UNIVERSITY OF VETERINARY MEDECINE OF BUDAPEST

DEPARTMENT OF PARASITOLOGY AND ZOOLOGY

Studies on Babesia infection of red foxes (*Vulpes vulpes*) shot in Hungary

By Perrine BALAYN

Supervisor:
Professor Dr. Robert Farkas

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1 Introduction

The red fox (*Vulpes vulpes*) has a huge population size and it is the most widespread wild carnivore species in Europe. In urban areas, the availability of food and resting places, human tolerance and the absence of predators and competitors, make the red fox a very successful synanthropic species (Otranto et al., 2015). The vaccination campaign against rabies and measures to protect wild fauna also contributed for the increasing fox population in Europe (Chautan et al., 2000).

Red foxes have been recognized as potential reservoirs of several parasites and pathogens transmitted by arthropod vectors and a source of infection for all susceptible species sharing the same habitats, including dogs and humans (Dusher et al., 2015). Reasons for that includes their proximity to urban areas, their susceptibility to relevant pathogens, their frequent exposure to arthropod vectors, like ticks, or their hunting preference for small mammals leading to ingestion of intermediate hosts (Duscher et al., 2015). Hunting practices as well as human encroachment in wildlife habitats (development of rural tourism facilities, craze for outdoor activities) may facilitate encounter between red foxes and domestic canids and therefore the flow of parasites.

Vector-borne diseases are caused by parasites, bacteria or viruses transmitted by the bite of hematophagous arthropods, mainly ticks and mosquitoes. The frequency of some vector-borne diseases of pets, like canine babesiosis, is increasing over the past few years, It has been shown that dogs can be infected by several *Babesia* species in Europe, the most significant being the large piroplasm *Babesia canis* (Solano-Gallego et al., 2016). Another large babesia species, *Babesia vogeli* and small piroplasms like *Babesia gibsoni* and *Babesia microti-like* also occur on the continent.

Some formerly unknown small babesiae were first described in a German dog returned back from Spain, showing clinical babesiosis characterized by lethargy, fever and anaemia (Zahler et al., 2000). These protozoa were closely related with *Babesia microti* of rodents by phylogenetic analysis, but did not segregate with parasites belonging to the *Babesia sensu stricto* group. Zahler et al. (2000) proposed at this time the name *Theileria annae.* Due to disagreement on its placement in the *Theileria* or *Babesia* genera, several names have been used for these parasites, including *Babesia* ‘Spanish dog isolate’, *Babesia microti*-like,

Babesia (*Theileria*) *annae* and *Babesia* cf. *microti*. However, no types were fixed for either of them, therefore these names must be considered *nomina nuda* and thus unavailable names.

Infection of red foxes (*Vulpes vulpes*) by *B. microti*-like piroplasms was first recorded in Spain in 2003 (Criado-Fornelio et al., 2003). Since then, other studies have shown increasing prevalence of *B. microti*-like infections of red foxes in almost all European countries, suggesting that red foxes are the natural reservoirs of this pathogen (Baneth et al., 2015). Baneth et al. (2019) officially established and described a species named as *Babesia vulpes* n. sp. among the *Babesia microti*-like spp., examining blood samples of dogs and red foxes from Portugal and Israel.

In Hungary, 404 blood samples collected from red foxes in 2011 were screened by PCR for *Babesia* parasites in order to compare their partial 18S rRNA sequences to those parasites of domestic dogs and wild canids from other countries (Farkas et al., 2015). Altogether 81 (20%) samples out of 404 were infected with piroplasms. Among those positive samples, 30 were sequenced and compared with sequences available in GenBank, and 14 of them were 100% identical to *B. microti-like* piroplasms isolated from foxes from Croatia or Italy.

The aim of the present study was to screen more blood samples also collected in 2011 and to compare their partial 18S rRNA sequences to those parasites of domestic dogs and wild canids from other countries, especially with sequences of *Babesia vulpes* n. sp. recently described by Baneth et al. (2019).

2 Literature reviews

2.1 General characteristics of *Babesia* species of domestic and wild canids

Babesiosis is a tick-transmitted disease caused by intraerythrocytic parasites of the genus *Babesia*, belonging to the family Babesiidae, order Piroplasmida. Traditionally, two *Babesia* spp. has been identified according to the size of their merozoites in the erythrocytes: *Babesia canis* as large *Babesia* (2.5-5 µm) and *Babesia gibsoni* as small *Babesia* (0.5-2.5 µm) (Beugnet, 2013). *B. canis* is a piriform- or teardrop-shaped organism and there is usually more than one merozoite in a single erythrocyte. *B. gibsoni* is a pleomorphic organism and is usually observed as a single form (Figure 1).

