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Babesia infection in naturally exposed pet dogs from a north-eastern state (Assam) of India: detection by microscopy and polymerase chain reaction

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Abstract The objective of the study was to detect *Babesia* infections in pet dogs of a north-eastern state of India. The diagnostic efficacy of *Babesia* infection by polymerase chain reaction (PCR) technique has been compared with micros-copy examination. For this, a total of 111 blood samples of pet dogs presented at clinical complex of the College of Veteri-nary Science, Guwahati, Assam with clinical signs suspected for *Babesia* infection subjected to the study. A total of 44 (39.63 %) dogs were diagnosed as positive for *Babesia* infections after microscopic examination. Among these, *Babesia canis* infection was diagnosed in 5 dogs (4.50 %) and *B. gibsoni* infection in 39 (35.13 %) dogs microscopically in Giemsa stained blood smears. Molecular diagnosis using PCR detected 63 (56.75 %) dogs positive for *Babesia* infection. Single infection with *B. canis* was found in 9 (8.10 %) dogs while *B. gibsoni* alone was detected in 3 (2.70 %) dogs. Mixed infections by both these species were detected in 51 (45.94 %) dogs. Overall, PCR detected 54 (48.64 %) dogs as *B. gibsoni* and 60 (54.05 %) dogs as *B. canis* positive.

Keywords *Babesia canis* · *Babesia gibsoni* · Detection · Dog · Microscopy · PCR · India

Introduction

Canine babesiosis is an emerging tick-borne life threaten-ing disease caused by the intra-erythrocytic protozoan parasites under the genus *Babesia* in many parts of the world including India. There are two species of *Babesia* viz; *Babesia canis* and *B. gibsoni* which are morphologi-cally differentiated on the basis of their size. *B. canis rossi*, *B. canis canis* and *B. canis vogeli* have been recognized so far as subspecies of *B. canis* on the basis of geographical distribution of vector tick, differences in pathological and clinical syndrome, antigenic property and molecular analysis (Boozer and Macintire 2003). Similarly recent molecular analyses have revealed three morphologically similar but genotypically distinct small *Babesia* of which *B. gibsoni* Asia type is endemic to Asia, North America, North and East Africa (Zahler et al. 2000). The identifi-cation of the causal agents involved in cases of canine babesiosis in a particular locality is important to define treatment planning for a successful outcome (Boozer and Macintire 2003). In India, the situation of canine babesiosis is not clear (Megat Abd Rani et al. 2010) except few reports (Sundar et al. 2004; Chaudhuri 2006; Chaudhuri and Varshney 2007; Senthil Kumar et al. 2009; Bala-chandran et al. 2010; Karunakaran et al. 2011) that are also based on conventional method of diagnosis and moreover very little study has been done in the North-Eastern region of India. The present communication reports the results of detection of *Babesia* infection in dogs of Assam, a north-eastern state of India, through conventional and molecular methods.

Materials and methods

Animals and sample collection

A total of 111 randomly selected pet dogs of either sex, different ages and breeds presented at the teaching veterinary

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clinical complex of the College of Veterinary Science, Gu-wahati, Assam during the year 2010–2011 and suspected for haemoparasite infection on the basis of clinical findings (depression, inappetance, lethargy, fever, abnormal colora-tion of urine and stool, anaemia) and history of tick exposure were included in the study. Blood samples were collected from all these dogs in vials containing EDTA (Ethylene diamine tetraacetic acid) as anticoagulant. A drop from each of well mixed fresh blood sample was used to prepare blood smear and rest was stored at −20 °C for extraction of DNA.

Diagnosis

Conventional method

Giemsa stained blood smears were examined under microscope for the presence of intra-erythrocytic *Babesia* organisms. In positive cases, species differentiation into *B. canis* and *B. gibsoni* was done on the basis of their size (large or small) and appearance inside the red blood cells (Soulsby 1982). Failure to detect parasite in a smear after evaluating at least 500 oil immersion fields in 20–30 min was declared as microscopically negative blood sample.

