were carried out in a final volume of 25 μL with 5× Green Reaction Buffer and GoTaq G2® Polymerase (Promega, Germany). Positive (specific pathogen DNA i.e. *B. canis*, *H. canis*, *L. infantum*, *A. phagocytophilum*, *E. canis*, *B. henselae*, *R. raoultii*, *D. repens*, *D. immitis*) and negative (PCR grade water) controls were included in PCR assays. The amplified products were electrophoresed on 2% agarose gel stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Germany), and purified and sequenced in both directions (Sanger sequencing method) by a company LGC Genomics, Germany. The sequences obtained were edited with the software BioEdit v.7.2.5 (Hall, 1999) and compared for identity with the sequences deposited in GenBank® nucleotide database using BLAST (Basic Local Alignment Search Tool) analysis (http://www.ncbi.nlm.nih.gov/BLAST).

Molecular detection of Hepatozoon canis *in foetal tissues*

A total of six foetuses from a pregnant fox that was found to be infected by *H. canis* (universal primers) were dissected and tested by PCR in order to investigate the possible transplacental infection. Briefly, the partially developed foetuses (5–6 cm in length) were cut independently in two halves by means of a sterile scalpel and all internal organs from abdominal and thoracic cavity were removed, transferred to a 50 mL plastic tube and then homogenized using a metal spatula. Standard precautions (e.g. separate gloves, sterile dissection tools, sterile tubes) were taken during dissection to avoid contamination and contact with infected blood from the vixen. Approximately 20 mg of foetal tissue homogenate was subjected to DNA extraction using the same kit (DNeasy® Blood & Tissue Kit, Qiagen). The DNA from the mother fox and all six foetuses were tested with genus-specific primers H14Hepa18SFw and H14Hepa18SRv (Hodžić *et al*. 2015), which amplify a 620 bp fragment of the 18S rRNA gene of *Hepatozoon* species (Table 1), and positive products were sequenced.

Phylogenetic analysis

For phylogenetic analysis, only those 18S rRNA nucleotide sequences of *Babesia* cf. *microti* and *H. canis* available in GenBank® database, which showed a minimum of 458 bp (86 sequences) and 413 bp (370 sequences) overlap respectively, with sequences generated herein were included in the overall alignment. Multiple sequence alignment was performed with ClustalW algorithm implemented in BioEdit v.7.2.5 (Hall, 1999) and the sequences were trimmed manually. Median Joining (MJ)

https://doi.org/10.1017/S0031182017001536 Published online by Cambridge University Press

Table 1. Primer sequences and PCR protocols used for molecular detection of arthropod-borne pathogens

Target organism	Genetic marker	Primer sequences (5′→3′)	Product size (bp)	Reference
Babesia spp./ *Hepatozoon canis*	18S rRNA	BTH-1F: CCTGAGAAACGGCTACCACATCT BTH-1R: TTGCGACCATACTCCCCCCA Nested PCR GF2: GTCTTGTAATTGGAATGATGG GR2: CCAAAGACTTTGATTTCTCTC	720/750 590/610	Zintl *et al.* (2011)
Hepatozoon canis	18S rRNA	H14Hepa18SFw: GAAATAACAATACAAGGCAGTAAAATGCT H14Hepa18SRv: GTGCTGAAGGAGTCGTTTATAAAGA	620	Hodžić *et al.* (2015)
Leishmania infantum	kDNA	RV1: CTTTTCTGGTCCCGCGGGTAGG RV2: CCACCTGGCCTATTTTACACCA	145	le Fichoux *et al.* (1999)
Anaplasmataceae	16S rRNA	EHR16SD: GGTACCYACAGAAGAAGTCC EHR16SR: TAGCACTCATCGTTTACAGC	345	Brown *et al.* (2001)
Anaplasma phagocytophilum	*groEL*	Apgroe02for: CGAAAGCTGCTGGATCTGA Apgroe02rev: TCCTTGAAGCCTTTGCTTTC	235	This study
Bartonella spp.	16S-23S rRNA	BA325s: CTTCAGATGATGATCCCAAGCCTTYTGGCG BA1100as: GAACCGACGACCCCCTGCTTGCAAAGCA	500–800	Diniz *et al.* (2007)
Rickettsia spp.	23S/5S rRNA	ITS-F: GATAGGTCGGGTGTGGAAG ITS-R: TCGGGATGGGATCGTGTG	342–533	Vitorino *et al.* (2003)
Filarioid nematodes	COI	H14FilaCOIFw: GCCTATTTTGATTGGTGGTTTTGG H14FilaCOIRv: AGCAATAATCATAGTAGCAGCACTAA	724	Hodžić *et al.* (2015)

