

THE DETECTION OF *Anaplasma phagocytophilum* AND *Babesia vulpes* IN SPLEEN SAMPLES OF RED FOX (*Vulpes vulpes*) IN SLOVENIA

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Abstract: The entrance of wild animals into human settings serves as the access of vector-borne pathogens to susceptible hosts. A red fox (*Vulpes vulpes*) frequently enters and is quite adapted to living in urban and periurban environments. Due to its living habits, it could be a possible source of tick-borne pathogens, but it could also transfer pathogens through bites. One hundred and ten spleen samples from red foxes were screened for the presence of the *Anaplasma phagocytophilum* and *Babesia vulpes* genomes with real-time and conventional PCR. Positive PCR products were further sequenced. A genotype of *A. phagocytophilum* was determined and species of *Babesia* spp. if possible. Five (4.5%) spleen samples from red fox were positive for *A. phagocytophilum* DNA. With nucleotide comparison, three genotypes from cluster I were detected. The detected prevalence of *B. vulpes* in red fox in Slovenia was 76.3%. The parasite was detected in all tested regions of the country. Data from our study suggest that the red fox may have only limited impact on the circulation of the zoonotic genotype of *A. phagocytophilum*, but it represents a risk of transmission of *B. vulpes* near human settings and consequently poses a threat to domestic animals.

Key words: red fox, *Vulpes vulpes*; *Anaplasma phagocytophilum*; *Babesia* spp.; *Babesia vulpes*; tick-borne pathogens; dogs

Introduction

Wild carnivores, often in contact with domestic carnivores, are considered the primary source of tick-borne pathogens to humans (1). As the human population grows, urban areas expand and, consequently, more and different animal species enter human habitats (2). The red fox (*Vulpes vulpes*) represents the most widely distributed species in the order Carnivora. It is present across the Northern Hemisphere, that is Northern America, Europe, Asia and some parts of Africa (3). The red fox lives in distinct ecosystems

and frequently enters human settings. It has adapted to the urban environment mainly due to the availability of food and lack of predators (1). Recently, it has been recognized as a potential reservoir of several vector-borne pathogens and, therefore, a source of infection for domestic and companion animals and humans (3). It represents a sentinel species for *Anaplasma phagocytophilum* (4, 5), *Babesia* spp. (3, 6), *Bartonella* spp. (7) and many other vector-borne pathogens (3). Due to its natural environment, it is frequently exposed to different arthropod vectors (2) and to a non-vector-borne transmission of pathogens through bites.

A. phagocytophilum is a well-known tick-borne pathogen that infects humans and animals. Many ecotypes of *A. phagocytophilum* exist in nature, but

not all are pathogenic for humans and companion animals (8). Sequences of *A. phagocytophilum* isolated from red foxes belong to ecotype I, and some genotypes in this cluster are zoonotic (9). In Slovenia, in wild and domestic animals, several genotypes have been detected that belong to three ecotypes (10). It has been suggested that wild boar (*Sus scrofa*) could serve as reservoir species for a genotype that infects humans and dogs in Slovenia (11), but this animal rarely enters human environments. In contrast, during the night time, the red fox frequently searches for easily available food.

Babesiae are parasitic intracellular microorganisms that infect erythrocytes. They are transmitted by hard ticks and represent an increasing global risk to both animals and humans (12). *Babesia* spp. parasites are present in the Slovenian area, in dogs, small mammals, and cervids (13–15). Recently, a new babesial pathogen of dogs and red foxes has been recognized elsewhere; it belongs to the group *Babesia microti* (small babesiae like) (16). It is reported under various names: *Theileria (Babesia) annae*, *Babesia* sp. 'Spanish dog' isolate, *Babesia microti*-like, *Babesia* cf. *microti*; so far no current valid name is agreed upon (17). As red foxes from distant regions are highly infected with this parasite, it is suspected that fox is a natural host for this parasite, and the name *Babesia vulpes* was proposed (18). Infections with the parasite cause anaemia, thrombocytopenia, and azotemia in dogs (18), although asymptomatic infection has been reported, too (19). Foxes usually have no apparent clinical signs of the disease (18). As the red fox is quite adapted to urban and periurban areas, it poses a risk of transmission of the infection to domesticated dogs.

Wild carnivores are rarely examined for vector-borne pathogens, although they are highly infested with ticks and fleas. Adding the factors of global warming, deforestation, urbanization, human outdoor activities, etc. to improved molecular and diagnostic tools more new, emergent and re-emergent vector-borne pathogens are detected. Therefore, wild animals represent an important source of zoonotic agents. Our study aimed to detect the presence of selected pathogens in Slovenian population of red foxes.

Materials and methods

One hundred and ten red foxes (*V. vulpes*) of both sexes were shot by professional hunters during regular hunting season from autumn 1996 to spring 2003 (Table 1). From each animal, a sample of spleen was collected by hunters and delivered to our laboratory. Samples were stored at -20°C until processing. DNA was extracted with QIAamp DNA blood mini kit, tissue protocol (Qiagen, Germany), according to manufacturer's instructions and stored at -20°C until further processing.

