Tropical Biomedicine 36(1): 304–309 (2019)

Short Communication

Molecular detection of *Babesia microti* in dogs and cat blood samples collected from Punjab (Pakistan)

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Received 10 December 2017; received in revised form 28 July 2018; accepted 31 October 2018

Abstract. *Babesia microti* (*B. microti*) is an intra erythrocytic protozoan that mainly infects red blood cells and causes babesiosis. Its frequent hosts are rodents, ticks and humans. Dog's blood samples (N = 150) were collected from three different districts in Punjab including Multan (N = 49), Islamabad/Rawalpindi (N = 49) and Lahore (N = 52) while 159 cat's blood samples were collected from Lahore (N = 159). Data on the epidemiological characters of all animals (including age, gender, breed, body temperature, deworming, vaccination, mucus membrane status, hydration status, presence of hematuria and tick infestation) was collected through questionnaire. Polymerase chain reaction (PCR) amplified a 238 base pair amplicon specific for 18S rRNA gene of *B. microti* in two (1.3%) dog and 21 (13.2%) cat blood samples. Amplified PCR products were confirmed by DNA sequencing and the four partial 18S rRNA gene sequences were submitted to the EMBL/GenBank. Among epidemiological factors, high body temperature ($P < 0.05$) and pale mucous membrane ($P < 0.05$) were the parameters associated with the presence of *B. microti* in infected dogs. Females were found to be more infected ($P = 0.05$) than males and incidence of *B. microti* infection was higher in cat samples collected during winter months ($P = 0.0001$) than in summer. In conclusion, we are reporting the prevalence of *B. microti* in blood samples of cats and dogs from Pakistan for the first time and recommending that this Protozoan parasite should be considered for screening in cats and dogs with compatible clinical signs.

INTRODUCTION

Cats and dogs are the most common pet animal throughout the world and their population vary from 8 to 11.6 million (Asher *et al.*, 2011). Due to affiliation between them and human, they have more exposure towards environment and they are reported to be infected with variety of pathogens including vector borne pathogens that are the main cause of mortality and morbidity in pets throughout the world (Torres and Oranto, 2014). Canines are reported to suffer from tick transmitted *Babesia* infection (canine babesiosis) that targets the red blood cells of the host. *Babesia vogeli*, *Babesia canis* and *Babesia gibsoni* are the three major species, which exhibit clinical signs and symptom in them. The severity of illness varies considerably depending on the species of *Babesia* involved, as well as the immune response of the infected animal (Zhou *et al.*, 2013). While *Babesia microti* causes human babesiosis and infection due to this intraerythrocytic apicomplexan piroplasm have been reported from United States where vector of this disease i.e *Ixodes scapularis* is most prevalent, while few cases have been

reported from Asia and Europe and *Ixodes ricinus* has been reported as the vector for babesiosis in Europe (Simoes *et al*., 2011). At the beginning of this century, a small piroplasmid was initially reported in a sick dog from Spain and shown to be the most closely related with *Babesia microti* by phylogenetic analysis and it was first referred to as *Babesia microti*-like species (Baneth *et al*., 2015). Shortly after its description, this pathogen was shown to cause severe disease with anemia, thrombocytopenia, and azotemia in 157 dogs from the Galicia region in northwestern Spain (Camacho *et al*., 2001) and has since then been identified as a cause of infection and/or disease in dogs in other areas of northern Spain, Portugal, Croatia, Sweden, and USA (Beck *et al*., 2009; Simoes *et al*., 2011). Recently Persichetti *et al*. (2016) has reported the presence of *B. microti* in the blood samples of cats, the unconventional host for this pathogen, collected from Italy. In Pakistan, to our knowledge there is no information available regarding the prevalence of *B. microti* in dogs and cats. All these reports lead us to design the present study during which we have used *B. microti* specific primers and used polymerase chain reaction (PCR) technique for the detection of *B. microti* in blood samples of dogs and cats collected from Lahore, Rawalpindi/ Islamabad and Multan districts of Punjab province in Pakistan.