networks from both datasets were calculated with Network software v.4.6.0.0 (available at: www.fluxus-engineering.com) applying the default parameters. Unnecessary median vectors were reduced in the post-processing step using Maximum parsimony (MP) option.

Statistical analysis

The Mann–Whitney-*U* test was employed to test the associations between pathogen occurrence and sex and age of the animals, whereas proportions of positivity between blood and spleen were compared with the Chi-Squared and Fisher's exact tests. Differences were considered statistically significant if $P < 0{\cdot}05$. All statistical analyses were performed with SPSS 20.0 statistical software. Samples positive for *Babesia canis*, *A. phagocytophilum*, *Candidatus* Neoehrlichia sp. and *Bartonella rochalimae* were excluded from the statistical analysis because of very low infection rates.

Ethical statement

All animals from the present study were killed legally during regular hunting events and under the Austrian hunting laws.

RESULTS

Pathogen diversity, prevalence and multiple infections

Overall 69·5% blood samples (244/351; 95% CI: 64·5–74·1%) and 56·7% spleen tissue samples (287/506; 95% CI: 52·4–60·1%) of red foxes from western Austria were found to be positive by PCRs and the following pathogens were identified: *B. canis*, *Babesia* cf. *microti* (syn. *Theileria annae*), *H. canis*, *A. phagocytophilum*, *Candidatus* Neoehrlichia sp. and *B. rochalimae* (Table 2). Multiple infections with two or three different agents were recorded in 33 (6·5%) and 1 (0·2%) animals, respectively, and co-infection with *Babesia* cf. *microti* and *H. canis* was most prevalent (31/506; 6·1%) followed by *H. canis* and *A. phagocytophilum* (2/506; 0·4%), and *Babesia* cf. *microti*, *H. canis* and *A. phagocytophilum* (1/506; 0·2%). DNA of *Babesia* cf. *microti* and *H. canis* was detected in both blood (50·7 and 18·5%, respectively) and spleen tissue (25·7 and 29·8%, respectively), whereas *A. phagocytophylum* (0·6%), *Candidatus* Neoehrlichia sp. (0·4%) and *B. rochalimae* (0·2%) could be amplified only in spleen samples. *Babesia canis* DNA from a single positive fox (0·3%) was found in blood, but not in spleen (Table 2). Furthermore, blood was significantly more frequently infected with *Babesia* cf. *microti* ($P < 0{\cdot}0001$) compared with spleen, but the positivity rate of *H. canis* infection was higher in spleen ($P < 0{\cdot}05$) than in blood (Table 2). However, no significant differences were observed between infection rates and gender or age ($P > 0{\cdot}05$) of the host.

