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Serological and Molecular Investigations of Babesia Microti in Dogs from Southern Italy

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Abstract

Piroplasmosis is now considered emerging tick-borne zoonosis worldwide and domestic animals have been proposed as potential reservoirs for some piroplasm infections. The aim of this research was to identify the frequency of *Babesia microti* infection in untravelled dogs from Southern Italy (Sicily). Blood samples from 89 dogs were examined for the presence of *Babesia microti* antibodies and DNA. The dogs were infested with ticks. Among the examined dogs only one (1.16%) had *B. microti* antibodies, associated to *B. canis*, *A. phagocytophilum* and *R. conorii* infections. In the PCR assay, the dog was also found positive for *B. microti* and *R. conorii* DNA, and negative for other microorganisms. The infected dog showed a non-specific flu-like syndrome, with depression, disorexia, hyperthermia (39.6°C), light dehydration, moderate lymphadenopathy and heavy tick infestation (>20). No significant changes were present in the cell blood count. To the best of our knowledge, this is the first report of serological and molecular identification of *B. microti* piroplasm in a dog from Southern Italy. Because specific antibodies and DNA were detected in an untraveled dog, babesiosis is probably due to an autochthonous tick infection. However, this study indicates that *Babesia microti* is not widely distributed in dog populations in Sicily, as demonstrated by low prevalence of infection. So, in Sicily the dog would not appear to represent a reservoir of infection, but rather an accidental host.

Keywords: *Babesia microti*; Dog; PCR; Southern Italy

Abbreviations:

CBC: cell blood count; C.R.A.Ba.R.T: National Reference Centre for Anaplasma, Babesia, Rickettsia and Theileria; ELISA: enzyme-linked immunosorbent assay; IFAT: immunofluorescent antibody test; IZS: Istituto Zooprofilattico Sperimentale; K3 EDTA: tri-potassium ethylenediaminetetraacetic acid; PCR: polymerase chain reaction; RBC: red blood cell; TBD: tick borne disease

Introduction

Babesiosis, otherwise known as piroplasmosis, is a blood-borne infection of particular concern due to the severity of the clinical signs, which infect a variety of domestic and wild animals, and humans [1,2]. Canine babesiosis is a tick borne disease caused by an obligate intraerythrocytic parasite, genus *Babesia* (Apicomplexa: Piroplasmida) [3]. After host invasion Babesia spp. sporozoites parasitize RBCs, evolve into ring-shaped trophozoites and then replicate forming merozoites that move freely in the host cell cytoplasm [4]. Merozoites may further divide forming 4-8 or more parasites in the same RBC and eventually destroying the cell and become free in the blood to invade new cells [2]. In most European countries, B. canis is recognized as the principal etiological agent in canine piroplasmosis, but molecular and phylogenetic analyses suggest the presence of other species of piroplasms [3].

Traditionally, it was assumed that the only *Babesia* species causing disease in dogs were *Babesia canis* and *Babesia gibsoni* [5], but recently published reports demonstrate that different piroplasm species can parasitize dogs [2,6-9].

B. rossi, *B. canis* and *B. vogeli*, previously considered as subspecies of *B. canis*, are identical morphologically but demonstrate tremendous variations in geographic distribution, vector specificity, genetic characteristics, and clinical signs which they induce in dogs. These parasites are currently considered as separate species [2].

In Italy, *B. canis* is mainly described in the North and less frequently in Central regions, while *B. vogeli* is predominantly found in Central and Southern Italy [10].

The development of molecular methods has demonstrated that more piroplasmid species infect dogs. These include the small piroplasms *Babesia conradae* [7,11], *Babesia microti*-like piroplasm [9], which is also referred to as *Theileira annae* [6,9] or "Spanish dog isolate" [8], and *Theileria* spp. [2,12].

Babesia microti is the most common cause of human babesios. It is endemic in North America and has recently emerged in Europe and East Asia. Infection usually causes asymptomatic to mild flu-like clinical manifestations including fever, sweat, chills and anemia. However, severe and sometimes fatal outcomes generally occur in immunocompromised or elderly individuals [13,14].

A new pathogenic *B. microti*-like species from dogs was first described in a German dog infected during a trip to Northwestern region of Spain [9,15].

