Ticks and Tick-borne Diseases 14 (2023) 102076

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Red fox (*Vulpes vulpes*) play an important role in the propagation of tick-borne pathogens

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ARTICLE INFO

Keywords:
Anaplasma
Babesia
Borrelia
Carnivores
Tick-borne pathogens
Vulpes vulpes
Candidatus Neoehrlichia
Ehrlichia

ABSTRACT

The red fox (*Vulpes vulpes*) is the most widespread free-living carnivore in the world. Over the years, foxes have been recognized as hosts for a number of tick-borne pathogens. However, their role as reservoirs for zoonotic tick-borne diseases is poorly understood. The aim of our study was to investigate tick-borne pathogens in the red fox population in the Czech Republic. Out of 117 red foxes, 110 (94.02%) individuals tested positive for the presence of at least one pathogen by the combined PCR and sequencing approach. *Hepatozoon canis* was the most frequently detected pathogen ($n = 95$; 81.2%), followed by *Babesia vulpes* ($n = 75$; 64.1%). *Babesia canis* was not detected in our study. Four (3.42%) red foxes were positive for *Candidatus* Neoehrlichia sp., 3 (2.56%) for *Anaplasma phagocytophilum*, and one red fox (0.85%) tested positive for the presence of *Ehrlichia* sp. DNA. Overall, DNA of spirochetes from the *Borrelia burgdorferi* s.l. complex was detected in 8.6% of the foxes and *B. miyamotoi* in 5.12% of the samples. As a carnivore found in all ecosystems of Central Europe, foxes obviously contribute to transmission of tick-borne pathogens such as *A. phagocytophilum, B. burgdorferi* s.l., and *B. miyamotoi*. In addition, foxes apparently harbour a community of pathogens, associated with this host in local ecological context, dominated *by H. canis* and *B. vulpes* (possibly also *Candidatus* Neoehrlichia sp.). These species have the potential to spread to the domestic dog population and should be included in the differential diagnosis of febrile diseases with hematologic abnormalities in dogs.

1. Introduction

The red fox (*Vulpes vulpes*) is the most widespread free-living carnivore in the world (Lindsø et al., 2022). The original range of this species includes most of the Northern Hemisphere, and introduced populations exist in parts of Australia and North America (Soe et al., 2017). In natural ecosystems, the red fox occupies wetland, shrubby grassland, deciduous and floodplain forests (Pyšková et al., 2018). This predator exhibits high ecological plasticity and it has adapted to different habitats over the years (Sakalauskas et al., 2020). In the early 1940s, sylvatic rabies began to appear among foxes in Europe, which had a significant negative impact on fox population. However, successful control of rabies through oral vaccination in most parts of Europe contributes to steep growth in the fox population. As a result, foxes have increasingly expanded their range to urban and suburban areas in recent decades (Deplazes et al., 2004), and the same pattern is observed in the Czech Republic (Pyšková et al., 2018).

Foxes harbour a wide range of ectoparasites (Kočišová et al., 2006); at least 16 tick species are known to feed on foxes, with regional variation (D'Amico et al., 2017). Foxes are infested by exophilic tick species: *Ixodes ricinus*, adult *Dermacentor reticulatus, D. marginatus, Rhipicephalus pusillus, R. sanguineus* s.l. or *Haemaphysalis* spp. But also several endophilic, nest-dwelling tick species such as *I. hexagonus* and *I. canisuga* were reported on foxes (Dwużnik et al., 2020; Lledó et al., 2016). The

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https://doi.org/10.1016/j.ttbdis.2022.102076
Received 5 May 2022; Received in revised form 22 September 2022; Accepted 20 October 2022
Available online 1 November 2022

range of ticks also includes other species whose ecology is not always fully understood, such as *I. crenulatus, I. rugicollis* and *I. kaiseri* (Karbowiak et al., 2020).

Among the tick-borne pathogens, *Hepatozoon canis,* the causative agent of canine hepatozoonosis in Europe, is one of the most common blood parasites found in foxes. It has been suggested that the spread of *H. canis* is related to expansion to its tick vector *R. sanguineus* s.l. (Acari, Ixodidae) (Baneth, 2011; Giannelli et al., 2017). However, the detection of *H. canis* in carnivores far outside the areas inhabited by *R. sanguineus*, e.g. Slovakia, Czech Republic, Austria, Hungary and Poland suggests that other tick species are also involved in the life cycle and transmission of this protozoan (Duscher et al., 2013; Majláthová et al., 2007; Mitková et al., 2016; Tolnai et al., 2015).