Sequence and phylogenetic analyses

A total of 244 blood and 281 spleen samples were positive by PCR performed with universal primer sets BTH-1R, BTH-1R and GF2, GR2, which

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Table 2. Prevalence of pathogens detected in blood and spleen samples of red foxes (*Vulpes vulpes*) in western Austria

Sample	Total examined	*Babesia canis*		*Babesia* cf. *microti*		*Hepatozoon canis*		*Anaplasma phagocytophilum*		*Candidatus* Neoehrlichia sp.		*Bartonella rochalimae*		Total	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Blood	351	1	0·3	178***	50·7	65	18·5	0	0·0	0	0·0	0	0·0	244	69·5
Spleen	506	0	0·0	130	25·7	151*	29·8	3	0·6	2	0·4	1	0·2	287	56·7

*$P < 0{\cdot}05$; ***$P < 0{\cdot}0001$.

amplify the 18S rRNA gene of piroplasmids and *Hepatozoon* species (Tables 1 and 2). However, only 378 (178 blood and 200 spleen) samples, which delivered a strong signal were sequenced, and the BLAST search revealed 99–100% identity to the following nucleotide sequences: *B. canis* (100%, e.g. GenBank® accession nos. KY447296, KT008057), *Babesia* cf. *microti* (99–100%, e.g. KY447297, KY486299, KT223483) and *H. canis* (100%, e.g. KU893127, FJ497011). The species differentiation from the remaining PCR products (66 blood and 81 spleen samples) was made based on the fragment length, as there is an approximately 30–40 bp difference between *Babesia* and *Hepatozoon* species (Table 1) (Modrý *et al.* 2017).

Three samples positive by Anaplasmataceae-specific PCR showed 100% identity to the 16S rRNA gene of *A. phagocytophilum* strains (e.g. KY458571, KX180948), whereas two nucleotide sequences belonged to the recently described sequence of *Candidatus* Neoehrlichia sp. [the finding of one of these samples has already been published in Hodžić *et al.* (2015*a*)]. *Anaplasma phagocytophilum* species validation and subtyping were done by PCR targeting *groEL* gene and sequence analysis revealed 100% identity to a genetic variant (so called G-variant, A → G) previously detected in humans (e.g. KT454993, KF015601), and domestic and wild animals (e.g. CP006618, JF494840, EU860090, DQ680012, KR092132). Furthermore, the spleen sample from one fox was infected by a zoonotic bacterium, *B. rochalimae* (99% identity to e.g. DQ683199, KX169194). None of the collected samples showed the presence of *Ehrlichia*, *Leishmania*, *Rickettsia*, *Dirofilaria* and other filarioid helminths DNA.

The MJ network constructed with 18S rRNA sequences of *Babesia* cf. *microti* obtained in this study and sequences available in GenBank® showed the existence of ten different haplotypes separated from one another by one to three nucleotide substitutions (Fig. 1). Almost all the sequences from our study, except one with a single nucleotide position difference, belong to the most dominant and centrally positioned haplotype, along with sequences previously reported in domestic and wild carnivores (Fig. 1A) from Europe, Asia and North America (Fig. 1B). The genealogic network of *H. canis* showed a considerable 18S rRNA gene nucleotide diversity compared to *Babesia* cf. *microti*, with a total of 56 individual haplotypes divided into several clades and with no obvious correlation between geographic locality, host species and haplotype composition (Figs 2 and 3). However, only one haplotype which grouped together with a haplotype described in other carnivores originating from several European countries was found circulating in the fox population in western Austria (Figs 2 and 3).