B. microti-like piroplasm had been molecularly sequenced from infected dogs with clinical babesiosis from Galicia northwestern Spain, [6] and in sick dogs from northern Portugal [16].

There are reports of infection in a few clinically healthy dogs: one from Tarragona, northeastern Spain [17], another one from Croatia [18] and an additional one from the US [8]. DNA of the *B. microti* or *B. microti*-like piroplasms has also been detected in several species: in foxes from Italy [19,20], Central and Northern Spain [21-23], Portugal [24], North America [15], and Croatia [25]; in cats from Portugal [26] and Italy [27,28]; in a donkey from Northern Spain [29]; in roe deers from Italy [30]; in feral raccoons from Japan [31]. Molecular analyses also detected the parasite DNA in *Ixodes ricinus* and *Rhipicephalus sanguineus* ticks from Italy [1,3], in *I. ricinus* and *Ixodes hexagonus* from Northern Spain [23] and in *I. ricinus* in Poland [32].

A potential role in the parasite transmission seems to be attributed to birds ticks. In fact, a recent research revealed positivity to *B. microti* infection in two larvae of bird-fed ticks [33].

The pathology that *Babesia* inflicts in the host varies considerably with the different species and sub-species involved, and also with the host's individual immune status, age, concurrent infections or illness and response to infection [34]. Haemolytic anemia and systemic inflammatory response syndrome leading to multiple-organ dysfunction syndrome account for most of the clinicopathological abnormalities observed in canine and feline babesiosis [35,36]. In general, *Babesia* species cause a haemolytic anemia which is multifactorial and is the predominant clinical manifestation inducing a number of immune responses that may have a devastating influence [37]. Haemolytic anemia can occur due to direct red blood cell lysis by replicating intracellular parasites which cause a combination of intravascular and extravascular haemolysis [2].

The most frequent clinical findings reported in dogs infected with B. microti-like piroplasm are weakness, fever, lethargy, tachycardia and tachypnea [2]. Azotemia, hyperphosfatemia, hypoalbuminemia, hypercholesterolemia, proteinuria and high urine protein/creatinine ratios suggest a glomerular component to the disease [38].

In light of these findings and since that only few studies investigating the presence of this parasite in dogs of the Mediterranean area are available, the aim of this work was to verify the prevalence of *B. microti* in untraveled dogs from several areas of Sicily.

Materials and Methods

The study was conducted on 86 dogs from different areas of Sicily (Southern Italy), during the period May-October 2005. All the subjects differed for breed, gender, age and management (kennel, farm and family dog). Relatively to the origin, the dogs were divided as follows: 26 were from Caltanissetta (37.49°N, 14.04°E; 597 m. a.s.l.), 7 from Catania (37.5°N, 15.09°E; 19 m. a.s.l.) and 53 from Messina (38.19°N, 15.55° E; 59 m. a.s.l.) (Table 1 and Figure 1).
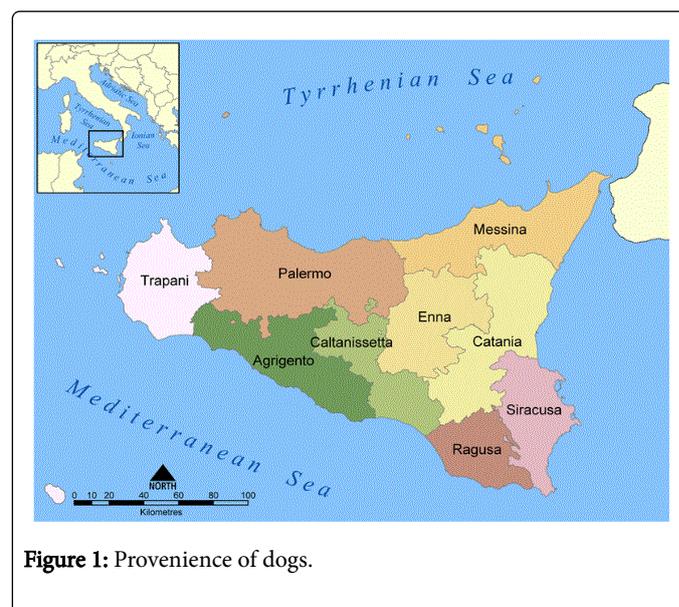
Figure 1: Provenience of dogs.