In Europe, four *Babesia* species: *B. canis, B. vogeli, B. gibsoni* and *B. vulpes* (also referred to as *Theileria annae, Babesia annae, B. microti*-like, *Babesia* Spanish dog isolate) (Alvarado-Rybak et al., 2016; Bartley et al., 2016; Medkour et al., 2020; Solano-Gallego et al., 2016), were reported from canids and may cause clinical babesiosis in dogs. The occurrence of babesiosis in dogs is associated with tick species shared by dogs and wild canids, such as *D. reticulatus, R. sanguineus* s.l. and *I. ricinus.* The dominant piroplasm in European populations of red fox is *B. vulpes* (Alvarado-Rybak et al., 2016; Baneth, 2011; Baneth et al., 2015, 2019). Although the pathogen causes sporadic severe disease in dogs, clinical cases in foxes are not reported. However, in postmortem analysis splenomegaly and kidney enlargement, was observed (Mierzejewska et al., 2021). The tick vector for *B. vulpes* has not yet been recognized. For *B. canis*, the main (if not the only) vector is *D. reticulatus* and disease in dogs is restricted to areas where this tick is common (Dwużnik-Szarek et al., 2021; Majláthová et al., 2011). Nevertheless, *B. canis* is detected only sporadically in red foxes. This pathogen has been detected with low prevalence in foxes from Portugal (1.1%), Bosnia (0.8%) (Cardoso et al., 2014; Hodžić et al., 2015) and Poland (2.4%) (Mierzejewska et al., 2021).

Bacteria from the *Anaplasmataceae* family such as *Anaplasma phagocytophilum* and *Ehrlichia canis* have been studied in foxes recently (Checa et al., 2018; Hodžić, et al., 2015; Medkour et al., 2020; Najm et al., 2014a, 2014b; Sgroi et al., 2021). *Anaplasma phagocytophilum* is the causative agent of granulocytic anaplasmosis in humans, horses, dogs, as well as tick-borne fever in ruminants. Based on the *groEL* gene, several ecotypes have been described that circulate in distant geographic and biotic niches (Jaarsma et al., 2019; Jahfari et al., 2014). Zoonotic potential has been described for the variant prevalent in Europe (Rar et al., 2021), previously referred to as ecotype I (Jahfari et al., 2014) or cluster 1 (Jaarsma et al., 2019). This zoonotic variant has been detected in a wide range of carnivores, including foxes (Sgroi et al., 2021). *Candidatus* Neoehrlichia sp., a new member of the family *Anaplasmataceae*, was first described in foxes from Austria (Hodžić et al., 2015). Data on this bacterium are sparse and the vector has not been recognized. The role of red foxes in the eco-epidemiology of spotted fever group rickettsiae is not fully recognized. Recently, the DNA of *R. sibirica* and *R. raoultii* was detected for the first time in the organs of red foxes from China (Liu et al., 2021). Antibodies against *Rickettsia conori* and *R. massiliae*/Bar29, *R. typhi, R. slovaca* were detected in Spanish foxes (Lledó et al., 2016; Ortuño et al., 2018)

The *Borrelia burgdorferi* sensu lato (s.l.) complex is a diverse group of globally distributed bacteria that includes 22 spirochete genospecies with and without confirmed pathogenicity to humans (Majerová et al., 2020). In Europe, the *B. burgdorferi* complex is transmitted by *I. ricinus* ticks (Rauter and Hartung, 2005) and is carried by a variety of hosts, including birds and small to medium-sized mammals (Gern, 2008; Gern et al., 2010). Approximately 240 animal species are recognized as hosts maintaining *I. ricinus* tick populations and are thus available as potential reservoir hosts for *Borrelia* (Gern, 2008). The abundance of small mammal hosts, which likely have the greatest impact on adult tick infection, may be responsible for the occurrence of Lyme borreliosis (LB) (Levi et al., 2012). It is surprising, but the occurrence of LB may be indirectly influenced by the absence of some predators that may control the abundance of key rodent reservoirs. The connection of red fox abundance and LB risk has been linked to fluctuations in populations of small mammal hosts controlled by red foxes. A decrease in the fox population could actually lead to an increase in LB in some areas (Levi et al., 2012).

Findings of canine tick-borne pathogens associated with red foxes remain rare in the domestic dog population in Central Europe, notwithstanding the ongoing synurbization of the red fox population. At the same time, the role of foxes as reservoirs for zoonotic tick-borne diseases is poorly understood. The aim of our study was to investigate tick-borne pathogens in the red fox population in the Czech Republic and to discuss the role of this common predator in their epidemiology.

2. Materials and methods

2.1. Study area and sampling

As part of a comprehensive survey of red fox parasites in the Czech Republic, 117 carcasses were collected nationwide between June 2014 and July 2015 in cooperation with local hunters (Fig. 1) and examined by complete necropsy. Samples of skin, blood, heart muscle, liver, lung and trachea, spleen, kidney, muscle, and macroscopic ecto- and endoparasites were collected. Tissue samples were stored at -20 °C for DNA isolation, and parasites were morphologically identified by genus and stored at -20°C in 96% ethanol.

2.2. DNA extraction, PCR protocols and sequencing

DNA was extracted from spleen samples using a commercial kit (QIAampDNA Blood & Tissue, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were tested for the presence of DNA from the major groups of tick-borne pathogens.

PCRs for detection of *A. phagocytophilum, B. canis, B. vulpes, Candidatus* Neoehrlichia spp. and *Hepatozoon* spp. were performed using 2 × PCRBIO Taq Mix Red (PCR Biosystems, UK). First round reactions in nested protocols were prepared in a total volume of 15 μl using 2 μl of template DNA. In the second round, 1 μl of the reaction from the first round was used as a template in a total volume of 25 μl. Details of each reaction can be found in Table S1. To determine the *groEL* ecotype of *A. phagocytophilum*, 1297 bp fragments of the *groESL* operon or (in the case of a missing amplicon) 407 bp of the *groEL* gene were amplified by nested PCR as previously described in (Hrazdilová et al., 2021).