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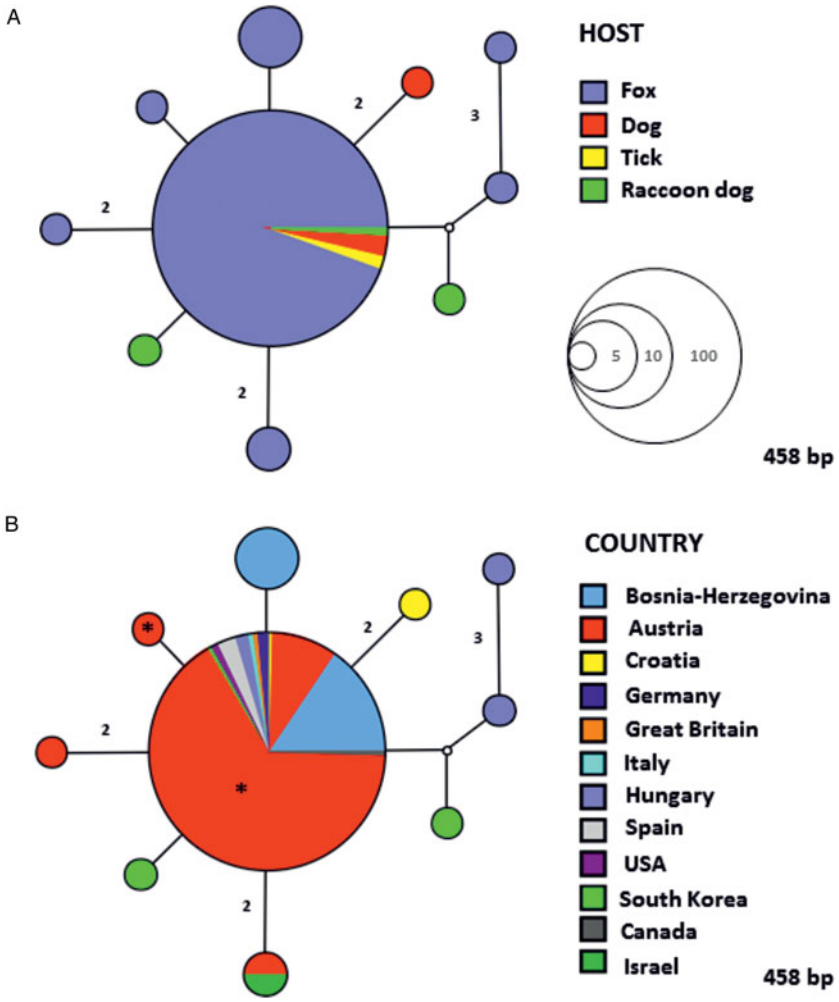

Fig. 1. Median Joining (MJ) network of the 18S rRNA gene (458 bp) of *Babesia* cf. *microti*, showing the relationships between haplotypes according to host (A) and geographic locality (B). The size of the circles in the network is proportional to the number of individuals sharing the same haplotype. Numbers along branches indicate the number of substitutions between haplotypes. An asterisk (*) in the circles represents sequences (haplotypes) obtained in the present study.

The representative sequences obtained in the present study have been deposited in GenBank® database and they are available under the following accession numbers: KY693669 (*B. canis*, 18S rRNA gene), KY693667–KY693668 (*Babesia* cf. *microti*, 18S rRNA gene), KY693670 (*H. canis*, 18S rRNA gene), KY693672 (*A. phagocytophilum*, 16S rRNA gene), KY693671 (*A. phagocytophilum*, *groEL* gene) and KY693673 (*B. rochalimae*, 16S-23S rRNA gene).

Detection of Hepatozoon canis *in fox foetuses*

Out of six foetuses retrieved from a *H. canis* infected vixen, tissues of two (33·3%) tested positive by *Hepatozoon*-specific PCR. DNA sequencing showed that both foetuses and the respective mother fox were infected with the same haplotype of *H. canis*, indicating possible intrauterine infection.

DISCUSSION

The present study reports a relatively high overall prevalence and diversity of pathogens transmitted by arthropod vectors in red foxes from western Austria. It also provides an overview of genetic variability, population structures, geographic distribution and host range for the two most frequently detected haematozoa (i.e. *Babesia* cf. *microti* and *H. canis*) in foxes worldwide. Moreover, the results of this study demonstrate, for the first time, the possible transplacental transmission of *H. canis* from a mother fox to its offspring.

