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Short communication

Clinicopathological findings, molecular detection and characterization of *Babesia gibsoni* infection in a sick dog from Italy

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ABSTRACT

A 4-year-old intact female American Pit Bull Terrier from Italy descendant of an American-born bitch was evaluated for anorexia, lethargy, weakness, and intermittent vomiting. On physical examination, the dog was dehydrated, had pale mucous membranes, hunched posture and abdominal pain. A moderate anemia was observed. Splenomegaly and hyperechoic regions suspected as infarcts in the spleen were seen on abdominal ultrasound. Based on the suspicion of splenic torsion, splenectomy was performed. After surgery, the clinical condition deteriorated. A follow-up complete blood count demonstrated severe macrocytic normochromic anemia with evidence of marked regeneration, left shift neutrophilia, monocytosis and marked thrombocytopenia. Blood smear evaluation revealed single to multiple, variable sized (1–3 μm in diameter), and round to oval to band-like piroplasms within many red blood cells consistent with small form *Babesia* spp. or *Theileria* spp. A partial segment of the 18S rRNA gene was amplified and the PCR product was analyzed by direct sequencing. The nucleotide sequence was completely identical to that of *Babesia gibsoni* present in GenBank®. This is the first molecular detection and characterization of *B. gibsoni* infection in a sick dog from Italy.

1. The study

Autochthonous canine babesiosis is mainly caused by *Babesia canis canis* (*B. c. canis*) in North and Central Europe and by *Babesia canis vogeli* (*B. c. vogeli*) in the Mediterranean basin (Cacciò et al., 2002; Solano-Gallego et al., 2008; Beck et al., 2009; Tabar et al., 2009). In addition, *Babesia microti*-like has been described in Northwestern Spain (Camacho-Garcia, 2006). In contrast, the epidemiology and clinical importance of *Babesia gibsoni* (*B. gibsoni*) infections in Europe are not well known. In fact, only few clinical cases with molecular evidence of *B. gibsoni* infection have been reported in dogs from Spain (Criado-Fornelio et al., 2003; Tabar et al., 2009) and Germany (Hartelt et al., 2007).

B. gibsoni is a tick-borne hematozoan parasite reported to cause severe hemolytic anemia in dogs (Taboada and Lobetti, 2006). This *Babesia* species is endemic in Southeast Asia (Taboada and Lobetti, 2006), North America (Birkenheuer et al., 2005) and Australia (Jefferies et al., 2007a). The clinical signs of *B. gibsoni* infection are variable. In some cases, the disease is fulminant with multiple organ failure and death. However, recent reports of *B. gibsoni* in the South Eastern United States documented cases of mild and, in some dogs, even inapparent disease (Meinkoth et al., 2002). The present case report describes the clinicopathological findings in a case of natural infection by *B. gibsoni* in a female American Pit Bull Terrier dog from Italy with detection and molecular identification of *B. gibsoni* by polymerase chain reaction-restriction fragment length

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polymorphism (PCR-RFLP) analysis and direct sequencing of an 18S rRNA gene fragment.

A 4-year-old intact female American Pit Bull Terrier was evaluated in December, 27th 2005 in Rome by a local veterinarian for anorexia, lethargy, weakness, and intermittent vomiting. She was born in Croatia from a bitch imported from the USA and transferred to Rome at 4 months of age. The dog lived mostly indoors, and had travelled only for a short time to Abruzzo in Central Italy. It had received current vaccination, had no history of previous serious illness, and had never received a blood transfusion. On physical examination, the dog had pale mucous membranes, hunched posture, abdominal pain, and dehydration.