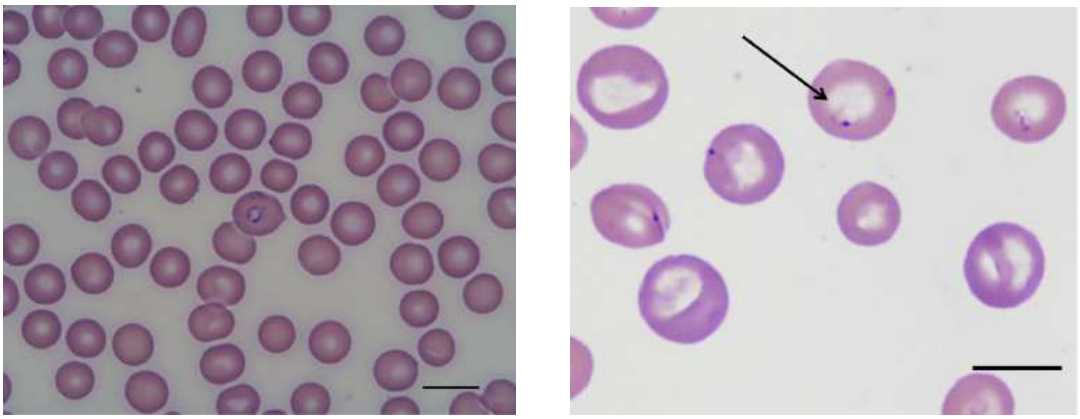


Figure 1: Larged-sized *B. canis* (left) and small-sized *B. gibsoni* (right) in Giemsa-stained canine blood smears. Scale-bar: 10 µm (Solano-Gallego et al., 2016)

Blood feeding female ticks ingest infected erythrocytes containing merozoites which are disintegrated in the tick's intestine (Figure 2). Whereas the merozoites are digested, ovoid gamonts resisting digestion form processes to differentiate into anisogametes (ray-bodies), which fuse to form a zygote. Zygotes enter intestinal cells, multiply and form sporokinetes which pass into the haemolymph and infect subsequently haemocytes and cells of various organs in which further multiplication occur. Following the infection of the ovaries, *Babesia* stages may be transmitted in the egg (transovarial transmission) to the next tick generation (vertical transmission). After hatching of a tick from an infected egg, sporokinetes infect salivary gland cells in which the infective stage, sporozoites develop (Deplazes et al., 2016).

Sporozoites are inoculated through the bite of ixodid ticks and infect erythrocytes of the host, where they develop to amoeboid trophozoites that multiply asexually by binary fission, forming mostly two drop-shaped merozoites. The host cells are destroyed by babesiae and the merozoites infect other erythrocytes.

Babesia spp. are transmitted transstadially (from one stage of the ticks to the next) and depending on the species they can be also transmitted transovarially (through tick's eggs).

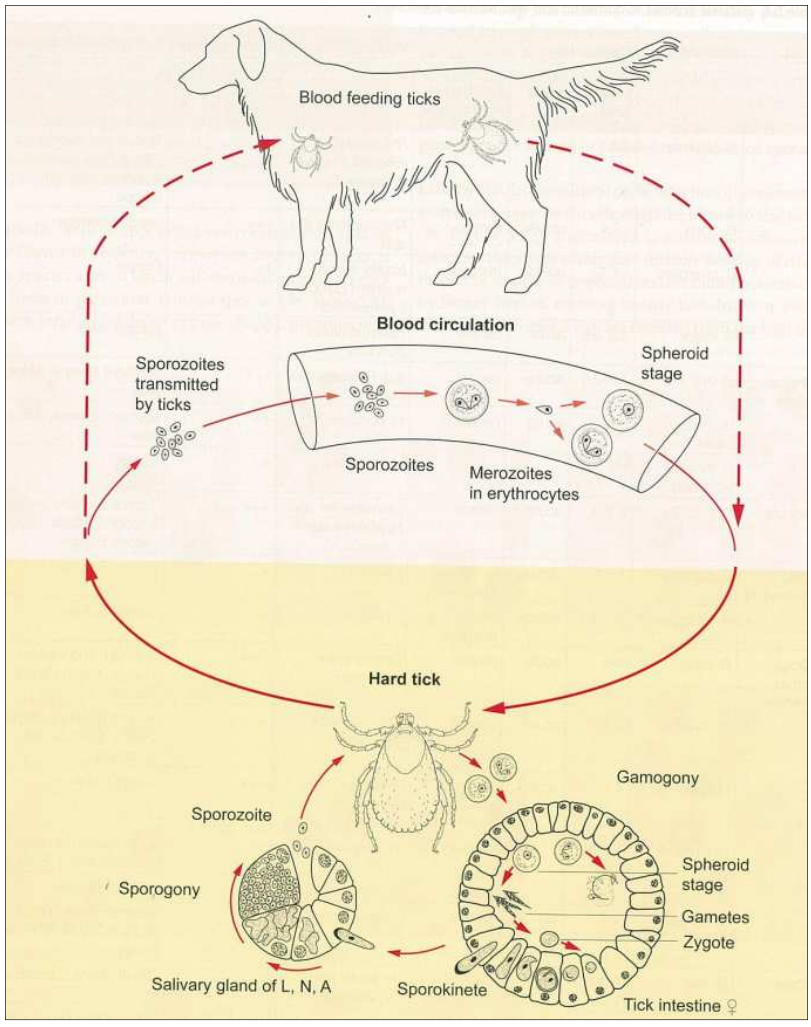


Figure 2: Developmental cycle of *Babesia vogeli* (Deplazes et al., 2016)

2.2 *Babesia* infection of domestic dogs in Europe

The large *Babesia* sp. previously considered to be *B. canis* currently includes *B. canis canis* (*B. canis*), *B. canis vogeli* (*B. vogeli*) *and B. canis rossi* (*B. rossi)* now considered to be three distinct species. Only the first two species have been detected in Europe.
There are two genetically and clinically distinct species of 'small' *Babesia* known to cause infection in dogs in Europe: *Babesia gibsoni* and *Babesia microti*-like species.
These species and their vectors are summarized in the Table 1 below.