Haematozoan parasites

Three species of apicomplexan haemoparasites namely *B. canis*, *Babesia* cf. *microti* and *H. canis* were identified in the current study. *Babesia* cf. *microti* (order Piroplasmida) is a tick-transmitted parasite affecting dogs and wild carnivores

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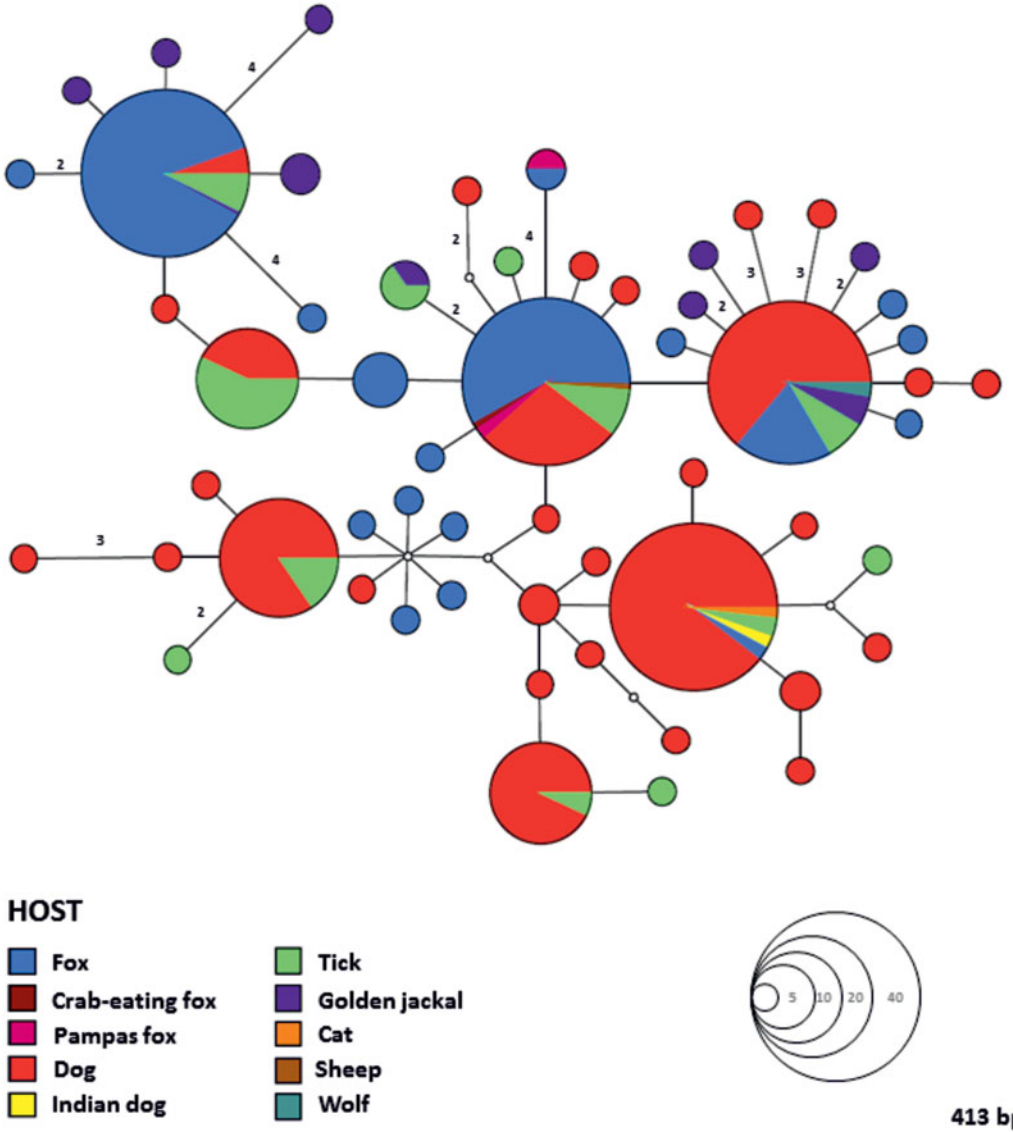

Fig. 2. Median Joining (MJ) haplotype network of the 18S rRNA gene (413 bp) of *Hepatozoon canis*. Colours indicate different hosts and the circle size in the network corresponds to the number of individuals sharing the same haplotype. Numbers along the branches indicate the number of substitutions between haplotypes.