Detection of spirochetes from the *B. burgdorferi* s.l. complex was performed by nested PCR of genes encoding outer surface protein C (*ospC*) and *flagellin* using previously established protocols (Bunikis et al., 2004; Clark et al., 2005; Rudenko et al., 2014; Sgroi et al., 2021), for details see Table S1. In the case of low sequence quality, a portion of the chromosomal gene encoding p66 was also amplified and sequenced (Bunikis et al., 2004; Rudenko et al., 2009).

The presence of *B. miyamotoi* was confirmed by nested PCR for the gene encoding glycerophosphodiester phosphodiesterase (*glpQ*) of *B. miyamotoi* using the protocol described previously (Fomenko et al., 2010) (Table S1). The reaction mix without DNA template was used as a negative control. DNA isolated from *B. carolinensis* cultured in MKP medium and total DNA from *I. ricinus* that was positive for the presence of *B. miyamotoi* were used as positive controls in the respective PCR assays. The detection of all *Borrelia* spp. was performed in a final volume

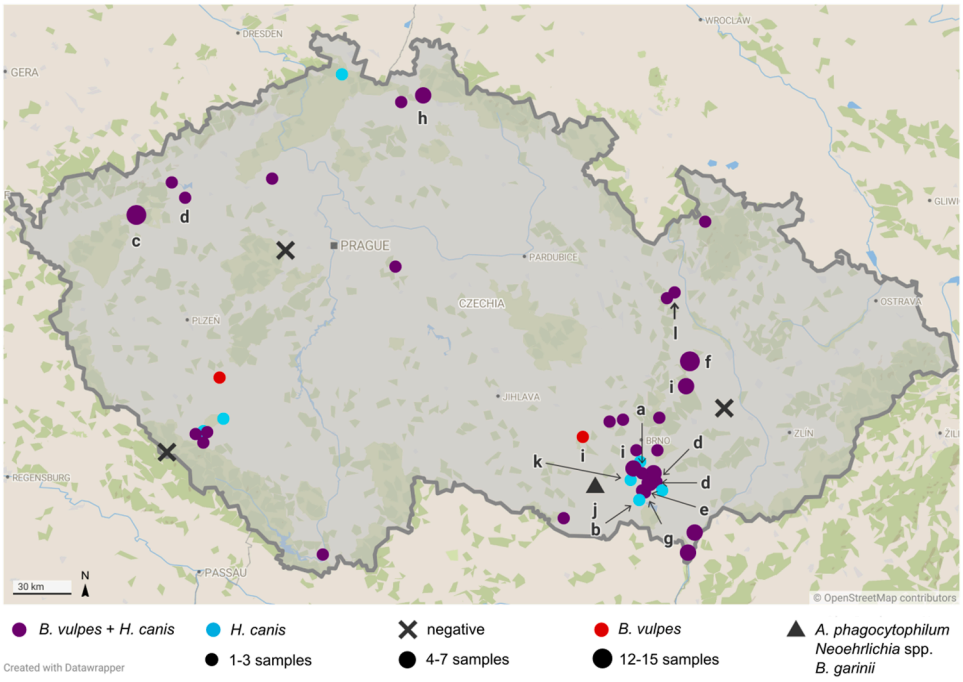

Fig. 1. Georeferenced collection sites of hunted foxes in the Czech Republic. Sites marked with cross, or triangle represent one fox each. Pathogens marked with letters: *Ehrlichia* sp. (a); *Anaplasma phagocytophilum* (b); *Borrelia burgdorferi* s.s. + *B. garinii* (c); *Candidatus* Neoehrlichia sp. (d); *B. burgdorferi* s.s. + *B. bissettii* (e); *B. burgdorferi* s.s. + *B. afzelii* + *B. miyamotoi* (f); *B. burgdorferi* s.s. + *B. garinii* + *A. phagocytophilum* (g); *B. burgdorferi* s.s.+ *B. miyamotoi* (h); *B. miyamotoi* (i); *A. phagocytophilum* + *Candidatus* Neoehrlichia sp. + *B. garinii* (j); *B. burgdorferi* s.s. + *B. afzelii* (k) and *B. burgdorferi* s.s. (l).

of 20 µl using 2x HotStarTaqPlus Master Mix (Qiagen).

Amplicons were separated by electrophoresis in a 1.5 % agarose gel stained with Midori Green Advance (Nippon Genetics Europe, Germany) or SYBR® Gold DNA gel stain (Invitrogen) and visualized under UV light. All PCR products of the expected size were excised from the agarose gels, purified, and sequenced in both directions using the amplification primers. Sequence analysis was performed by SeqMe (Czech Republic) or by Macrogen Capillary Sequencing Services (Macrogen Europe, the Netherlands). The sequences obtained were processed using the Geneious 11.1.4 software (Kearse et al., 2012) and compared with those available in the GenBank™ dataset by Basic Local Alignment Tool (BLAST).