Table 1: *Babesia* species detected in dogs in Europe and their vectors (Beugnet et al., 2013)

Species	Vector
Babesia canis	*Dermacentor reticulatus* *Rhipicephalus sanguineus*? *Ixodes ricinus?*
Babesia vogeli	*Rhipicephalus sanguineus* *Ixodes ricinus*?
Babesia gibsoni	*Rhipicephalus sanguineus* *Haemaphysalis longicornis* *Haemaphysalis bispinosa* *Dermatocentor variabilis*
Babesia microti-like / Babesia cf. Microti / Theileria annae / B. vulpes	*Ixodes hexagonus*? *Ixodes canisuga?*

The frequency of arthropod-borne diseases is increasing in Europe. Climate change (especially the reduction of the winter period allowing ticks to be active all year round over an increasing area for example) and movements of production animals affect arthropod vector density, geographical distribution and vectorial capacity. The founding of the European Union facilitated pet travel and importation of dogs by private persons and animal welfare organisations, enabling the introduction of vector-borne infections to non-endemic regions.

Landscape change such as the creation of recreational parks, private gardens or artificial lakes facilitates the establishment of tick populations close to human habitation (Beugnet et al., 2009).

Epidemiological surveillance data on disease occurrence, as well as on both ticks and vertebrate host population dynamics, are required to map regional risk and predict future *Babesia* infection scenarios. Several studies have shown that the geographical occurrence of *Babesia* species infections of dogs in Europe is highly variable and mostly depends on the distribution of the competent tick vector (Figure 3).

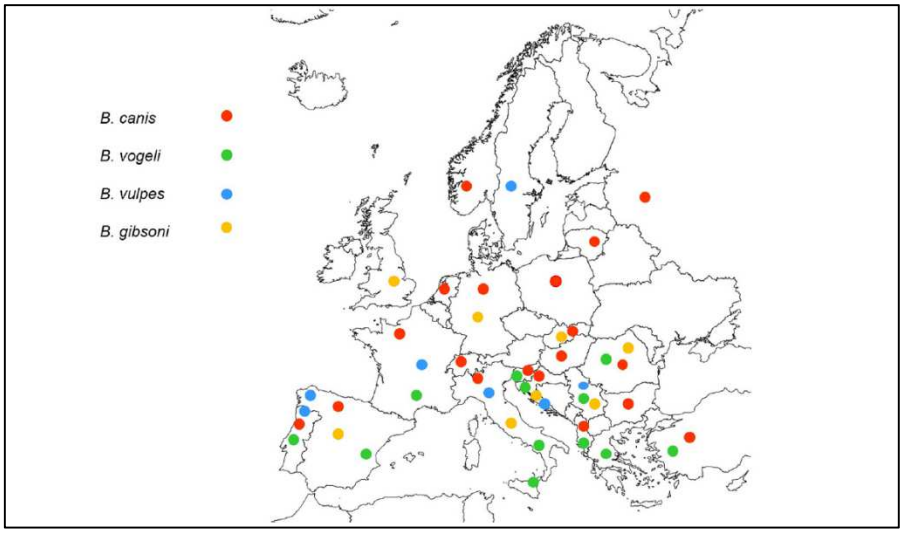


Figure 3 : Distribution of canine *Babesia* species in Europe in 2016 (Solano-Gallego et al., 2016)

2.2.1 Large *Babesia* spp.

.2.2.1.1 *Babesia canis*

This species is the most common agent of canine babesiosis in Europe. Although it has been reported that it was detected in *Ixodes ricinus* (Cieniuch et al., 2009) and *Rhipicephalus sanguineus* ticks (Cassini et al., 2009), its known vector is *Dermatocentor reticulatus* (Beugnet et al., 2013). It is assumed that this is the most pathogenic large-sized species in Europe. Acute babesiosis caused by *B. canis* in dog is associated with haemolytic anaemia (pale mucous membrane, jaundice), apathy, high fever, anorexia, vomiting, splenomegaly and lymphadenomegaly (Solano-Gallego et al., 2016).

The prevalence for *B. canis* was 2.3% in Italy (Cassini et al., 2009), 4.6% in Slovenia (Duh et al., 2004), 25.3% in Poland (Welc-Falęciak et al., 2009) and 50% in Hungary (Hamel et al., 2012).

.2.2.1.2 *Babesia vogeli*

This species is transmitted by *Rhipicephalus sanguineus*, *Ixodes ricinus* could also be implicated in its transmission (Cassini et al., 2009). It is morphologically similar to *B. canis*, but it is less pathogenic. *B. vogeli* infections are often asymptomatic or accompanied by mild fever and weak anaemia.

The prevalence of dog babesiosis caused by this species was 0.9 % in France (Criado-Fornelio et al., 2009) and 1.3 % in Slovenia (Hamel et al., 2012).