worldwide (Alvarado-Rybak *et al*. 2016). It is also known as *Theileria annae*, *Babesia annae*, *Babesia* sp. 'Spanish dog isolate' and *Babesia vulpes*. However, all these names are considered unavailable as the formal description of the agent is still lacking (Harris, 2016). The piroplasm is genetically most closely related to zoonotic *Babesia microti* (Piroplasmida, Babesiidae), therefore in this study we refer to it as '*Babesia* cf. *microti*' (*cf*. is an abbreviation for the Latin word *confer* which means compare) as recommended by Harris (2016). *Babesia* cf. *microti* has been reported to cause severe illness in dogs (Zahler *et al*. 2000; Camacho, 2006; Falkenö *et al*. 2013; Miró *et al*. 2015), but foxes are considered as major natural reservoir hosts for this species (Otranto *et al*. 2015; Alvarado-Rybak *et al*. 2016). The pathogen has been found infecting foxes in Europe, Asia and North America (Alvarado-Rybak *et al*. 2016) and the prevalence rates range from 0·98% in Italy (Zanet *et al*. 2014) to 69·2% in Portugal (Cardoso *et al*. 2013). The prevalence of 50·7% (in blood) herein observed is the same as that previously reported in the north-eastern part of Austria (50%; Duscher *et al*. 2014). However, only 36 foxes were examined, representing the only currently existing data on haematozoan parasites in Austrian foxes. The hedgehog tick *Ixodes hexagonus* (Acari, Ixodidae) was assumed to be the main vector candidate for *Babesia* cf. *microti*, since it was the most frequently reported tick species in infected dogs from hyperendemic regions in Spain (Camacho *et al*. 2003). Nevertheless, the piroplasmid DNA has recently been found in questing *Dermacentor reticulatus* in Austria, suggesting the possible implication of this tick in the life cycle and transmission of

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Fig. 3. Median Joining (MJ) haplotype network of the 18S rRNA gene (413 bp) of *Hepatozoon canis*. Colours indicate different geographic locality and the circle size in the network denotes the number of individuals sharing the same haplotype. Numbers along the branches indicate the number of substitutions between haplotypes. An asterisk (*) in the circle represents sequences (haplotypes) obtained in the present study.

Babesia cf. *microti* (Hodžić *et al*. 2017). Although the vector competence has not yet been proven for this tick, the existence of the same nucleotide haplotype circulating between foxes and ticks, not only in Austria but all over the Europe, supports this hypothesis.

Another canine babesial species, *Babesia canis* (Piroplasmida, Babesiidae), was molecularly confirmed in the blood of a single fox. Given that only two cases have been previously reported in foxes in Portugal (Cardoso *et al*. 2013) and Bosnia and Herzegovina (Hodžić *et al*. 2015), it can be concluded that they are not suitable hosts for *B. canis*, with hardly any impact as reservoir or spreader (Hodžić *et al*. 2015).

Hepatozoon canis (Eucoccidiorida, Hepatozoidae) is a protozoan parasite of domestic and wild carnivores. In general, the geographical distribution of *H. canis* is related to the distribution of its major tick vector *Rhipicephalus sanguineus* sensu lato (Acari, Ixodidae), also known as the brown dog tick (Baneth, 2011; Giannelli *et al*. 2017). However, this protozoan has also been reported in areas lacking *R. sanguineus* and these include Austria (Duscher *et al*. 2013, 2014), the Czech Republic (Mitková *et al*. 2016), Hungary (Tolnai *et al*. 2015) and Slovakia (Majláthová *et al*. 2007; Miterpáková *et al*. 2017). In addition to the main vector in Europe, a recent study has demonstrated the sporogonic development of *H. canis* in *R. turanicus* collected from a naturally infected fox in Italy, confirming its role as a definitive host (Giannelli *et al*. 2017). *Hepatozoon canis* is one of the most prevalent parasite species found in foxes and the