To detect anaplasma or babesial species, screening PCR was performed. For detection of *A. phagocytophilum*, a real-time PCR for the *msp2* gene was performed on all samples (20). Positive samples were further amplified with conventional nested PCR to detect a part of the *ankA* gene of *A. phagocytophilum* (10) and subjected to sequencing to determine the genotype. A conventional PCR with primers PiroA and PiroB that target the hypervariable region of the 18S rRNA gene was used to detect different species of *Babesia* sp. (14). All samples were also tested using a specific conventional PCR with primers for a part of beta-tubulin gene of *B. vulpes* (21). Amplified PCR products of conventional PCRs were separated on gel electrophoresis. All positive samples were purified and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences obtained were edited using the software CLC Main Workbench 7 (CLCBio, Qiagen, Germany) and compared with sequences available in GenBank.

Results

The DNA of *A. phagocytophilum* was detected in five out of 110 (4.5%) spleen samples from red foxes by real-time PCR for the *msp2* gene, ct values ranged from 23.65 to 28.93 (Table 1). All five samples were subjected to a conventional PCR for a part of *ankA* gene amplification and subsequent sequencing of a PCR product (523 bp). We detected three different sequences that belong to Cluster I (Table 1). One genetic variant (three foxes) had 100% nucleotide identity to a variant that was previously detected in human patients and dogs in Slovenia (acc. No. JQ347533). The second variant (one fox) had 100% nucleotide identity to a

variant detected in a dog in Switzerland (acc. No. KF242674) and Slovenia (not deposited in GenBank). The third genetic variant was 99% similar to a variant detected in a horse from Germany (acc. No. MH973605) but was not previously detected in Slovenia.

Among 110 DNA isolates, 97 (88.2%) were positive with a screening PCR for 18S rRNA gene (377 bp) for *Babesia* sp. Sequencing of PCR product of 18S rRNA gene revealed 80 sequences were identical with each other and to a published sequence of *Babesia vulpes* isolate 03/00349 from a red fox from Spain (Acc. No. KT223483) (Table 1). For 17 PCR products, the nucleotide sequence could not be determined. Therefore, all

samples were subjected to a specific conventional PCR for a part of the beta-tubulin gene of *B. vulpes* (510 bp). Eighty-four (76.3 %) samples were positive and subjected to sequencing. Eighty PCR products were, again, confirmed to be 99% similar to *B. vulpes*. Sequences of four additional PCR products, which previously could not be determined by sequencing of 18S rRNA gene, were now 99% similar to *B. vulpes*. A sequenced part of a beta-tubulin gene from Slovenian red fox babesiae differed from the most similar sequence in a GenBank (*B. microti* isolate SN87-1, fox, USA, AY144707) for one or two nucleotides (99% similarity). *B. vulpes* was detected in samples of red fox from different regions of Slovenia.

Table 1: Detected *A. phagocytophilum* and *B. vulpes* in spleen samples from red fox (*V. vulpes*) in Slovenia

YEAR OF COLLECTION	REGION	NO. OF SAMPLES	<i>B. vulpes</i>	<i>A. phagocytophilum</i>	
			positive	positive (ct value)	<i>ankA</i> genotype (Acc. No.)
1996	Central Slovenia	1	1	0	
1997	Upper Carniola	3	1	1 (27,2)	human, Slovenia (JQ347533)
	Central Slovenia	1	1	0	
1998	Upper Carniola	2	2	0	
	Southeast Slovenia	5	5	0	
	Central Slovenia	6	4	0	
	Lower Sava	4	4	1 (26,75)	dog, Switzerland (KF242674)
	Littoral-Inner Carniola	3	2	0	
	Central Sava	2	2	0	
	ND	6	3	0	
1999	Upper Carniola	1	1	0	
2000	Upper Carniola	5	2	0	
2002	Upper Carniola	2	2	0	
	Southeast Slovenia	6	5	0	
	Central Slovenia	18	16	1 (27,38) 1 (28,93)	human, Slovenia (JQ347533) horse, Germany (MH973605)
	Savinja	1	1	0	
	Central Sava	1	0	1 (23,65)	human, Slovenia (JQ347533)
2003	Upper Carniola	1	1	0	
	Southeast Slovenia	10	6	0	
	Central Slovenia	3	3	0	
	Central Sava	1	0	0	
ND	Upper Carniola	1	1	0	
	Southeast Slovenia	5	2	0	
	Central Slovenia	11	10	0	
	Mura	2	1	0	
	Littoral-Inner Carniola	1	1	0	
	ND	8	7	0	
TOTAL		110	84 (76,3%)	5 (4,5%)	



Figure 1: Statistical regions of Slovenia (https://en.wikipedia.org/wiki/Statistical_regions_of_Slovenia)