MATERIALS AND METHODS

Sample and Data Collection

Blood samples were collected from apparently healthy or diseased client-owned dogs (N = 150) and cats (N = 159), presented to the Veterinary Clinics in three cities, Lahore (N = 52); Rawalpindi/Islamabad (N = 49) and Multan (N = 49), of Punjab Province while cat blood samples were exclusively collected from Veterinary Clinic of the University of Veterinary and Animal Sciences (UVAS) in Lahore, Pakistan. All the subjects were physically examined by the professional veterinarians and a questionnaire was filled by them at the spot in order to gather data for the epidemiological study of risk factors associated with babesiosis. None of the enrolled dogs and cats was diagnosed with babesiosis. Blood samples were collected from the jugular vein of the animals and immediately preserved in EDTA quoted tubes.The body of each enrolled subject, with special attention to the ears, was examined for the presence of ticks. If present, they were removed with forceps and placed in bottles with moistened cotton wool and transferred to the laboratory for identification by using taxonomic keys. All the animal handling procedures and lab protocols were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan (Application number ETH/Zool/2014/Para76-2).

DNA Extraction

An inorganic method of DNA extraction was used as previously described elsewhere (Saeed *et al*., 2015).

PCR amplification

A set of oligonucleotide primers, Forward Bab1: 5´-CTTAGTATAAGCTTTTATACAGC-3´ and Reverse Bab4: 5´-ATAGGTCAGAAAC TTGAATGATACA-3´ was used to amplify 18S rRNA gene sequences of *B. microti* producing a 238 bp fragment as previously reported by Persing *et al*. (1992). An ultimate reaction volume of 50 ul was prepared containing 200 mM deoxynucleotide triphosphate (dNTPs), 50 mM Tris (pH 8.3), 50 pmol of each primer, 1.5 mM of divalent cation $MgCl_2$, 5µl of DNA template, 0.25 U of replication starting enzyme, Taq DNA polymerase (Vivantis, UK). *B. microti* negative and positive samples were also amplified as negative and positive controls during PCR amplification.

Amplification of DNA was carried out by a DNA thermal cycler (MultiGene OptiMax PCR system 1402015). For the present study, a thermo profile as employed Persing *et al*. (1992) was followed; *B. microti* amplification was accomplished by an overall denaturation for 5 min at 94°C accompanied by 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 2 min at 72°C and overall

extension was achieved at 72°C for 7 min. PCR products were held at 4°C until separated by electrophoresis on a 2% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

DNA Sequencing

To confirm PCR results, both positive PCR products of dog and five randomly selected PCR products of cat were sent for DNA sequencing. The generated DNA fragments were purified with a PCR purification kit (GF-1 96-well PCR Clean-up kit, Vivantis, UK) and sent to a commercial laboratory (Macrogen, Korea) for sequencing. DNA sequences obtained were evaluated with Chromas Lite software, version 2.1.1 (Technelysium Pty Ltd., Australia) and compared for similarity to registered sequences in GeneBank (http://blast.ncbi.nml.nih.gov/).

Statistical Analysis

All the data was shown as Mean ± Standard Error. Results were statistically analyzed by Statistical package Minitab (version 16). Significance level was set at $P < 0.05$. Comparison of parasite prevalence among various sampling sites and between various cats and dog breeds was made by using one way analysis of variance (ANOVA). Various studied epidemiological factors were correlated with the presence of *B. microti* through the Fischer's exact test.