All the animals present at the moment of the inspection of the facilities (kennels, farms or veterinary clinics) were enrolled for the study. Animal shelters and pet owners consented to have their animals involved in this study.

Provenience	Facilities	Nr.	Breed	Age (years)	Gender	
					Male	Female
Caltanissetta	Kennel 1	14	Mongrel	3-5	2	12
	Kennel 2	8	Mongrel	3-5	2	6
	Owned dogs	2 2	Corso dog Mongrel	>1	2	2
Catania	Sheep Farm	7	Mongrel	1-4	3	4
Messina	Kennel 3	16 1 1 1	Mongrel Pointer English Setter Syberian Husky	4-10	10	9
	Kennel 4	7 2	Mongrel English Setter	4-8	6	6

		1	Cirneco of Etna			
		1	German Sheperd			
		1	Shi tzu			
	Veterinary Hospital	8	Mongrel			
		2	Labrador Retriever			
		2	German Sheperd			
		2	Italian Hound			
		1	Epagneul Breton			
		1	Rottweiler	4-10	12	10
		1	Shit-tzu			
		2	German Hound			
		1	Dachsund			
		1	Syberian Husky			
		1	Samoeido			
Total		86		1-10	37	49

Table 1: Group of examined dogs.

All the subjects after a general physical examination and a measurement of rectal temperature were venipunctured from cephalic or saphenous vein and blood samples were collected. Immediately after collection, the blood was stored at refrigerating temperature into tubes containing K3 EDTA and into tubes containing separating gel pending analysis. On the blood samples, within 8 hr from collection, a complete CBC was performed by means of an automated analyzer (Hemat 8, Seac, Florence, Italy).

On individual sera, IFAT for the research of anti-*B. microti* antibody was performed.

On each wool blood samples, *B. microti* DNA was detected using a PCR.

Each sample was simultaneously checked for *B. canis*, *Ehrlichia canis*, *Rickettsia conorii* and *Anaplasma phagocytophilum*.

The serological and molecular investigations were performed by the C.R.A.Ba.R.T. of the IZS "A. Mirri" of Sicily.

Serological and molecular assays

IFAT kits for *B. microti* (Fuller Laboratories, Fullerton, CA, USA) were used following manufacturer's recommendations. An antibody titre of 1:64 or greater was considered positive for *B. microti* antigens.

Molecular analysis

DNA was extracted using the PureLink Genomic Mini kit (Applied Biosystem Corporation) and quantified by Nanodrop ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Nucleic acids were analyzed by PCR to detect the presence of Babesia microti DNA. PCRs were assessed using GoTaq Polymerase (Promega), as previously reported [39].

The amplification of 18S rRNA region of *B. microti* was carried out using the primers BAB1 (5'-CTTAGTATAAGCTTTTATACAGC-3') and BAB4 (5'-ATAGGTCAGAAACTTGAATGATACA-5'). The amplicon expected length was 238 bp for Babesia microti.

PCRs were carried out in buffer $1 \times$, 0.4 µM of each primer, 1.5 mM of MgCl2, 0.2 mM of dNTPs, 1.25 U of Taq Polymerase (Promega, GoTaq DNA Polymerase).

The thermal profile of PCR was: 4 minutes at 94°C for denaturation followed by 35 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 50 seconds, and extension at 7°C for 1 minute. The final extension step was 5 minutes at 72C. In each reaction a positive control, consisting of pathogen DNA, and a negative control, in which DNA was replaced by water, were used. PCR products were visualized after electrophoretic migration on agarose gel containing ethidium bromide 10 µg/ml. PCR products were purified by a PCR and gel purification kit (Promega Wizard SV Gel and PCR Clean-up System), cloned into a cloning vector (Invitrogen pCR2.1 TOPO TA cloning kit), quantified and sent for sequencing (Macrogen, Inc., Seoul, South Korea). Obtained sequences were aligned using Bioedit software (Tom Hall, Ibis Biosciences) and ClustalW 2.0.10 and analysed for nucleotide sequence identity by comparing them with reference strains.

Results

The general clinical examination of the recruited dogs showed that all were infested with ticks.