2.2.2 Small *Babesia* spp.

.2.2.2.1 *Babesia gibsoni*

In Europe, canine babesiosis caused by *B. gibsoni* is rare, and its known vector on the continent is *Rhipicephalus sanguineus*. Infected animals may show fever, anaemia, apathy, thrombocytopenia and haemoglobinuria. There is a tendency to a chronic course of the disease (Solano-Gallego et al., 2016). Epidemiological data of prevalence of clinical illness or subclinical infection are limited in Europe. In Croatia, molecular data have revealed a prevalence of 0.7% for *B. gibsoni* (Beck et al., 2009).

.2.2.2.2 *Babesia microti*-like

Some formerly unknown small *babesiae* were first described in a German dog returned back from Spain, showing clinical babesiosis characterized by lethargy, fever and anaemia (Zahler et al., 2000). These protozoa were closely related with *Babesia microti* of rodents by phylogenetic analysis, but did not segregate with parasites belonging to the *Babesia sensu stricto* group. Zahler et al. (2000) proposed at that time the name *Theileria annae.* Since then *B. microti*-like spp. have been identified as a cause of infection and/or disease in dogs in Croatia (Beck et al., 2009), USA (Yeagley et al., 2009), Portugal (Simoes et al., 2011) and Sweden (Falkenö et al., 2013).

Due to disagreement on their placement in the *Theileria* or *Babesia* genera, several names have been used for these parasites, including *Babesia* ‘Spanish dog isolate’, *Babesia microti*-

like species, *Babesia* (*Theileria*) *annae*, *Babesia* cf. *microti* and *Babesia vulpes*. However, no types were fixed for either of them, therefore these names must be considered *nomina nuda* and thus unavailable names. Baneth et al. (2019) officially established and described a species named as *Babesia vulpes* n. sp. among the *Babesia microti*-like spp., examining blood samples of dogs and red foxes from Portugal and Israel.

Dogs infected with the *Babesia microti*-like piroplasms had a syndrome clinically more severe than those infected with *B. canis*, including severe haemolysis, intense regenerative haemolytic anaemia, thrombocytopenia and azotaemia related to renal failure which was the main cause of death implicated in Spanish dogs (Camacho et al., 2001; 2003; 2004).

The modes of transmission and tick vectors of *B. microti*-like spp. have not been determined yet. It has been suggested that the hedgehog tick, *Ixodes hexagonus* is a vector of this parasite (Camacho et al., 2003; Checa et al., 2018), however this hasn't been proven. Furthermore, *Babesia microti*-like species infection has been detected in areas where this tick species has not been reported (Birkenheuer et al., 2010). DNA of *B. microti*-like spp. has been detected in several tick species including *I. hexagonus*, *I. ricinus* (Najm et al., 2014; Lledó et al., 2014), *I. canisuga* (Najm et al., 2014), and *Rhipicephalus sanguineus* (Iori et al., 2010). These findings do not prove the capacity of these ticks to act as competent vectors but they might suggest that these parasites can be transmitted by different tick species. It has been suggested that other non-vectorial modes of natural transmission described for canine *Babesia* species, including transplacental transmission from dam to pups and direct infection by bite wounds, as in the case of *B. gibsoni*, can also be valid for *B. microti*-like spp. (Birkenheuer et al., 2010).

In Croatia, molecular data have revealed a prevalence of 0.1% for *Babesia microti*-like spp. in dogs (Beck et al., 2009). The occurrence of *Babesia microti*-like spp. have also been detected in dogs in Serbia (Gabrielli et al., 2015), Sweden (Falkenö et al., 2013), France (René-Martellet et al., 2015) and especially in northern Portugal (Simões et al., 2011) and Galicia, Spain (Camacho et al., 2001; Miró et al., 2015).

2.3 *Babesia* infection of domestic dogs in Hungary

2.3.1 Etiology

Babesiosis of dogs is endemic in Hungary and it has been demonstrated with morphological analysis that the causative agent is *B. canis*, transmitted by *D. reticulatus* (Horváth et al., 1996). In 2002, small *babesiae* were identified for the first time in two dogs, based on the size of the intracellular parasites observed in their blood smears. The clinical pictures were not consistent with the infection caused by *B. gibsoni*, therefore the authors suggested that another small *Babesia* species might have caused the infection (Farkas et al., 2004).

The first molecular survey on canine babesiosis in Hungary attempting to identify and characterize the subspecies of *B. canis* in dogs was made in 2005 (Földvári et al., 2005). The piroplasm-specific PCR's results were positive for 39 out of the 44 blood samples of dogs showing clinical signs of babesiosis (88.6%) and 5 samples were chosen randomly for sequencing and showed 99.8–100% similarity with *Babesia canis canis* (Földvári et al., 2005).

2.3.2 Clinical manifestation

A study about clinical manifestation of canine babesiosis in Hungary showed that uncomplicated babesiosis was diagnosed in half of the cases, in younger animals mainly. Symptoms were similar to those published from other parts of the world: lethargy, fever, splenomegaly, pallor, icterus, haemoglobinuria and presence of ticks were the most common observations. Thrombocytopenia, lymphopenia and neutropenia were frequent haemogram changes (Máthé et al., 2006). The other half demonstrated babesiosis with complications (older individuals): hepatopathy (44%), pancreatitis (33%), acute renal failure (ARF; 31%) and disseminated intravascular coagulopathy (DIC; 24%).