Complete blood count (CBC), biochemistry profile and serum protein electrophoresis were performed on admission by Ematos Vet Lab (Rome) on December, 28th 2005 and the results are reported in Table 1 (day 0). On abdominal ultrasound, splenomegaly and hyperechoic regions suspected as infarcts in the spleen were seen. Splenectomy was performed on December, 29th 2005 based on the suspicion of splenic torsion. Histopathological evaluation of the spleen revealed massive necrosis, hemorrhages, associated with vascular thrombosis and a diffuse granulocytic infiltrate. White and red pulp hyperplasia were present. The histopathological findings were suggestive of splenic infarct.

Serial CBCs were performed after the dog's first clinical presentation (Table 1). Eight days after surgery, the clinical condition deteriorated. Another CBC was performed 10 days after splenectomy (Table 1). Eighteen days after surgery, the dog's clinical condition was critical. A CBC demonstrated severe macrocytic normochromic anemia with marked regeneration. Mild leukocytosis with left shift neutrophilia, monocytosis and marked thrombocytopenia were observed (Table 1, day 19). Blood smear evaluation revealed variable sized (1–3 μm in diameter), single to multiple, and round to oval to band-like piroplasms within many red blood cell (RBC) consistent with small form *Babesia* spp. or *Theileria* spp. (Fig. 1). The serology tests performed (01/18/2006) by Ematos Vet Lab revealed that the dog was positive for *Ehrlichia canis* (1:640) and *Babesia canis* antibodies and negative for *Rickettsia rickettsii* and *Leishmania infantum* antibodies measured by immunofluorescent assays.

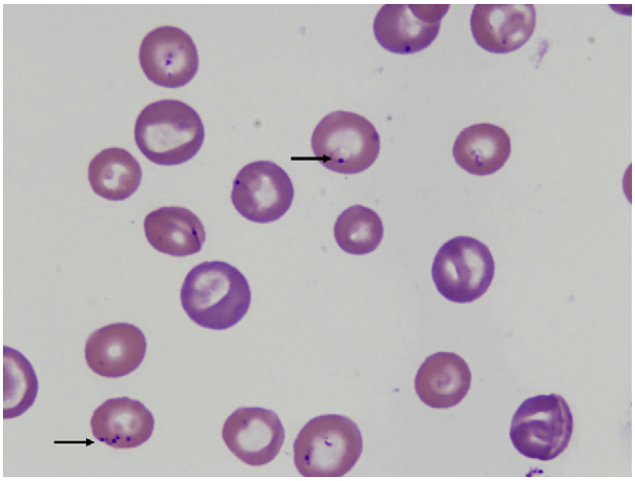

Fig. 1. *Babesia gibsoni* (arrows) in erythrocytes in a blood smear stained with modified Wright technique (1000×).

DNA extraction was performed from EDTA–whole blood sample by the High Pure PCR Template Preparation Kit (Roche Applied Science) in accordance with the manufacturer's protocol with some modifications. Partial 18S rRNA gene was amplified and PCR product was analyzed by RFLP analysis as previously described (Solano-Gallego et al., 2008). The PCR-RFLP showed a typical pattern for *B. gibsoni*. The amplified PCR product of 412 bp was used for partial 18S rRNA gene sequencing. The direct sequencing was performed by BMR Genomics srl (Padua, Italy). Consensus sequence was compared to the sequences deposited in GenBank® using the basic local alignment search tool (BLAST). The sequence revealed an identity of 100% with sequences of *B. gibsoni* 18S rRNA gene present in GenBank®. The sequence obtained was submitted in GenBank® database with accession number FJ554534. The sequence alignments were constructed using Clustal W in the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequence obtained was compared to corresponding sequences from other canine piroplasms and phylogenetic analysis of DNA sequences was performed using Mega version 4.1 (Tamura et al., 2007) (Fig. 2). The *B. gibsoni* from Italy clustered together with *B. gibsoni* from infected dogs of different areas of the world. *Babesia canis canis*, *B. c. vogeli* and *B. c. rossi* clustered together in a separate clade. *Babesia conradae* formed a separate branch (Fig. 2).