Among the examined dogs only one (1,16%) had *B. microti* antibodies, associated to B. canis, *A. phagocytophilum* and *R. conorii* infections. In the PCR assay, the dog was also found positive for *B. microti* and R. conorii DNA, and negative for other microorganisms. *B. microti* isolate showed 100% homology with the GenBank closest sequence AY693840.1, the *B. microti* isolate Gray 18S ribosomal RNA gene, described by Slemenda SB, Herwaldt BL. and Pieniazek NJ. in the USA in 2004 (unpublished data) and isolated from human.

Physical examination of the infected dog revealed: depression, disorexia, hyperthermia (39.6°C), light dehydration, moderate lymphadenopathy and heavy tick infestation (>20). The dog was a 2-year-old female autochthon sheepdog lived in an organic sheep dairy farm located in Ramacca municipality (province of Catania; Oriental

Sicily; Southern Italy). The six other consanguineous dogs living in the farm was variously positive against TBDs but negative for the research of *B. microti* antibodies and DNA. In Table 2 is reported the haematological pattern of the infected dog.

Parameter	Observed values	Reference values*
Red blood cells (10^6/µL)	6.22	5.50-7.90
White blood cells (10^3/µL)	13.27	6.0-16.0
Haemoglobin (g/dL)	13.60	12-18
Haematocrit (%)	40.10	37-55
MCV (fL)	64.50	60-76
MCH (Pg)	21.95	20-27
MCHC (g/dL)	34	32.0-38.0
PLT (10^3/µL)	380	240-400
MPV (fL)	9.55	4.9-7.0
PCT (%)	0.38	0.150-0.300
RDW (%)	13.05	12-16

Table 2: Haematological parameters of the *B. microti* infected dog. *Hemat 8, Seac, Florence, Italy.

The Figure 2 shows the electrophoresis of PCR amplification product from the *B. microti* positive sample.


Figure 2: Ethidium bromide-stained 1.5% agarose gel electrophoresis of amplification products from a *B. microti* positive sample (lane 1), negative control (lane 2), positive control (lane 3); lane 4: 100-bp ladder. N.B. bp: base pair.
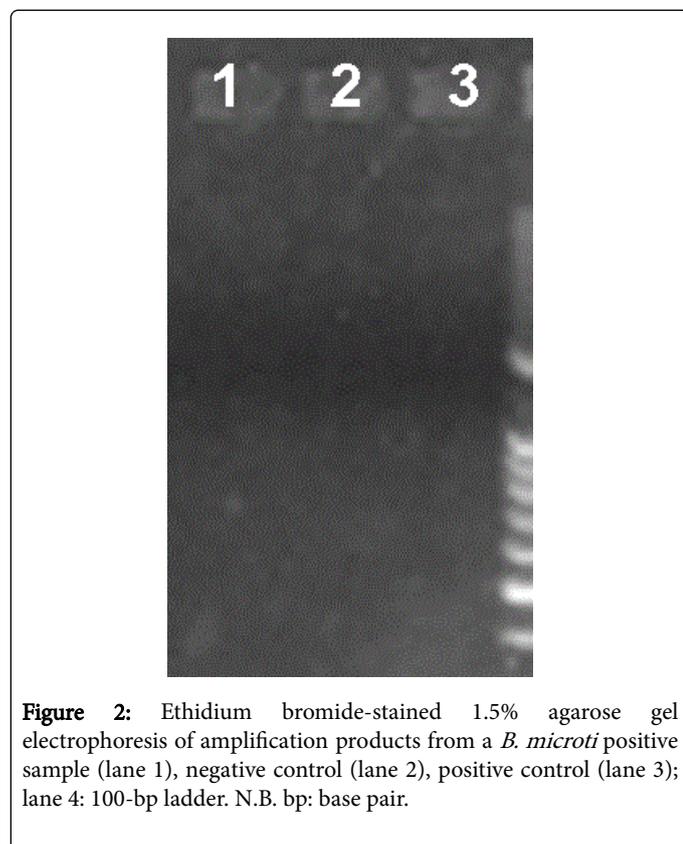
Discussion

To the best of our knowledge, this is the first report of serological and molecular identification of *B. microti* piroplasm in a dog from Southern Italy. Because specific antibodies and DNA were detected in an untraveled dog, babesiosis is probably due to an autochthonous tick infection.