Molecular method

For diagnosis of infection by molecular technique, poly-merase chain reaction (PCR) method was used. DNA was extracted from all blood samples using DNA extraction kit (Genei^TM Blood Genomic DNA purification kit, Genei Bangalore, India) as per manufacturer's instruction. PCR of all blood samples was done using *B.canis* and *B.gibsoni* specific primers as presented in Table 1.

For molecular diagnosis of *B. canis*, PCR was set up in 50 μl reaction mixture consisting 25 μl of 2× PCR Master Mix (Promega) containing 50U/ml *Taq* Polymerase, 400 μM each dATP, dGTP, dCTP, dTTP and 3 mM Mgcl$_2$, 25 pmol each (0.25 μl) of forward primer and reverse primer (GCC), 2 μl of Template DNA and 22.5 μl of autoclaved triple-distilled water. Amplification was per-formed in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystem) with cyclic conditions comprising

initial denaturation at 94 °C for 10 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s followed by final extension at 72 °C for 5 min (Foldvari et al. 2005).

In the case of molecular diagnosis of *B. gibsoni*, PCR was set up in 25 μl reaction mixture which consisted 12.5 μl of 2× PCR Master Mix, 12.5 pmol each (0.125 μl) of forward and reverse primer, 5 μl of Template DNA and 7.25 μl of autoclaved triple-distilled water. Amplification was performed with cyclic conditions comprising initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s followed by final extension at 72 °C for 5 min (Inokuma et al. 2004).

Electrophoresis of amplified DNA was done in 1.5 % agarose gel using 100 bp DNA marker for 2 h at 80 V using Tris Acetate EDTA (TAE) running buffer. The gels were stained with ethidium bromide (0. 5 μg/ml) and viewed in UV transilluminator for the expected size product.

For all amplifications, known positive and negative samples were used as controls.

Results

The results of blood examination through microscopy and PCR analysis are summarized in Table 2.

Microscopic examination of blood revealed 5 (4.50 %) and 39 (35.13 %) dogs positive to *B. canis* (Fig. 1) and *B. gibsoni* (Fig. 2) respectively, with a total 39.63 % *Babe-sia* positive. *Rhipicephalus sanguineus* ticks were identified from the body of the infected dogs. Molecular diagnosis using PCR detected 63 (56.75 %) dogs as positive to *B. canis* and *B. gibsoni* infections. Single infection with *B. canis* was found in 9 (8.10 %) dogs while single infection with *B. gibsoni* was detected in 3 (2. 70 %) dogs. Mixed infections by both these species were detected in 51 (45.94 %) dogs. In case of *B. canis* an expected PCR product of 450 bp band size (Fig. 3) and in case of *B. gibsoni* an expected PCR product of 671 bp band size (Fig. 4) were observed. Overall, PCR detected 54 (48.64 %) dogs as *B. gibsoni* and 60 (54.05 %) dogs as *B. canis* positive.

Table 1 Primer sets used for molecular diagnosis of *B. canis* and *B. gibsoni* infections using PCR

Primer name	Primer sequence	Target parasites	Expected amplicon size	Reference
PIRO-A1	F5′AGGGAGCCTGAGAGACGGCTACC3′	*B. canis*	450 bp	Foldvari et al. (2005)
PIRO-B	R5′TTAAATACGAATGCCCCCAAC3′			
Gib 599	F5′CTCGGCTACTTGCCTTGTC3′	*B. gibsoni*	671 bp	Inokuma et al. (2004)
Gib 1270	R5′GCCGAAACTGAAATAACGGC3′			

Table 2 Results of examination of Giemsa stained blood smears and molecular diagnosis using PCR for detection of canine Babesiosis

Total no. of samples tested	Nos. positive after microscopical examination			Nos. positive after molecular diagnosis using PCR					
	B. gibsoni	B. canis	Total	B. gibsoni (single)	B. canis (single)	Mixed	Total	Overall B. gibsoni	Overall B. canis
111	39 (35.13 %)	5 (4.50 %)	44 (39.63 %)	3 (2.70 %)	9 (8.10 %)	51 (45.94 %)	63 (56.75 %)	54 (48.64 %)	60 (54.05 %)