RESULTS AND DISCUSSION

Analysis of the results revealed that polymerase chain reaction (PCR) had amplified a 238 base pair amplicon specific for 18S rRNA gene of *B. microti* in two (1.3%) out of 150 dog blood samples collected during the present study, one from Islamabad/Rawalpindi (N = 1; 2%), one from Lahore (N = 1; 1.9%). None of the blood samples collected from Multan was found positive for *Babesia microti*. Results of one way ANOVA test revealed that prevalence of parasite varied non-significantly when compared between the three sampling sites ($P = 0.6$). Partial 18S rRNA gene sequences of both positive samples of *B. microti* were submitted to the EMBL/GenBank database under the Accession numbers KY709689 (dog from Lahore district) and KY709690 (dog from Rawalpindi district). A BLAST search indicated that the sequences were 99-100% identical to the *B. microti* isolates registered in GenBank.

In case of cats, analysis of the results revealed that polymerase chain reaction (PCR) had amplified a 238 base pair amplicon specific for 18S rRNA gene of *B. microti* in 21 out of 159 (13.2%) cat blood samples collected during present study. A BLAST search indicated that the sequences were 99-100% identical to the *B. microti* isolates registered in GenBank. Two amplified partial 18S rRNA gene sequences of *B. microti* from analyzed cats were submitted to the EMBL/GenBank database under accession numbers MF401440 and MF 401441.

Analysis of data revealed that both dogs positive for *B. microti* were German shepherd and 7.7% of them were parasite positive while *B. microti* was not detected in the blood of other 13 dog breeds (Boxer, Labrador, stray, cross, Bully, Pug, German Spitz, Russian, Shih Tzu, Pointer, Shiba Inu, Rottweiler and Sptzier) included in the present study. Statistical analysis of the data indicated that parasite prevalence was not limited to a particular breed ($P = 0.7$). Analysis of results indicated that body temperature ($P < 0.05$) and appearance of mucous membrane ($P < 0.05$) were the only parameters, which were found associated with the presence of *B. microti* in dogs. Both the infected dogs were suffering from fever and had pale mucous membrane. All dogs were examined for the presence of *Ixodid* ticks. Ten of 150 dogs (6.67%) were infested with adult and nymphal *Rhipicephalus sanguineus*. Forty-eight ticks (29 nymphs, 19 adults) were removed from dogs. All other studied parameters varied non-significantly ($P > 0.05$) when compared between parasite positive and negative animals (Table 1).

Our results revealed that *B. microti* was detected in three cat breeds. Maximum parasite prevalence was observed in stray cats (28%) followed by cross (19%) and pure

Table 1. Association of presence of *Babesia microti* with the studied parameters describing animal characters collected during the present study from three sampling sites. N represents total number of samples. Prevalence (%) of *Babesia microti* is given in parenthesis. P-value indicates the results of Fischer exact test for each parameter

Parameters	N	*Babesia microti* positive dog samples	P - value	N	*Babesia microti* positive cat samples	P - value
Gender						
Male	82	0 (0%)	0.2	66	05 (8%)	0.05*
Female	68	2 (2.9%)		77	15 (19%)	
Age						
>1 year	38	1 (2.63%)	0.99	103	13 (13%)	0.4
<1 year	112	1 (0.89%)		43	08 (19%)	
Body Temperature						
Normal	115	0 (0%)	0.05*	128	18 (14%)	0.8
Fever	35	2 (5.7%)		31	03 (10%)	
Mucous Membrane						
Normal	122	0 (0%)	0.03*	146	18 (12%)	0.4
Pale	28	2(7.1%)		13	03 (23%)	
Hematuria						
Present	24	1 (4%)	0.3	01	00 (0%)	1
Absent	125	1 (0.8%)		158	21 (13%)	
Vomiting						
Present	39	1 (2.5%)	0.5	19	01 (5%)	0.5
Absent	111	1 (0.9%)		140	20 (14%)	
Vaccinated						
Yes	93	2 (2%)	0.5	73	13 (18%)	0.2
No	56	0 (0%)		86	08 (9%)	
Dewormed						
Yes	57	1 (1.75%)	1	67	12 (18%)	0.2
No	93	1 (1.08%)		92	09 (10%)	
Tick Infestation						
Present	10	0 (0%)	1	21	03 (14%)	1
Absent	140	2 (1.4%)		138	18 (13%)	
Season						
Winter	–	–	–	59	16 (27%)	0.0001***
Summer				98	05 (5%)	

P > 0.05 = Non-significant, P < 0.05 = Least significant (*), P < 0.001 = Highly significant (***)

breed cat (5%). *B. microti* was not detected in the blood of other three cat breeds (Persian, Siames and local) included in the present study (Data not shown here). Statistical analysis of the data indicated that parasite prevalence was not limited to a particular breed (P = 0.18) during present study.