Climatic conditions in Sicily make ectoparasitic infestations as endemic, especially in kennels. In Italy, dogs and cats living in public shelters may be at high risk of acquiring vector-borne pathogens, mainly because they are often untreated against ectoparasites, so representing an easy feeding source for them [40].

The positive animal was a shepherd dog with a high burn of tick infestation. The dog lived in an organic sheep dairy farm, in which a significant presence of ticks was detected. The type of farming has certainly favored the proliferation of parasites, since these farms not allowed the use of pharmacological treatments both on animals and environment. In addition, in this habitat there was a greater chance of being exposed to small rodents, considered to be the main reservoirs of the infection [41].

A recent study seems to confirm that not all the ticks are capable to improve the piroplasm diffusion. *R. sanguineus* is a well-known vector of piroplasms affecting farm animals and pets [1] and it is one of the most widespread ticks in Sicily.

The low prevalence recorded in the present study is in agreement with previous research conducted in Italy, in which were not found serological and molecular positivity to the parasite in dogs [42]. This finding is much different, however, from what has been observed in Germany, where 15% of the examined dog population showed seropositivity to *B. microti* [43]. Our results differs also from that observed in other animal species in Sicily, where the parasite DNA has been found in 30% of examined cats (6 out of 20) [27], and in 11% of foxes (1 of 9) [19], although the latter data have not been confirmed by subsequent studies, in which no foxes (0 of 13) were infected [44]. In Sicily, the dog, unlike the cat, would not appear to represent a reservoir of infection, but rather an accidental host.

In *B. microti* infection seems that seroconvertion appears prior to the onset of parasitemia, and since antibodies persist even after parasites are cleared, IFAT and ELISA often fail to distinguish an active from a resolved infection [4].

Moreover, with serological methods, a cross-reaction between different *Babesia* types is possible, as showed from other authors [45,46]. Our dog was also seropositive to B. canis, but no specific DNA was detected suggesting a possible cross reactivity with *B. microti* and other *Babesia* spp. [47].

In the present study, the definitive diagnosis of *B. microti* piroplasm infection was achieved only by PCR results. Sensitive molecular detection and species identification are important for the selection of the appropriate therapy and for prognosis, as well as for the screening of subclinical infections and of blood donors [16,48].

The infected dog showed a specific clinical picture, without significant changes in the CBC.

Although in humans a similar symptomatology is described [49,50], the clinical findings may be reported generically to tick-borne diseases, also associated. Moreover, the clinical signs found in the present case could be attributed to the simultaneous presence of other pathogens, which infections could be favored by the rural housing conditions. In

fact, the dog was also seropositive for *R. conorii, A. phagocytophilum* and *B. canis,* although DNA was found only for *R. conorii* than *B. microti.*

Even if the CBC of the infected dog did not show any relevant changes, anemia and moderate to severe thrombocytopenia are common findings among dogs infected with *B. microti*-like piroplasm [16,51,52]. The mechanisms related with severe hemolytic anemia may be more dependent on the host immune response than on the direct destruction of RBC by the piroplasm [53]. Mechanisms of local or systemic intravascular coagulopathy, immune-mediated destruction or splenic sequestration may be implicated in severe thrombocytopenias. On the other hand, the almost constant presence of macroplatelets in blood smears is associated with a bone marrow regenerative response to platelet consumption, as well as sequestration or destruction [53]. Camacho-García [6] describes that half the dogs with piroplasmosis due to B. microti-like piroplasm had normal leukocyte counts. Nevertheless, the values may range between leukopenia and leukocytosis, with the latter reaching extreme counts compatible with a leukemoid response in immune-mediated hemolytic anemia [52].

The risk of *B. microti* spreading infection to other European countries via dog travel or infected vector transport should increase the efforts to prevent the infection [16].

Since *B. microti* and B. microti-like were found in several European territories, the level of attention to these pathogens must remain high and further studies must be conduct with a larger sample size using diagnostic methods based on the DNA sequencing.

Acknowledgements

Conflict of interest

The authors declare that no competing financial interests exist.

Ethical statement

All treatments, housing and animal care reported above were carried out in accordance with the standards recommended by the EU Directive 2010/63/EU for animal experiments.

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