Discussion

The red fox is an animal species that lives in distinct ecosystems and frequently enters human settings. Consequently, it serves as a possible source of numerous vector-borne pathogens for humans and domestic animals. It has been proven that red fox serves as a species infected with genotypes of *A. phagocytophilum* that are zoonotic (9). Slovenian foxes also harbour genotypes that are pathogenic for humans and dogs, in addition to a newly detected genotype that has been detected before in a German horse. Previously, it has been proposed that wild boars might serve as a reservoir for a human pathogenic strain of *A. phagocytophilum* in Slovenia. Although wild boars are now also entering urbanized areas, red foxes are already adapted to living there. Therefore, a red fox might be the wild species that could serve as a carrier of zoonotic strains of *A. phagocytophilum* to human settings. Although only 4.5% of the samples were detected positive in this study, a rather low number of samples were tested during

each year. The prevalence in foxes may be low, but this species could be a reservoir of pathogens and thus an important source of infection. The infection rate of foxes in Slovenia is in concordance with other European countries (0.8–16.6%) (1). Due to this low infection rate, the red fox may have only limited impact on the circulation of zoonotic genotypes of *A. phagocytophilum* in periurban and urban areas.

According to reports from various countries, *B. vulpes* is prevalent in European countries (14.5–69.2%) (3, 22–27), in North America (37%) (6) and Canada (28). Slovenia is one of the countries with the highest prevalence (75.5%), comparable with reports from NW Spain (Galicia, 72.2%) and from Portugal (69.2%) (24, 29), respectively. Differences in the prevalence among countries might be due to different samples tested or due to different PCR assay used, but also due to different vector species present. In NW Spain and Portugal, the tick *Ixodes hexagonus* is most abundant and was proposed as a vector species (30). In contrast, *B. vulpes* was also discovered in North America, where

I. hexagonus is not endemic (6). It was suggested that *I. ricinus* also might be a vector species for *T. annae*, along with *I. canisuga* (26). The role of non-vector-borne transmission has been suggested, as this is one of the primary routes of transmission for babesiosis in dogs in America (21). Barash et al. found a high prevalence of *B. vulpes* in American Staffordshire and Pit Bull Terrier type dogs that were rescued from dog fighting (21).

Due to the high prevalence of infected red foxes in all regions of Slovenia and due to the fact the red fox frequently enters human settings, this carnivore species poses a threat to domestic dogs. Therefore, veterinarians should be aware of possible infection of a dog with *B. vulpes* as this parasite belongs to the small babesiae group.

Conclusions

Red foxes are infected with a tick-borne zoonotic genotype of *A. phagocytophilum* in Slovenia. Due to its low prevalence, a fox could have only limited impact on the circulation of genotypes pathogenic to human and domestic animals. The prevalence of infection with *B. vulpes* in red foxes in Slovenia is high. That said, red foxes pose a threat to dogs with transmitting this vector-borne pathogen near human settings through the vector of ticks or bites. Veterinarians should be aware of possible small-babesiae infections.

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UGOTAVLJANJE ANAPLAZME (*Anaplasma phagocytophilum*) IN BABEZIJE (*Babesia vulpes*) V VZORCIH VRANIC PRI RDEČIH LISICAH (*Vulpes vulpes*) V SLOVENIJI

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Izvleček: Prehajanje divjih živali v urbana okolja omogoča prenos klopno prenosljivih patogenih mikroorganizmov do dovzetnih oseb in živali. Navadna rdeča lisica (*Vulpes vulpes*) pogosto prehaja v okolico bivališč ljudi, prav tako se je že dobro privadila življenju v bližini ljudi. Zaradi svojih življenjskih navad predstavlja možen vir klopno prenosljivih patogenov preko okuženih klopov, lahko tudi preko ugriza živali. 110 vzorcev vranic navadne rdeče lisice smo pregledali na prisotnost genoma bakterije *Anaplasma phagocytophilum* in parazita *Babesia* spp. Pozitivnim vzorce smo nato določili zaporedje DNK in določili genotip *A. phagocytophilum* oz. vrsto babezije, kadar je bilo to mogoče. Pet vzorcev vranic (4.5 %) navadne rdeče lisice je bilo pozitivnih na prisotnost genoma *A. phagocytophilum*. Z določitvijo zaporedja DNK smo določili tri genotipe. Prevalenca okužbe z *B. vulpes* pri slovenskih lisicah je 76.3 %, določili pa smo jo v vseh statističnih regijah Slovenije. Navadna rdeča lisica ima zelo omejen vpliv na kroženje zoonotskega genotipa *A. phagocytophilum*. Kljub temu pa predstavlja verjeten vir prenosa parazita *B. vulpes* v urbana področja in posledično nevarnost za domače živali.

Ključne besede: navadna rdeča lisica; *Vulpes vulpes*; *Anaplasma phagocytophilum*; *Babesia* spp.; *Babesia vulpes*; klopno prenosljivi patogeni mikroorganizmi; psi