After the diagnosis of *B. gibsoni*, the dog was immediately treated with imidocarb dipropionate, doxycycline, and prednisone (Table 1). After 5 days the dog condition did not improve, a CBC was done (Table 1, day 27) and piroplasms were detected again on blood smear evaluation. The dog was treated with azithromycin with partial clinical improvement. After atovaquone was added (Birkenheuer et al., 2004), piroplasms were not observed on blood smear and the dog's clinical condition markedly improved (Table 1, day 43) although blood PCR remained positive during all the follow-up period. Unexpectedly, although dog showed clinical improvement, the owner elected euthanasia.

This manuscript reports the first description of *B. gibsoni* infection with molecular detection and characterization from a dog living in Italy. A dog from the South of Italy was previously diagnosed with *B. gibsoni* by only blood smear examination but molecular characterization was not performed (Casapulla et al., 1998). In Europe, cases of *B. gibsoni* infection have been recently described with molecular detection and identification of parasites: two dogs in Germany (Hartelt et al., 2007) and four dogs in Spain (Criado-Fornelio et al., 2003; Tabar et al., 2009). Limited clinical data is available from previous cases reports (Criado-Fornelio et al., 2003; Hartelt et al., 2007; Tabar et al., 2009). Recently, six dogs from Croatia were diagnosed with *B. gibsoni* subclinical infection (Beck et al., 2009). Interestingly, the majority of the dogs previously reported in Europe were American Pit Bull Terriers as the dog described in the present clinical case.

Table 1
Serial CBC including blood smear evaluation, biochemical profile, PCR-RFLP results and treatments instituted to the dog with babesiosis due to *B. gibsoni* infection.

Parameters (reference intervals)	Day 0 (28/12/05)	Day 10 (07/01/06)[a]	Day 19 (16/01/06)	Day 22 (19/01/06)	Day 27 (24/01/06)	Day 30 (27/01/06)	Day 36 (03/02/06)	Day 43 (11/02/06)
RBC (5.5–8.5 × 10^6/μL)	3.18	3.29	0.8	1.81	1.01	1.84	2.58	3.17
Hemoglobin (12.0–19.5 g/dL)	8.23	8.62	3.18	5.0	3.32	5.32	6.28	7.53
Hct (37.0–54.5%)	24.0	26.0	9.4	16.0	10.6	17.6	20.4	23.1
MCV (62–74 fL)	75	79	117	88	105	96	79	73
MCH (22–27 pg)	26	26	40	28	33	29	24	24
MCHC (32–36%)	34	33	34	31	31	30	31	33
RDW (16–24%)	22.9	21.4	15.7	48	20.0		29.2	40.4
NRBC/100 WBC	5	10	18	30	15	20	2	0
Reticulocytes (0.1–2.0%)			38.0	38	40	30	12	7
Absolute reticulocytes/μL			304,000	687,800	404,000	552,000	309,600	221,900
CRP (0–1.5%)			7.92	13.51	9.42	11.73	5.44	3.59
IR (0–1)			2.85	5.51	3.46	4.95	2.44	1.71
WBC (5.8–14.0 × 10^3/μL)	37.0	32.7	14.3	19.8	18.9	12.5	11.0	9.38
Segmented neutrophils (3800–8800/μL)	28,490	23,544	8866	14,652	13,230	8500	6820	5815
Bands neutrophils (0–300/μL)	740	0	572	396	0	0	0	0
Lymphocytes (1300–4100/μL)	3700	4578	2002	3168	3402	1750	2200	2063
Monocytes (200–800/μL)	3700	2616	2860	1584	1890	2250	1540	750
Eosinophils (150–1100/μL)	370	1962	0	0	378	0	440	750
Platelets (120–450 × 10^3/μL)	129	40	12	56	90	189	1525	1009
Presence of piroplasms on blood smear	No	No	Yes	Yes	Yes	Yes	No	No
Biochemical abnormalities[b]	↑ ALP (651 U/L), hyperproteinemia (8.8 g/dL), hypoalbuminemia (2.3 g/dL) and hyperglobulinemia (6.5 g/dL), ↑ α1-globulins (4.29 g/dL), ↑ γ-globulins (33.67 g/dL)	NP	↑ CK (292 U/L), AST (170 U/L), ALT (404 U/L), ALP (750 U/L), bilirubin (0.65 mg/dL), urea (75 mg/dL), iron (577 μg/dL), C-reactive protein (0.18 mg/dL)	NP	NP	NP	NP	NP
PCR-RFLP results	NP	NP	NP	POS	POS	POS	POS	POS
Treatment	C + E	C + E	C + E	I + D + P	D + P + AZ	D + P + AZ	AT + AZ	AT + AZ