Analysis of results indicated that gender (P = 0.05) and season (P = 0.0001) were associated with the presence of *B. microti* in cats (Table 1). It was observed that *B. microti* infection was more frequent in female cats than male and also cat samples collected in winter had higher prevalence of *B. microti* than those collected in summer months.

Since year 2000, there are a number of reports that dogs are infected with *B. microti* like pathogens (Beck *et al.*, 2009; Baneth *et al.*, 2015; Camacho *et al.*, 2001; Persichetti

et al., 2016; Simoes *et al*., 2011). To our knowledge, no prior report is available in literature regarding the molecular detection of *B. microti* in blood samples of dogs from Pakistan. Dogs are the accidental host of *B. microti* as reported by Spada *et al*. (2014). *B. microti* usually parasitizes rodents and human so parasite may have been shifted from rodents to dogs and cats through tick bite as Lampereur *et al*. (2011), Potkonjak *et al*. (2015) and Stensvold *et al*. (2015) recovered *B. microti* infected ticks from dogs. Only one previous report is available regarding the presence of *B. microti* in dogs by Gabrielli *et al*. (2015) who analyzed 158 dogs blood sample from Southern Serbia i.e Prokuplje and Nis and Northern Serbia i.e Durdevo and Pancevo and overall prevalence of *B. microti* was 1.9% detected through PCR. As cats are unconventional hosts for this parasite, so limited information is available in literature regarding their prevalence in cats. Recently, Persichetti *et al*. (2016) had analyzed 42 cats blood samples from Southern Italy and found 10 of them positive for *B. microti* by seroprevalence and overall prevalence was 23.8%. They used real time PCR to confirm the seroprevalence but were unable to confirm the presence of *B. microti* in cat or in their vectors. Contrary to their results, we have reported comparatively higher prevalence of *B. microti* in cat blood samples from Pakistan and amplified products from 18S rRNA gene were confirmed by DNA sequencing.

Statistical analysis revealed that prevalence of *B. microti* varied non significantly when compared between three sampling sites (P = 0.6) (Table 1). These results are in contrast with Gabrielli *et al*. (2015) who have reported a significant variation in prevalence of *B. microti* among different sampling sites of Serbia. This difference in results could be explained on the basis of fact that the number of animals collected during present study was low and hence prevalence of *B. microti* in sampled dog population from Punjab was very low without any significant association with sampling sites. Analysis of risk factors indicated that none of the gender and age group was significantly associated with the presence of *B. microti* in case of dogs (Table 1). Similar observations were reported by Gabrielli *et al*. (2015). Both *B. microti* infected dogs were suffering from fever (P = 0.05) and had pale mucous membrane (P = 0.03). According to Acosta *et al*. (2013) and Gabrielli *et al*. (2015), fever is the main symptom of infection with *B. microti*. So, these phenotypes are in agreement with the molecular detection of parasite in dogs.

In conclusion we have used a previously reported molecular protocol for the amplification of 18S rRNA gene of *B. microti* in the blood of dogs and cats. For the first time from Pakistan, we are reporting, the presence of *B. microti* in dogs and cats and recommending that this agent should be considered in dogs and cats from Pakistan presented with compatible clinical signs.

Conflict of interest

Authors declare no conflict of interest of any sort with anyone.

Author's contributions

FI designed the study and revised the manuscript, AKM and AA collected the samples, INA and TP conducted the lab experiments, analyzed the data and prepared the manuscript.

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