Discussion

In the present study a high percentage (39.63 %) of clinically ill dogs was found positive for *Babesia* infection after microscopical examination. Species-wise, the percentage of infection with *B. gibsoni* was more (35. 13 %) than that of *B. canis* (4.50 %). The prevalence of canine babesiosis in Assam has been reported as 21.7 % by Chaudhuri and Varshney (2007) without mentioning the species. The incidence reported from Northern part of India varied between 0.66 and 8.9 % (Varshney and Dey 1998; Chaudhuri 2006) while from Southern India Senthil Kumar et al. (2009) recorded 11.6 % prevalence of haemoprotozoa and among haemoprotozoa *B. canis* and *B. gibsoni* were recorded 3.9 % and 84.9 %, respectively. Wide variation in climatic conditions prevailing in different parts of India might be responsible for varying percentage of these tick borne infections.

On the basis of present findings microscopy could detect 39.63 % cases as *Babesia* positive against 56.75 % detection by PCR. So 17.12 % cases remained false negative to *Babesia* during microscopy. Higher detection of *Babesia* in PCR compared to microscopy as observed in the present study was also reported by several authors (Birkenheuer et al. 2003; Gotsch et al. 2009; O'Dwyer et al. 2009). The prevalence pattern of this two species recorded in the present study was found similar to those microscopic and serologic findings reported by other workers (Bansal et al. 1985; Varshney et al. 2003; Senthil Kumar et al. 2009).

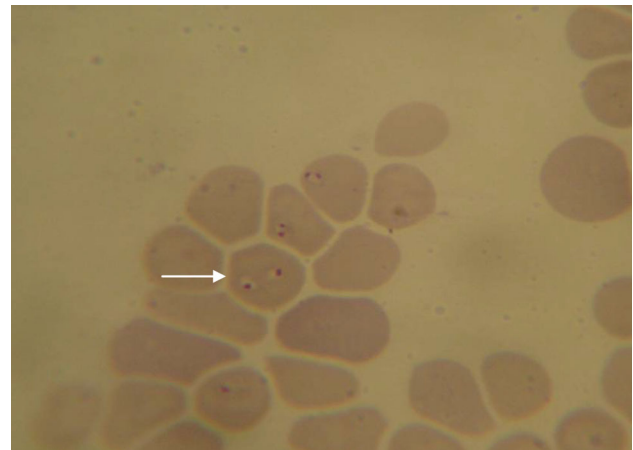**Fig. 1** *B. canis* within RBC in Giemsa stained blood smear of a dog (×1,000)

Fig. 2 *B. gibsoni* within RBC in Giemsa stained blood smear of a dog (×1,000)
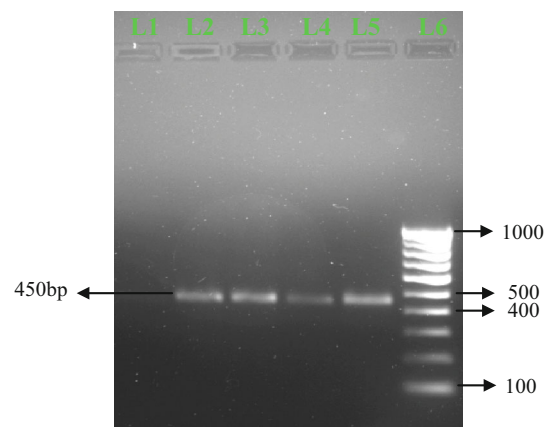

Fig. 3 Electrophoresis gel (1.5 % agarose, stained with ethidium bromide), showing lanes from left to right: *L1*, Negative Control; *L2* to *L5*, PCR product showing positive for *B. canis* (450 bp); L6, 100 bp DNA ladder

PCR analysis of the present study was found more sensitive than microscopy. This is in agreement with Matsuu et al. (2005) who reported detection limit of light microscopy as approximately 0.001 % parasitaemia compared to PCR capable of detecting 9 parasites per µl of blood similar to 50 organisms/ml of blood reported by Birkenheuer et al. (2003).