2.3. Phylogenetic analysis

The GenBank database was searched for the 16S rRNA gene sequences of *Candidatus Neoehrlichia* spp. and *Ehrlichia* spp. Sequences were sorted by host, country of origin and size, whereby only entries >1000 nt were considered for further analysis. The final data set for phylogenetic analyses for *Candidatus Neoehrlichia* spp. and *Ehrlichia* spp. was assembled from 147 GenBank sequences and 2 representative sequences obtained in this study for *Candidatus Neoehrlichia* sp. and *Ehrlichia* sp., respectively. The resulting 1040 nt long alignment was calculated with the MAFFT algorithm using the MAFFT online service (Katoh et al., 2019). The phylogenetic tree was constructed using the maximum likelihood method by IQTREE v. 1.6.5 (Nguyen et al., 2015). The best-fitting evolutionary model was selected based on the Bayesian information criterion (BIC) calculated by the implemented ModelFinder (Kalyaanamoorthy et al., 2017). Branch support was assessed by the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013) and the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010).

The phylogeny of *A. phagocytophilum* was constructed using unique *groEL* haplotypes detected in this study along with 121 sequences from GenBank, representing four ecotypes described by Jahfari et al. (2014) and five sequences from *A. platys* used as outgroup. Due to unequal sequence lengths, the alignment was calculated in two steps using the MAFFT algorithm 'Auto' strategy for sequences >1000 nt and the –add function for implementing sequences <1000 nt in the alignment. The phylogenetic tree was constructed using the MAFFT online service (Katoh et al., 2019), using the Neighbour-Joining method, and the Jukes-Cantor model, and the branch support was assessed using 1000 bootstrap repeats. Trees were visualised and graphically processed using FigTree v1.4.1 and Inkscape v0.91.

2.4. Statistical analysis

A chi-square test was used to assess statistical differences between males and females and site of the infection prevalence. A value of $P < 0.05$ was considered significant. Statistical analysis was performed using the online software Epitools - Epidemiological Calculators (Sergeant, 2018).

3. Results

The results were based on individual samples from 117 red foxes (75 males and 38 females, sex determination was impossible in 4 animals). One hundred and ten (94.02%) red foxes tested positive for the presence of at least one pathogen by the combined PCR and sequencing approach (Fig. 1, Table S2). *Hepatozoon canis* was the most frequently detected pathogen ($n = 95$; 81.2%), followed by *Babesia vulpes* ($n = 75$; 64.1%). *Babesia canis* was not detected in our study. Four (3.42%) red foxes were positive for *Candidatus* Neoehrlichia sp., three (2.56%) for *A. phagocytophilum*, and one red fox (0.85%) tested positive for the presence of *Ehrlichia* sp. DNA. No pathologic changes associated with *Babesia* spp. infection or *Babesia* spp. and *Hepatozoon* spp. coinfection were noted in the necropsies. There were no macroscopic changes suggestive of bacterial or viral infections, and although various gastrointestinal, respiratory, or urinary bladder parasites were detected, no or only mild tissue impairment was observed.

Spirochete DNA from the *B. burgdorferi* s.l. complex was detected in 10 individuals. Four different genospecies of the *B. burgdorferi* s.l. complex were identified by sequencing the genes encoding *Fla B* and *osp C* - *B. burgdorferi* s.s., *B. afzelii, B. garinii*, and *B. bissettii*. While three samples represented single spirochete infection with *B. burgdorferi* s.s., two with *B. garinii*, another four contained co-infection with *B. burgdorferi* s.s. and *B. garinii* (2 samples), *B. burgdorferi* s.s. and *B. afzelii* (1 sample), and *B. burgdorferi* and *B. bissettii* (1 sample). The remaining one

Table 1
Infections detected in this study and number of animals presenting the coinfection.

Status	Pathogens	No. of infected animals	% of infected animals
Monoinfection	*H. canis*	28	23.9%
	B. vulpes	10	8.5%
Coinfection	*H. canis* + *B. vulpes*	52	44.4%
	H. canis + *B. vulpes* + *Candidatus* Neoehrlichia sp.	3	2.5%
	H. canis + *B. vulpes* + *Ehrlichia* sp.	1	0.85%
	H. canis + *B. vulpes* + *B. burgdorferi* s.s. + *B. bissettii*	1	0.85%
	H. canis + *B. vulpes* + *B. burgdorferi* s.s. + *B. miyamotoi*	1	0.85%
	H. canis + *B. vulpes* + *B. burgdorferi* s.s.	1	0.85%
	H. canis + *B. vulpes* + *B. burgdorferi* s.s. + *B. afzelii*	1	0.85%
	H. canis + *B. vulpes* + *B. miyamotoi*	1	0.85%
	B. vulpes + *B. burgdorferi* s.s.	1	0.85%
	B. vulpes + *B. miyamotoi*	2	1.7%
	H. canis + *B. miyamotoi*	2	1.7%
	H. canis + *B. garinii*	1	0.85%
	H. canis + *B. burgdorferi ss* + *B. afzelii*	1	0.85%
	H. canis + *B. burgdorferi ss* + *B. garinii*	1	0.85%
	B. vulpes + *A. phagocytophilum* + *B. burgdorferi* s.s. + *B. garinii*	1	0.85%
	H. canis + *A. phagocytophilum*	1	0.85%
	Candidatus Neoehrlichia sp.+ *A. phagocytophilum* + *B. garinii*	1	0.85%
Total		110	94.1%