2.3.3 Prevalence

A serological analysis by IFAT (Indirect Fluorescence Antibody Test) of 651 canine blood samples from Hungary was made in 2006 and showed 5.7% positivity to *B. canis*. Furthermore, the prevalence of antibodies to *B. canis* was significantly higher among german

shepherds and komondors, suggesting for the first time a breed predisposition (Hornok et al., 2006).

Another study was made in 2012, 78 canine blood samples of dogs from Hungary were screened for *Babesia* spp. by indirect (IFAT) and direct methods (PCR) (Hamel et al., 2012). A total of 50% of dogs (39/78) were tested positive for *B. canis* by PCR, 1.3% (1/78) for *B. vogeli* and 1.3% (1/78) for *B. gibsoni*. This prevalence of *B. canis* is the highest one recorded in a dog population in Europe so far. Only 11.5% (9/78) were tested positive for *B. canis* spp. by IFAT, suggesting that the molecular method used for the detection influences the results and should be chosen accordingly.

2.4 *Babesia* infection of wild canids in Europe

Five species of wild canids are known to occur in Europe, the most abundant being the red fox, followed by the racoon dog, the wolf, the golden jackal and the artic fox. The red fox (*Vulpes vulpes*) is a unique example of a species with a very wide distribution range and with a huge population size (Otranto et al., 2015). The adaptation of foxes to urban environments and their increasing number result in an increased risk of pathogen transmission to human and domestic animals (Barandika et al., 2016). In particular, foxes are parasitized by several tick species and directly exposed to several vector borne pathogens, including *Babesia* spp. Suspected of being potential reservoirs of vector borne pathogens, several molecular surveys, using PCR and sequencing of the 18S rRNA, have been carried out in red foxes all over Europe in the last few years.

2.4.1 Large *Babesia* spp.

In Europe, *B. canis* was detected by molecular method only in one fox from Portugal (Cardoso et al., 2013), one fox from Bosnia and Herzegovina (Hodžić et al., 2015), one fox from Austria (Hodžić et al., 2018) and recently in one fox from Serbia (Juwaid et al., 2019). These studies are summarized in the Table 2 bellow. All infected red foxes were apparently healthy when being shot. It can be concluded that red foxes are not suitable hosts for *B. canis*, with hardly any impact as reservoir or spreader. *B. vogeli* has never been reported in wild carnivores so far.

Table 2: Prevalence of *B. canis* in red foxes in Europe

Country	Sample type	Positive/surveyed	% infected	Reference
Portugal	Bone marrow	1/70	1.4%	Cardoso et al., 2013
Bosnia and Herzegovina	Spleen	1/119	0.8%	Hodžić et al., 2015
Austria	Blood	1/351	0.3%	Hodžić et al., 2018
Serbia	Spleen	1/129	0.8%	Juwaid et al., 2019

2.4.2 Small *Babesia* spp.

.2.4.2.1 *Babesia gibsoni*

B. gibsoni has been detected in blood samples of red foxes but only based on morphological characteristics (Penzorn, 2006), not by molecular method so far.

.2.4.2.2 *Babesia microti*-like

Infection of red foxes by *B. microti*-like piroplams was first recorded in Spain when studying DNA samples obtained from the spleen of ten foxes captured between 1997 and 1999 (Criado-Fornelio et al., 2003). Since then, several studies with PCR and sequencing of the 18S rRNA have shown high prevalences of *B. microti*-like piroplams infection in red foxes in several European countries (Table 3) , suggesting that red foxes are the natural host of this pathogen (Baneth et al., 2015). Baneth et al. (2019) officially established and described a species named as *Babesia vulpes* n. sp. among the *Babesia microti*-like spp., examining blood samples of dogs and red foxes from Portugal and Israel.

Table 3: Prevalence of *B. microti*-like piroplasms in red foxes in several European countries

Country	Sample type	Positive/surveyed	% infected	Reference
Northern Spain, Burgos	Blood	1/5	20.0%	Gimenez et al., 2009
Croatia	Spleen	10/191	5.2%	Dežđek et al., 2010
Poland	Spleen	1/138	0,7%	Karbowiak et al., 2010
Portugal	Blood	53/64	82.8%	Cardoso et al., 2013
	Bone marrow	18/70	25.7%	
Germany, Thuringia	Spleen	121/261	46.4%	Najm et al., 2014
Italian Alps	Spleen	2/205	1.0%	Zanet et al., 2014
Austria	Spleen	13/35	76.5%	Duscher et al., 2014
	Blood	11/17	31.4%	
Bosnia and Herzegovina	Spleen	38/119	31.9%	Hodžić et al., 2015
Hungary	Blood	81/404	20.0%	Farkas et al., 2015
Great Britain	Lung exudate	46/316	14.6 %	Bartley et al., 2016
Germany, Brandenburg	Spleen	91/195	47.5%	Liesner et al., 2016
Northern Spain	Spleen	22/48	45.8%	Barandika et al, 2016
Central Italy	Spleen	35/153	22.8%	Ebani et al., 2017
Western Austria	Blood	178/351	50.7%	Hodžić et al., 2018
	Spleen	130/506	25.7%	
Slovakia	Spleen	29/300	9.7%	Koneval et al., 2017
NW Spain, Galicia	Spleen	171/237	72.2%	Checa et al., 2018
Romania	Blood	70/347	20,2%	Daskalaki et al., 2018
Serbia	Spleen	37/129	28.7%	Juwaid et al., 2019
Southern Italy	Spleen	36/82	43.9%	Santoro et al. 2019

The prevalence of *B. microti*-like piroplasms in red foxes ranges from 0.7 % in Poland to a maximum of 82.8% in Portugal.