[a] CBC performed after splenectomy.
[b] Biochemical parameters reference intervals: total protein (5.8–8.0 g/dL), albumin (2.6–3.8 g/dL), globulins (2.6–4.5 g/dL), α1-globulins (1.50–4.20 g/dL), γ-globulins (6.0–15.0 g/dL), creatinine phosphokinase (CK) (40–150 U/L), aspartate aminotransferase (AST) (15–40 U/L), alanine aminotransferase (ALT) (25–65 U/L) alkaline phosphatase (ALP) (20–130 U/L), bilirubin (0.15–0.28 mg/dL), urea (18–43 mg/dL), iron (81–220 μg/dL), and C-reactive protein (0.0–0.15 mg/dL).
NP = not performed, POS = positive. Treatment: C: cephalexin (10 mg/kg, BID *per os*), E: enrofloxacin (5 mg/kg, SID *per os*), I: imidocarb dipropionate (5 mg/kg, sc), D: doxycycline (10 mg/kg, SID, *per os* for 14 days), P: prednisone (2 mg/kg, SID, *per os* for 7 days), AT: atovaquone (13.3 mg/kg, TID, *per os*), AZ: azithromycin (10 mg/kg, SID *per os*).

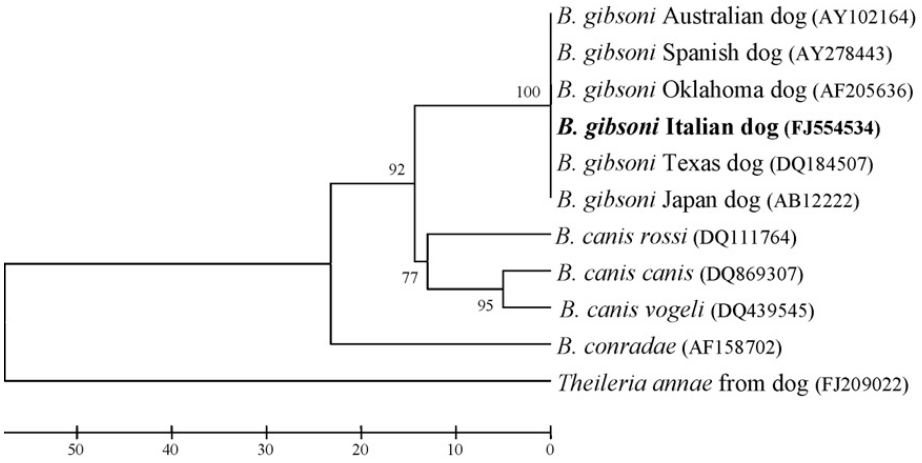

Fig. 2. Phylogenetic analysis based on the partial 18S rRNA gene sequences of several canine *Babesia* species and of *Babesia gibsoni* sequence from Italy (GenBank accession number in brackets). The phylogenetic tree was constructed by the Neighbor-Joining method, with nucleotide number of differences and bootstrap analysis with 1000 replicates. Numbers on branches indicate support for each clade ≥70%. The tree was rooted using *Theileria annae* from dog as outgroup.