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prevalence in Europe reaches up to 77% (Cardoso *et al*. 2014). The infection rate of *H*. *canis* assessed in this study (18·5% blood and 29·8% spleen samples tested positive) is noticeably lower than that recently reported in foxes from north-eastern Austria (58·3%, Duscher *et al*. 2014). Moreover, an extremely low level of genetic variability in the 18S rRNA gene of *H*. *canis,* represented by only one haplotype circulating in foxes, was observed in western Austria. Conversely, four haplotypes have been described in 21 foxes in the north-eastern part of the country (Duscher *et al*. 2014), whilst a single golden jackal (*Canis aureus*) from an area close to Vienna was found bearing six different genetic variants of *H*. *canis* (Duscher *et al*. 2013). The high mountain ranges (Central Eastern Alps) which separate western Austria i.e. the western provinces of Tyrol and Vorarlberg from the rest of the country could act as a physical geographic barrier inhibiting gene flow and leading to increased inbreeding frequency. All these factors may subsequently contribute to the low degree of genetic variations of *H*. *canis* in the studied area. Furthermore, the relatively low prevalence observed in this study compared with other parts of Europe and the existence of only one *H*. *canis* haplotype may indicate that the pathogen has recently been introduced into the studied area.

To evaluate which is the most suitable tissue for molecular detection of haematozoa in foxes, blood and spleen were tested and the proportion of positivity was calculated. Our results indicate the blood is better for *Babesia* cf. *microti* detection, whereas the spleen is a more sensitive tissue substrate for detection of *H*. *canis* DNA, most likely due to the occurrence of merogony and subsequently higher parasite load in the spleen (Cardoso *et al*. 2014).

Possible evidence for vertical transmission of Hepatozoon canis

Hepatozoon canis has a rather unique transmission mode as it, unlike any other vector-borne pathogen, needs to be ingested by a vertebrate intermediate host. Ingestion of the tick vector (definitive host) containing mature oocysts by the vertebrate host represents a main route of infection (Baneth, 2011). Nevertheless, vertical transmission from infected dogs to their puppies has been demonstrated (Murata *et al*. 1993). This non-vectorial way of infection has been hypothesized to occur in foxes, but it has never been confirmed. In this study, we detected *H*. *canis* DNA in tissues of two out of six foetuses retrieved from an infected vixen, and the same nucleotide sequence was confirmed in both foetuses and the mother fox, indicating the possible intrauterine infection. The existence of a transplacental route of infection in foxes could explain the high prevalence of *H*. *canis* in an area, including Austria, where *Rhipicephalus* tick vectors are not present. It would thus seem that female foxes play an important role in the maintenance of *H*. *canis*, as has already been suggested (Hodžić *et al*. 2015).

Arthropod-borne bacteria and zoonotic implications

In recent years, bacteria transmitted by blood-feeding arthropods have attracted the attention of many researchers since they were recognized as important causative agents of human and animal diseases (Hodžić *et al*. 2015). Among wild carnivores, foxes display a limited impact on the circulation of emerging zoonotic bacteria such as *A*. *phagocytophilum*, *Borrelia burgdorferi* sensu lato and *B*. *rochalimae* due to very low infection rates, but they may still represent a potential source of human infections (Henn *et al*. 2009; Dumitrache *et al*. 2015). Results from our study could support this as four foxes were infected with the same strains of *A*. *phagocytophilum* and *B*. *rochalimae* previously reported in human patients with apparent signs of illness (Petrovec *et al*. 1999; Eremeeva *et al*. 2007; Markowicz *et al*. 2016). However, data on the prevalence of *A*. *phagocytophilum* and *B*. *rochalimae* in foxes in Europe is scant and limited to very few countries such as the Czech Republic (Hulínská *et al*. 2004), Germany (Härtwig *et al*. 2014), Italy (Ebani *et al*. 2011), Poland (Karbowiak *et al*. 2009), Romania (Dumitrache *et al*. 2015) and France (Henn *et al*. 2009).