Differences between the prevalence levels reported in the studies above may occur due to employed methodology (type and specificity of the primers used for PCR, protocols), sample size (from 5 to 506 samples), sample type (blood or spleen samples mostly, but also bone marrow or lung exudate samples), geographical location, etc. However, the influence of these factors has never been evaluated.
Furthermore, only part of the positive samples is often sequenced due to financial reason, but the results of the sequencing are sometimes extrapolated to the entire positive samples.

Relatively high prevalence levels and good body condition of the positive red foxes might be indicative of a low pathogenicity of *B. microti*-like piroplams in this host. So far, clinical cases have only been described in few naturally infected red foxes from Canada (Clancey et al., 2010).

2.5 *Babesia* infection of wild canids in Hungary

Among wild canids, babesiosis due to *B. canis* has been molecularly detected in two captive grey wolves (*Canis lupus*) found dead in Hungary, with severe jaundice (Erdélyi et al., 2014). This sudden death could be, according to the authors, secondary to the immunosuppression related to captivity, which probably lead to the clinical manifestation.

Farkas et al. (2015) carried out the first investigation of *Babesia* infection in red foxes shot in Hungary in 2011. In total, 404 blood samples collected were screened for *Babesia* parasites by PCR and their partial 18S rRNA gene sequences compared with those of parasites of domestic dogs and wild canids from other countries. Altogether, 81 red foxes out of 404 (20.0%) were found to be infected with piroplasms. Among those positive samples, 30 were sequenced and compared with sequences available in GenBank, and 14 of them were 100% identical to *B. microti-like* piroplasms isolated from foxes from Croatia or Italy.

3 Materials and methods

3.1 Collection of samples

Blood samples were collected from 222 red foxes originating from all the 19 Hungarian counties a few years ago. The foxes were shot, and the carcasses were sent to the Veterinary Diagnostic Directorate, National Food Chain Safety Office, Budapest, as part of a control program on oral immunization of foxes against rabies. After opening the thoracic cavity of foxes, blood samples were obtained via cardiac puncture from the right atrium or chest cavity and were then frozen at −20°C until further processing. Gender of foxes was not recorded. The study was carried out in compliance with the ethical guidelines for study of wildlife animals in Hungary, and in agreement with the national animal welfare regulations (28/1998).

3.2 Molecular biological method

3.2.1 DNA extraction

DNA was extracted from each blood sample using the QIAamp DNA Mini Kit (QIAgen GmbH., Hilden, Germany) following the ‘‘Blood and body fluid” protocol instructions by the manufacturer.

3.2.2 Amplification and sequencing

A conventional single step PCR was used to amplify a 487 bp long fragment of the 18S rRNA gene of piroplasms with primers BJ1 [5’-GTC TTG TAA TTG GAA TGA TGG-3’] and BN2 [5’- TAG TTT ATG GTT AGG ACT ACG-3’]. Reaction mix contained 15.8 µl PCR water, 2.5 µl of 10× concentration of CoralLoad Buffer (15 mM MgCl2 included), 0.5 µl 10 mM dNTP, 0.5 µl of each primer (50 µM) and 0.2 µl (5 U/µl) of HotStarTaq Plus DNA Polymerase in a final volume of 25 µl containing 5 µl DNA. Amplification was performed with a BIOER Gene- Pro BIOER TC-E-BD device (Bioer, Hangzhou, PR China). Initial denaturation at 95°C for 10 minutes was followed by 40 cycles of denaturation at 95°C for

30 seconds, annealing at 54°C for 30 seconds and elongation at 72°C for 40 seconds. The thermal program was finished with 5 minutes of final elongation at 72°C. In each reaction set, a positive control and a negative control with no DNA were included.

PCR products of each reaction were electrophoresed in 1.5% agarose gel (100V, 40 minutes), stained with ethidium-bromide and visualized under ultra-violet light.