sample that was positive for the presence of *B. burgdorferi* s.s. DNA was also positive for *B. miyamotoi*, revealing a case of coinfection of red fox with spirochetes from two groups - LB and relapsing fever. Another five samples were positive only for *B. miyamotoi* DNA. Overall, DNA of spirochetes from the *B. burgdorferi* s.l. complex was detected in 8.6% of the samples and *B. miyamotoi* in 5.12% of the samples.

The simultaneous presence of *H. canis* and *B. vulpes* was the most frequently detected coinfection ($n = 52$, 44.4%). Three animals were infected with *Candidatus* Neoehrlichia sp., *B. vulpes* and *H. canis* and four individuals were infected with four different pathogens, mostly *H. canis, B. vulpes* and two different genospecies of *B. burgdorferi* s.l. or *B. miyamotoi*. Complete list of observed co-infection is presented in Table 1.

Based on the nested PCR assay targeted at a ~1530 bp fragment of 18S rDNA region, 95 (81.2%) out of 117 foxes were found positive for the presence of *H. canis*. Six randomly selected samples were sequenced in both directions. Two sequence variants (Hc1, Hc2) differing in a single nucleotide (> 99.9% similarity) showed 100% identity with *H. canis* sequences previously detected in foxes from the Czech Republic and European jackals (*Canis aureus*) from Romania in BLASTn analysis (Table 2). Amplification of a short (~697 bp) 18S rDNA fragment of *B. vulpes. vulpes,* yielded a product of expected size in 75 foxes. We identified three unique variants (Bv1, Bv2, Bv3) (Table 2). The most common variant (Bv1) was detected in 73 individuals (97.3%) and is identical to *B. vulpes* isolated from dogs (Spain) and foxes (United Kingdom, Italy and Spain) according to BLAST (Table 2). The remaining two variants (Bv2, Bv3) were found in one fox each. The unique variants differ in two nucleotide positions (99.7% similarity). In the case of *A. phagocytophilum*, nested PCR resulted in 3 sequences (366-1078 bp). Three unique genotypes (Ap1, Ap2, Ap3) characterized by 99.2–99.7% sequence similarity, were each detected in one animal (Table 2). In the phylogenetic analysis, sequences clustered in the clade representing the European ecotype I (Jahfari et al., 2014) closely related to the USA *A. phagocytophilum* strains. Variants Ap3 and Ap2 were grouped in the subclade with the European human cases and strains from horses, wild boars, ticks and carnivores. Genotype Ap1 grouped among sequences in great majority coming from different ungulates (Fig. 2).

Candidatus Neoehrlichia sp. was detected using nested PCR designed to amplify 1271 bp fragment of the 16S rDNA gene. This resulted in two unique genotypes detected in 1 and 4 individuals, respectively. BLASTn analysis of the single sequence revealed 99.91% similarity (with 99 % of query coverage) with *Ehrlichia* sp. isolated from *Haemaphysalis flava* from China (KX987318). The remaining four sequences were 100% identical to each other and to *Candidatus* Neoehrlichia sp. (MN155788-92) from *V. vulpes* from Poland, and 99.91% identical with *Candidatus* Neoehrlichia lotoris (MH020204) from *Canis familiaris* from Hungary. The phylogeny was constructed using 147 sequences of the genera *Ehrlichia* spp. and *Candidatus* Neoehrlichia spp. from GenBank, unique sequences from this study and 3 sequences of *A. phagocytophilum* as an outgroup (Fig. 3). Two groups strongly supported by bootstrap values were formed. The sequence Esp1 obtained in this study belongs to the subgroup formed by *E. ewingii*, which originated from dogs in the USA,

Table 2
Unique variants of pathogens detected in 110 V. vulpes (red fox) from the Czech Republic, the nearest BLAST hits and accession numbers.

Pathogen & gene	Variant/ Genotype	Name in phylogeny tree	No. of animals with detected variant	The closest BLAST hits	GenBank Acc. Number
H. canis[a] 18S rRNA	Hc1	-	3	100%, KU893121 (*V. vulpes*, Czech Republic), KU893127 (*C. familiaris*, Czech Republic)	ON128259, ON128261, ON128263
	Hc2	-	3	100%, KU893118, KU893119, KU893122, KU893123 (*V. vulpes*, Czech Republic)	ON128260, ON128262, ON128264
B. vulpes 18SrRNA	Bv1	-	73	100%, KT223483 (*V. vulpes*, Spain)[b]...	ON128284
	Bv2	-	1	99.85%, KT580785 (V. vulpes, Great Britain)[b]...	ON128285
	Bv3	-	1	99.84%, MK591948 (*C. familiaris*, Spain)[b]...	ON128283
A. phagocytophilum groESL/groEL	Ap1	Ap1_Vv39	1	100%, KJ832471 (horse, France); AY281844 (I. *ricinus*, Germany)	ON153216
	Ap2	Ap2_Vv4	1	100%, MK069873 (I. ricinus, Norway)...	ON153214
	Ap3	Ap3_17	1	99.92% AF033101 (*H. sapiens*, Slovenia); MT498616 (*S. scrofa*, Czech Republic); AY529490 (horse, Sweden); AF482760 (horse, Germany)	ON153215
Candidatus Neoehrlichia sp16S rRNA	CNsp1	CNsp1_Vv98	4	100% MN155788 (*V. vulpes*, Poland)[b]…	ON130254
Ehrlichia sp 16S rRNA	Esp1	Esp1_Vv14	1	99.74% KY046299 (*H. bispinosa*, Malaysia); KY046298 (*R. microplus*, Malaysia)	ON127918