Moreover, two foxes were found to be positive for a potentially new species of a recently specified cluster *Candidatus* Neoehrlichia, which is phylogenetically more closely related to a raccoon-associated species *Candidatus* Neoehrlichia lotoris from North America, than to zoonotic *Candidatus* Neoehrlichia mikurensis (data published in Hodžić *et al*. 2015*a*). The same sequences of *Candidatus* Neoehrlichia sp. (16S rRNA and *groEL* genes) were found later in a fox from the Czech Republic (Hodžić *et al*. 2017*a*) and in an European badger (*Meles meles*) from Hungary (Hornok *et al*. 2017), expanding our knowledge on geographic distribution and host association for this enigmatic bacterium. Regarding rickettsial species from the spotted fever group, they have been molecularly confirmed in arthropods collected from foxes (Marié *et al*. 2012; Torina *et al*. 2013), but never in this animal species itself (Duscher *et al*. 2014; Hodžić *et al*. 2015), which opens the question about the ability of foxes to serve as hosts for these emerging zoonotic bacteria. However, most of the studies conducted on foxes used either blood or spleen for molecular detection of rickettsial DNA, but other tissues (e.g. skin) might be more suitable for detection consistency, as seen in guinea pigs experimentally infected with *Rickettsia rickettsii* (Zemtsova *et al*. 2015). Large-scale studies employing several gene loci, more

https://doi.org/10.1017/S0031182017001536 Published online by Cambridge University Press

sensitive diagnostic tools and/or even *in vitro* culturing are needed to shed more light on the possible involvement of foxes in the transmission cycles of zoonotic bacteria transmitted by arthropods.

Filarial nematodes and Leishmania infantum

Neither blood nor spleen samples from animals tested in this study were PCR positive for filarial nematodes and *Leishmania* species. In general, *Dirofilaria immitis* and *D. repens* as well as *Leishmania infantum* have been molecularly confirmed in foxes in Europe (Magi *et al*. 2008; Penezić *et al*. 2014; Tolnai *et al*. 2014; Cardoso *et al*. 2015; Karayiannis *et al*. 2015), however their impact in the transmission of these zoonotic parasites still remains unclear. Infection with *D. repens* has occasionally been reported in dogs and humans (Fuehrer *et al*. 2016), and mosquitoes in Eastern Austria (Silbermayr *et al*. 2014). Nevertheless, *L. infantum* DNA was recently detected in *Phlebotomus mascittii* sand flies and in a dog from Austria (Obwaller *et al*. 2016). All these findings may indicate that the infections, in particular those caused by *D. repens* became endemic in this country (Fuehrer *et al*. 2016). However, further monitoring of animals and arthropod-vectors from all regions in Austria is required in order to investigate the distribution and potential risk of infection.

Concluding remarks

This work represents the first comprehensive molecular study on pathogens transmitted by blood-feeding arthropods in Austrian foxes, and the results obtained reveal the frequency of occurrence and diversity of protozoan and bacterial agents in the sampled area. Moreover, this study further demonstrates that foxes may play a pivotal role in maintaining, circulating and spreading of *Babesia* cf. *microti* and *H. canis*. However, none of these haematozoan parasites have yet been diagnosed in dogs in Austria, most likely because they could easily have been overlooked, due to a very low parasitaemia and absence of specific clinical signs (Mitková *et al*. 2016). Molecular diagnostic tools are therefore to be highly recommended for the detection and identification of these parasites in domestic dogs and other wild carnivores as potential hosts for these agents.

ACKNOWLEDGMENT

The authors would like to thank Barbara Eigner (University of Veterinary Medicine Vienna, Austria) for her help in laboratory work, and to Prof. Dr David Modrý and Barbora Mitková (University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) for helpful discussions and support. The work of Adnan Hodžić, Georg G. Duscher and Hans-Peter Fuehrer was conducted under the frame of EurNegVec COST Action TD1303.

FINANCIAL SUPPORT

This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

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