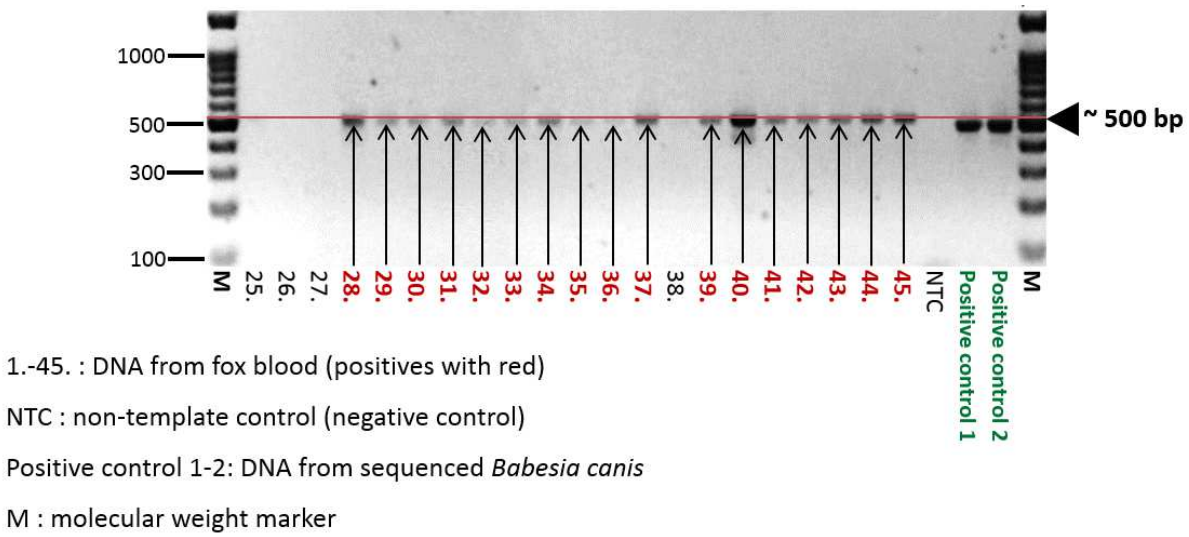
The PCR products were cleaned and sequenced at the Hungarian Academy of Sciences, Biological Research Centre, Szeged, Hungary. The obtained sequences were edited, aligned and compared to reference GenBank sequences by the nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov).

3.2.3 Statistical analysis

Confidence intervals (CI) for the prevalence rates were calculated at the level of 95%.

4 Results

Altogether 85 foxes out of 222 (38.3%; 95% CI: 31.9-44.7%) were found to be infected with piroplasms after PCR amplification and electrophoresis (Figure 4).



1.-45. : DNA from fox blood (positives with red)

NTC : non-template control (negative control)

Positive control 1-2: DNA from sequenced *Babesia canis*

M : molecular weight marker

Figure 4: Electrophoresis gel of piroplasma spp.

The PCR positive animals were shot in 17 of the 19 Hungarian counties (Table 1), except Heves and Nógrád located in the northern part of the country close to Slovakia. The highest prevalence levels were found in Somogy (100%), Tolna (100%) and Baranya (86.7%) counties (Table 4).

Table 4: Prevalence of piroplasm infection of red foxes shot in Hungary

County	Positive/examined	Prevalence (%)	95% CI
Baranya	13/15	86.7	69.5-100
Baz	5/8	62.5	29.0-96.0
Békés	9/14	64.3	39.2-89.4
Bács-Kiskun	2/36	5.6	0-13.0
Csongrád	5/6	83.3	53.5-100
Fejér	5/34	14.7	2.8-26.6
Györ-Moson-Sopron	1/2	50.0	0-100
Hajdú-Bihar	5/10	50.0	19.0-81.0
Heves	0/6	0	0
Jász- Nagykun- Szolnok	7/8	87.5	64.6-100
Komárom- Esztergom	1/3	33.3	0-86.7
Nógrád	0/5	0	0
Pest	3/36	8.3	0-17.4
Somogy	8/8	100	100
Szabolcs- Szatmár- Bereg	13/18	72.2	51.5-92.9
Tolna	2/2	100	100
Vas	2/6	33.3	0-71.1
Veszprém	1/1	100	100
Zala	3/4	75	32.6-100
TOTAL	**85/222**	**38.3**	**31.9-44.7**

When the 18S RNA gene fragments from 37 positive foxes were sequenced and compared by BLAST with 18S RNA sequences available in GenBank, all of them were 99-100% identical to *B. vulpes* n. sp. detected in Italy (GenBank: MK742780), Serbia (GenBank: MH699396) and Slovakia (GenBank: KY175167).

Eleven sequences have been deposited in GenBank database, the accession numbers are: MK937700- MK937711; 18S rRNA.

5 Discussion

Red foxes are parasitized by several tick species and directly exposed to vector borne pathogens, including *Babesia* spp. In Europe, the most prevalent *Babesia* spp. infecting red foxes are the *B. microti*-like piroplasms, reported in almost all European countries under different unavailable names, with prevalence levels ranging from 0.7 % in Poland (Karbowiak et al., 2010) to a maximum of 82.8% in Portugal (Cardoso et al., 2013). Baneth et al. (2019) recently officially established and described the new species *Babesia vulpes* n. sp. and it is very likely that the *B. microti*-like piroplasms formerly detected in red foxes are *B. vulpes* n. sp.

Babesia canis was molecularly detected only in four red foxes in Portugal (Cardoso et al., 2013), Bosnia and Herzegovina (Hodžić et al., 2015), Austria (Hodžić et al., 2018) and in Serbia (Juwaid et al., 2019). The known vector of this large *Babesia* species is *Dermacentor reticulatus* (Beugnet et al., 2013), but it was also detected in *Rhipicephalus sanguineus* (Cassini et al., 2009) and *Ixodes ricinus* (Cieniuch et al., 2009) ticks.
The worldwide distributed small babesia of dogs, *B. gibsoni* has been detected in blood samples of red foxes but only based on morphological observations (Penzorn, 2006), not by molecular method so far.