B. gibsoni infection in dogs is endemic in Asia (Inokuma et al., 2004) and it is supposed to be tick-transmitted by *Haemaphysalis bispinosa* and *Rhipicephalus sanguineus* (Taboada and Lobetti, 2006). However, definitive proof identifying the vectors in this infection is lacking (Taboada and Lobetti, 2006). It is assumed that the initial reports of *B. gibsoni* infection in countries outside Asia (Australia and USA) were the result of its introduction by subclinical carrier dogs travelling from endemic regions. Numerous cases have reported in fighting dogs breeds such as the American Pit Bull Terrier and American Staffordshire Terrier in the Southeastern and the Midwestern regions of the USA (Meinkoth et al., 2002; Birkenheuer et al., 2005) and in Australia (Jefferies et al., 2007a) similar to the breed reported in the present case. The higher prevalence of *B. gibsoni* infection among these particular breeds suggested breed susceptibility and environmental factors that lead to high exposure to vector ticks (Taboada and Lobetti, 2006). In addition, blood exchange during fighting may play a role in transmission through bite wounds, saliva or ingested blood (Birkenheuer et al., 2005; Jefferies et al., 2007a). However, other non-tick modes of *B. gibsoni* transmission have been suggested. Transplacental transmission of *B. gibsoni* is thought to occur as demonstrated in experimental conditions (Fukumoto et al., 2005). In addition, transmission of *Babesia* spp. by blood transfusion is well documented in dogs (Stegeman et al., 2003). Due to the fact that the present case was an American Pit Bull Terrier descendant of an American-born bitch, vertical transmission is likely in the absence of history of fights and blood transfusion in a dog living in Italy. However, tick or dog-to-dog transmission cannot be ruled out in the present clinical case and the definite source of infection remains unclear.

The clinicopathological findings observed in this case included severe regenerative hemolytic anemia, lethargy, anorexia, marked splenomegaly and thrombocytopenia as previously described (Taboada and Lobetti, 2006; Jefferies et al., 2007b). The dog was splenectomized, and after surgery the clinical condition of dog deteriorated. It has been documented that spleen has an important function in controlling babesiosis. Splenectomized dogs that were experimentally infected with *B. canis* rapidly developed parasitemia and clinical disease and reached high parasitemia levels (Vercammen et al., 1995) and similar findings are also observed in dogs infected with *B. gibsoni* (Wozniak et al., 1997). Splenectomy has also been documented to be associated with clinical natural canine babesiosis caused by *B. microti*-like piroplasm (Camacho et al., 2002) and *B. c. vogeli* (Solano-Gallego et al., 2008).

In the present case, the therapy instituted with common antibabesial drugs was unsuccessful and piroplasms were observed on the blood smear evaluation during and after treatment. Only when atovaquone and azithromycin were administered, the dog's clinical condition improved and piroplasms were not seen on blood smear, although PCR remained always positive. Some antibabesial drugs such as a combination of atovaquone and azithromycin can reduce the severity of clinical signs, the number of circulating parasites and the mortality associated with natural and experimental disease but *B. gibsoni* infection is commonly not completely eliminated (Birkenheuer et al., 2004; Jefferies et al., 2007b).

In conclusion, we reported a clinical case of *B. gibsoni* infection in a dog from Italy. On the basis of this clinical case and recent reports in Europe (Criado-Fornelio et al., 2003; Hartelt et al., 2007; Tabar et al., 2009), infection by *B. gibsoni* should be included in the differential diagnosis list of lethargy, anorexia, weight loss, weakness, splenomegaly, hemolytic anemia and thrombocytopenia. In addition, this infection should be highly suspected if the patient is an American Pit Bull Terrier or a related dog breed. Accurate molecular detection and species identification are important for the selection of correct therapy, for predicting the course of disease in dogs with babesiosis and for the evaluation of subclinical infections and blood donor status.

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