[a] Out if 95 positive animals six randomly chosen samples were analyzed.
[b] Higher number of hits with the same % of similarity.

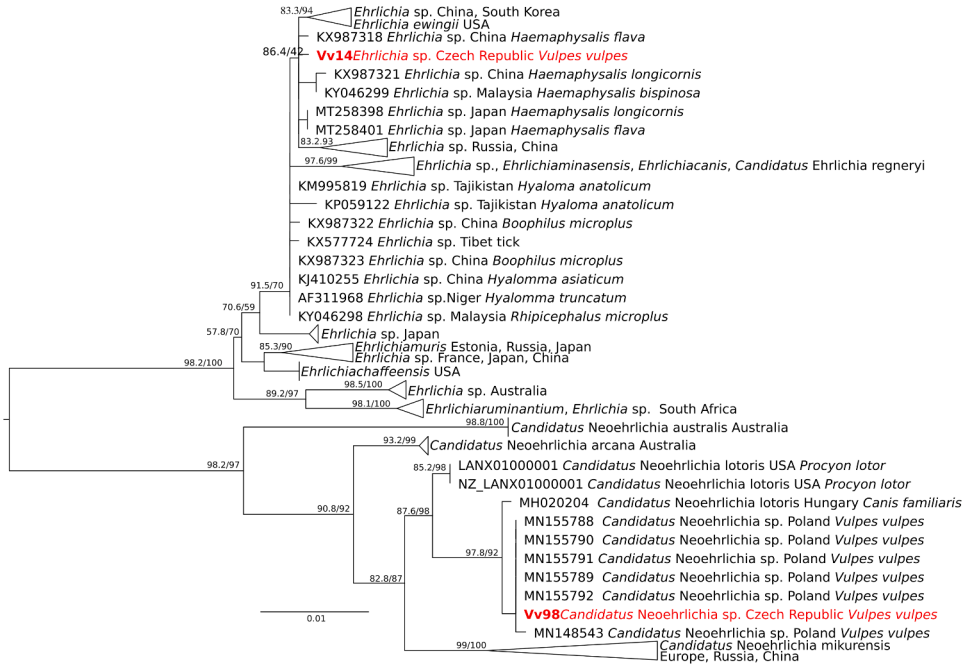

Fig. 2. Schematic representation of the maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of *Ehrlichia* sp. and *Candidatus* Neoehrlichia sp. Best-fit model TN+F+I+G4 chosen according to BIC; bootstrap values (SH-aLRT/UFB) are displayed; three sequences of *A. phagocytophilum* used as an outgroup are not displayed. The sequences acquired from the GenBank database are marked by their accession number, species of pathogen, country of origin and host. Sequences from this study are marked in red. The scale bar indicates the number of nucleotide substitutions per site.

and sequences designated as *Ehrlichia* sp. from a variety of tick species from Asia (Fig. 3). Our sequence CNsp1 belongs to the group formed by sequences of *Candidatus* Neoehrlichia sp. from foxes from Poland, in a sister position to *Candidatus* N. lotoris from North American raccoons (Fig. 3). The representative sequences from this study were deposited in GenBank with accession numbers listed in Table 2.

4. Discussion

The increasing distribution and growing abundance of the red fox in urban and suburban areas makes this species a bridge between natural ecosystems, humans and their companion animals, and an important element in the triad of vector, host and pathogen. The present study reports a high prevalence of *H. canis* and *B. vulpes* and data on the occurrence and genetic diversity of *A. phagocytophilum, Candidatus* Neoehrlichia sp., *B. burgdorferi* s.l., *B. myiamotoi*, and *Ehrlichia* sp. in red foxes.

The high prevalence of *H. canis* and *B. vulpes* indicates a key role played by foxes in the circulation of these pathogens. Our result is consistent with the high prevalence of *H. canis* (28-92%) reported in foxes from Central and Eastern Europe (Cardoso et al. 2014; Medkour et al. 2020; Sgroi et al. 2021; Mierzejewska et al. 2021; Najm et al., 2014b; Gimenez et al. 2009). The presumed main tick vector for *H. canis* is *R. sanguineus* s.l., which is absent from the Czech Republic and the surrounding areas. Repeated reports of *H. canis* in areas where *R. sanguineus* is not present (Hodžic et al., 2018; Tolnai et al., 2015) suggest the involvement of other tick species commonly found in foxes, such as the nidicolous ticks *I. hexagonus, I. canisuga* and *I. crenulatus* (D'Amico et al., 2017; Mitková et al., 2016). In the Czech Republic, *H. canis* was previously reported from domestic and hunting dogs (Mitková et al., 2016; Mitkova et al., 2017) with evidence of endemic infection in *Rhipicephalus*-free areas.