The present study reports a relatively high prevalence (38.3%, 8/222) of piroplasmosis in red foxes in Hungary. In a survey of piroplasmosis in 404 local red foxes previously conducted by Farkas et al. (2015) its prevalence was 20.0%. Blood samples were screened for babesial parasites by PCR and the partial 18S rRNA gene sequences of some of the positive animals were compared to those parasites of domestic dogs and wild canids from other countries. The positive animals were shot in 17 of the 19 Hungarian counties, except Heves and Nógrád, and only *B. vulpes* species was detected. This and the former study confirm that *Babesia* infections are widespread in red foxes in the country.
Furthermore, the results of the present examination demonstrate for the first time the occurrence of *B. vulpes* n. sp. proposed to be accepted as a new species internationally (Baneth et al., 2019). Although the presence of this *Babesia* species was only confirmed in the blood samples of 37 red foxes by sequencing of the PCR products probably the other 48 infected red foxes were very likely to be *B. vulpes* n. sp as well.

The detection of the *B. vulpes* n. sp in such considerable proportion of the apparently healthy foxes sampled suggests that a sylvatic life cycle of this protozoan exists in fox populations and that foxes are the major reservoir host for this parasite. Although it is suspected that *Ixodes hexagonus* and *Ixodes canisuga* are its vector, the tick vect still remain unknown (Camacho et al., 2003; Checa et al., 2018). It has been suggested that other non-vectorial modes of natural transmission described for canine *Babesia* species, including transplacental transmission from dam to pups and direct infection by bite wounds, as in the case of *B. gibsoni*, can also be valid for *B. vulpes* n. sp. (Birkenheuer et al., 2010).

So far, no clinical case of *Babesia* infection caused by any species in red foxes has been described in Europe. It is still unknown whether *B. vulpes* n. sp can be pathogenic for red foxes, other wild canids or dogs. *B. vulpes* n. sp was only detected in a dog once in Portugal so far (Baneth et al., 2019).

A recent survey of *Babesia* infection in 1,311 hunting dogs in the Campania region of southern Italy revealed only the presence of *B. canis*, *B. gibsoni*, and *B. vogeli* (Veneziano et al., 2018). So despite the frequent contacts occurring between hunting dogs and red foxes, and the few ixodid tick vectors they share, this suggests that at least in this region they tend to acquire different *Babesia* spp.

Research are still needed to better understand the role of red foxes and wild carnivores in general in the epidemiology of *B. vulpes* n. sp. The gaps in the knowledge include:

- The identity of the vector(s) of *B. vulpes* n. sp, its ways of transmission and whether or not it can be transmitted in between dogs and red foxes;
- The pathogenicity of *B. vulpes* n. sp in canids;
- The ability of ticks to serve as reservoirs in the absence of the vertebrate host;
- The confirmation of suspected wild canid reservoirs, like golden jackals, and the competence to infect ticks;
- The investigation of alternative ways of transmission (transplancental, direct) and its role in the maintenance of *Babesia* spp. in the wild in the absence of a tick vector.

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8 Abstract

Red foxes have been recognized as potential reservoirs of several parasites and pathogens transmitted by arthropod vectors and a source of infection for all susceptible species sharing the same habitats, including dogs and humans (Dusher et al., 2015).
Several studies have shown increasing prevalence of *Babesia microti*-like infections of red foxes in almost all European countries, suggesting that red foxes are the natural reservoirs of this pathogen (Baneth et al., 2015). Baneth et al. (2019) officially established and described a new species named as *Babesia vulpes* n. sp. among the *Babesia microti*-like spp.

In Hungary, blood samples collected from red foxes in 2011 were screened by PCR for *Babesia* parasites (Farkas et al., 2015). Altogether 20% of the red foxes sampled were infected with piroplasms, and the positive samples sequenced were 100% identical to *B. microti-like* piroplasms isolated from foxes from Croatia or Italy.
The aim of the present study was to screen more blood samples also collected in 2011 and to compare their partial 18S rRNA sequences to those parasites of domestic dogs and wild canids from other countries, especially with sequences of *Babesia vulpes* n. sp. recently described by Baneth et al. (2019). DNA was extracted from each blood sample and a conventional single step PCR was used to amplify a fragment of the 18S rRNA gene of piroplasms with primers BJ1 and BN2. PCR products were electrophoresed in agarose gel, stained and visualized under ultra-violet light.
Altogether 85 red foxes out of 222 (38.3%; 95% CI: 31.9-44.7%) were found to be infected with piroplasms. The positive animals were shot in 17 of the 19 Hungarian counties, except Heves and Nógrád. The 18S RNA gene fragments from 37 positive foxes were sequenced and compared by BLAST. All of them were 99-100% identical to *B. vulpes* n. sp. This is the first time that infection of red foxes by *B. vulpes* n. sp was detected in Hungary.

Further studies are needed to identify the tick vectors involved in its transmission, the mechanisms of transmission, its pathogenicity and whether or not it can be transmitted in between dogs and red foxes.

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