This is the first study reporting the presence of *B. vulpes* in the Czech red fox population. Animals infected with this piroplasm were found across the country. *Babesia vulpes* is known to cause clinical disease in dogs (Alvarado-Rybak et al., 2016; Matijatko et al., 2012), however, no clinical cases (caused by *B. vulpes*) have been reported in dogs from the Czech Republic. The eco-epidemiology of *B. vulpes* remains unclear. Similar to *H. canis, I. canisuga, I. hexagonus* and *I. ricinus,* which are commonly found on foxes (D'Amico et al., 2017) may also play a role in the transmission of this piroplasm. However, their competence to serve as vectors of *B. vulpes* has not been proven, and the presence of DNA in the ticks could be a result of the current blood meal (Najm et al., 2014a). The lack of clinical cases of *H. canis* and *B. vulpes* infections in dogs in the Czech Republic contrasts with the high prevalence in foxes. This discrepancy suggests that there are ecological barriers between foxes and dogs and limited exchange of ticks. The lack of transovarial transmission in the tick vector may also play an important role (Aktas and Özübek, 2017). However, it is also possible that both pathogens are simply underdiagnosed as *H. canis* is not considered to be highly pathogenic, which may also explain the lack of clinical cases.

Vertical transmission of *H. canis* infection has been demonstrated in dogs (Murata et al., 1993) and foxes (Hodžic et al., 2018) and may contribute to its high prevalence in areas outside the distribution of *R. sanguineus* s.l. Alternative modes of transmission have also been described for a few species of the genus *Babesia*, including transmission by blood transfusion, fighting/biting (Jefferies et al., 2007; Levin and Krause, 2016) and transplacental transmission from females to offspring (Adaszek et al., 2016; Fukumoto et al., 2005; Mierzejewska et al., 2014; Yeagley et al., 2009).

Within the broad spectrum of tick-borne pathogens naturally harbored by the red fox, the spirochetes of the *Borrelia burgdorferi* s. l. complex, together with *A. phagocytophilum*, are among the pathogens of medical interest. The spirochetes detected in our study, *B. burgdorferi* s. s., *B. afzelii, B. garinii, and B. bissettii,* are species with demonstrated zoonotic potential. Although *B. afzelii* and *B. garinii* are recognized as the two major spirochete species in Europe (Rauter and Hartung, 2005; Rudenko et al., 2011; Marques et al., 2021), the involvement of *B. burgdorferi* s.s. and *B. bissettii,* species with lesser abundance in Europe, in human LB is confirmed. The two species mentioned above, which are "insignificant" to Europe, were until recently considered to be purely North American taxa, and their distribution in Europe was more focal in nature (Rudenko et al., 2011; Hacker et al., 2018). Recently, *B. burgdorferi* s.s. was discovered as one of the main species infecting Eurasian red squirrels or northern, white-breasted hedgehogs (Majerová et al., 2020). The diversity of genospecies identified in Czech foxes corresponds to the range of LB spirochetes detected in various European countries (Dumitrache et al., 2015; Mysterud et al., 2019; Sgroi et al.,

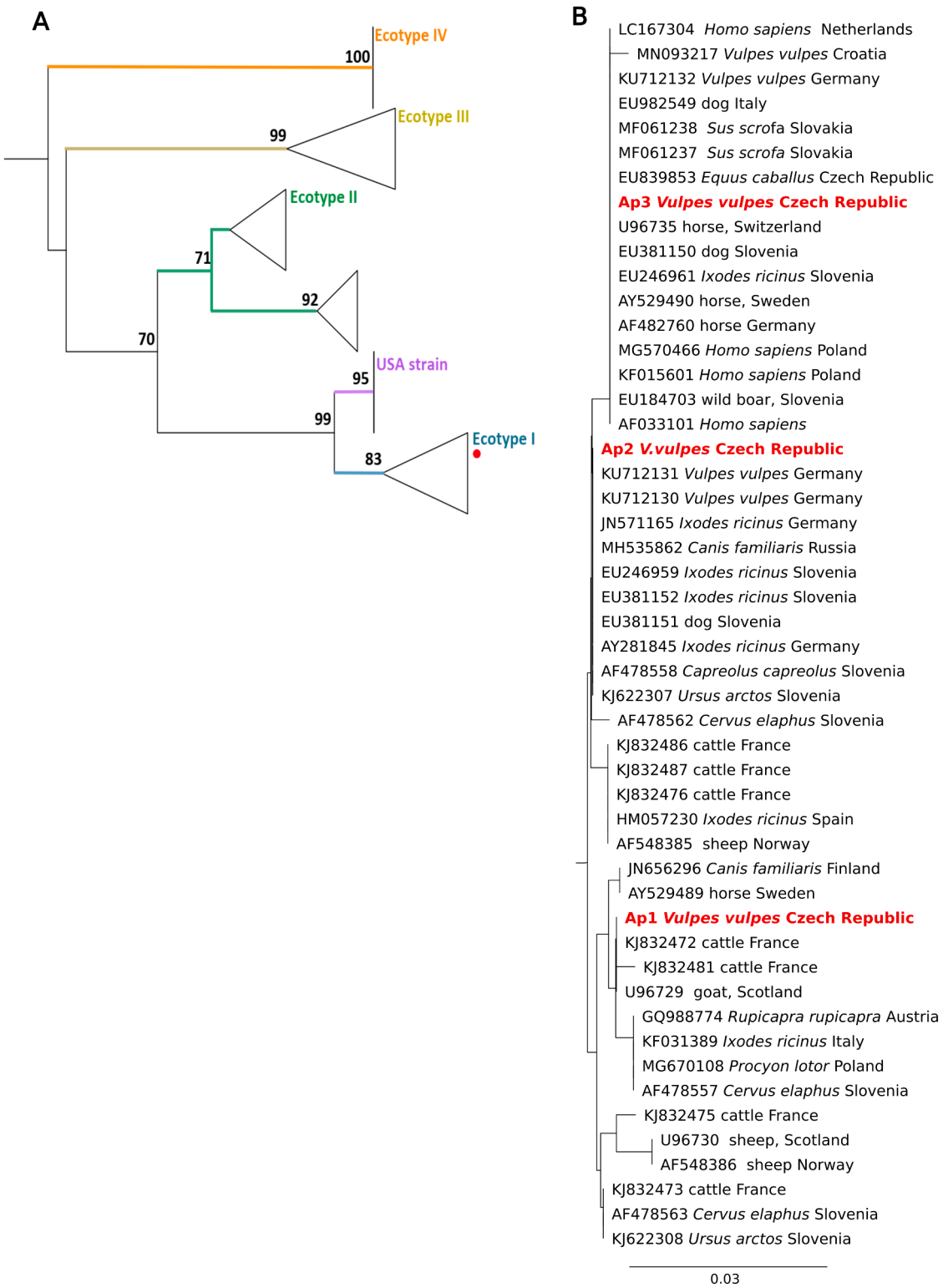

Fig. 3. Visual representation of phylogenetic tree. A - graphical scheme of tree; B - A single clade, described as Ecotype I (Jahfari et al. 2014) from the phylogenetic tree conducted for *Anaplasma phagocytophilum* based on the *groEL* partial gene; the tree was computed based on the neighbour-joining method and Jukes-Cantor model. Branch supports were assessed by 1000 bootstrap replicates. Sequences acquired from the GenBank database are marked by their accession number, host, and country of origin. Sequences from this study are marked by the red dot. Sequences of *A. platys* used as an outgroup are not displayed as well as the branch support.

2021). The results of this study revealed a frequent occurrence of *B. miyamotoi* and its coinfection with *B. burgdorferi* s.s. in red foxes. The prevalence of *A. phagocytophilum* in red foxes corresponds well with the prevalence (0.65–16.6%) reported in other European countries (Ebani et al., 2011, 2017; Härtwig et al., 2014; Hulínská et al., 2004; Karbowiak et al., 2009; Sgroi et al., 2021), suggesting foxes as a reservoir, including the zoonotic variant described as Ecotype I/Cluster I (Jaarsma et al., 2019; Jahfari et al., 2014). *Candidatus* Neoehrlichia sp. was first

detected in foxes from Austria (Hodžić et al., 2015), followed by detection in a fox from the Czech Republic (Hodžić et al., 2017) and in European badger from Hungary (Duscher, 2017). To date, only 59 sequences (16S DNA, *groEL*, and *gltA*) identified as *Candidatus* Neoehrlichia sp., from different countries and hosts, have been deposited in the GenBank. The vector for this pathogen is not yet confirmed. In our study, we found a fox that was also positive for unidentified *Ehrlichia* sp. with unclear phylogenetic position. The polytomies in the tree suggest missing data and thus more *Ehrlichia* spp. to be described and sequenced.

As a carnivore dominant in ecosystems of Central Europe, foxes obviously enter the cycle of tick-borne pathogens such as *A. phagocytophilum, B. burgdorferi* s.l., and *B. myiamotoi*. In addition, foxes apparently harbour a community of fox-associated pathogens dominated *by H. canis* and *B. vulpes* (possibly also *Candidatus* Neoehrlichia sp.). These species have the potential to spillover to the domestic dog population and should be included in the differential diagnosis of febrile diseases with hematologic abnormalities in dogs. Nidicolous ticks such as *I. hexagonus, I. canisuga*, and *I. crenulatus*, which are common in foxes, deserve future attention because they may play a role as vectors of these fox-associated pathogens.

Ethical statement approval

Not included - our work does not include any experimental work on animals. The samples used in the study we obtained from authorized hunters and the samples originated from culled individuals.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgment

This study is part of the project QK1920258, financed by the Ministry of Agriculture of the Czech Republic and is further supported by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601). KH was supported by the project National Institute of Virology and Bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) - Funded by the European Union - Next Generation EU.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102076.

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