Microscopic detection of *B. gibsoni*, though smaller in size than *B. canis*, was easier due to its frequent appearance
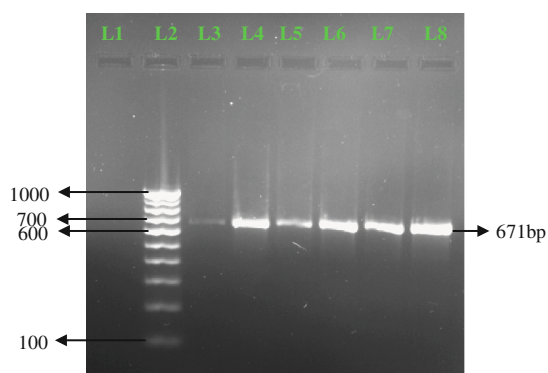
Fig. 4 Electrophoresis gel (1.5 % agarose, stained with ethidium bromide), showing lanes from left to right : L1, Negative Control; L2, 100 bp DNA ladder; *L3* to *L8*, PCR product showing positive for *B. gibsoni* (671 bp)

in circulating blood as evidenced by 35.13 % positivity against 48.64 % molecular detection, thus raising its false negative as 13.51 % by microscopy, although they showed clinical manifestations. This might be due to parasitaemia beyond the level of microscopic detection during very early or carrier stage (Birkenheuer et al. 2003; Bourdoiseau 2006; Irwin 2009). However in the present study, it was a difficult task to find *B. canis* during microscopy which showed only 4.50 % cases against 54.05 % positivity in PCR; thus raising its false negativity to 49.55 % by microscopy. Very less detection of *B.canis* similar to the present finding was also reported by Senthil Kumar et al. (2009) who recorded only 3.9 % *B.canis* against 84.9 % occurrence of *B. gibsoni* in haemoparasite positive dogs. Higher percentage of false negativity in the case of *B. canis* might be due to its transient appearance in circulating blood during febrile stage of acute infection only as reported by Bourdoiseau (2006) who failed to observe the parasite during chronic infection. Schalm et al. (1975) also reported demonstration of *B. canis* under microscope as a fortunate occurrence. Baneth et al. (1998) failed to diagnose *B. canis* in blood smears although 90 % dogs remained sero-reactive in subclinical state of infection. False negativity in the present microscopic study was found more with *B. canis* than in *B. gibsoni*. Scrutiny of the results of microscopy and PCR analysis of individual blood sample in the present study revealed that out of 39, 5 microscopic positive *B. gibsoni* cases remained negative in PCR and the false positivity was 12.82 %.

Confirming the vector specific geographic distribution (Uilenberg et al. 1989; Zahler et al. 1998) of the subspecies of *B. canis*, several workers reported significant association of *Dermacentor reticulatus* with *B. canis canis* prevalent in Europe (Caccio et al. 2002; Porchet et al. 2007; Zygner et al. 2008; Welc-Faleciak et al. 2009) and *Rhipicephalus sanguineus* with *B. canis vogeli* in tropical and subtropical

countries (O'Dwyer et al. 2009; Gotsch et al. 2009). Using PIRO-A1 and PIRO-B primers Foldvari et al. (2005) reported *Babesia* DNA having 99.8 or 100 % similarity with *B. canis canis* in sequencing. On the contrary O'Dwyer et al. (2009) using the same sets of primers reported PCR products 100 % identical to *B. canis vogeli*. Although no attempt was made in the present study to characterize *B. canis* at the subspecies level, in view of detection of *R. sanguineus* ticks in the body of suspected dogs during the present investigation, it is suggested that *B. canis* might belong to *B. canis vogeli*. Megat Abd Rani et al. (2010) opined that *B. vogeli* and *B. gibsoni* were likely co-endemic in India. Our study also revealed that a large number of *Babesia* positive cases had co-infections with *B. gibsoni* and *B. canis*.

Although parasite detection by microscopy is a gold standard in terms of specificity (Ravindran et al. 2007), this method in the present study failed to detect *B. canis* in blood smear thus giving an incorrect diagnosis in maximum cases. Molecular analysis of dog's blood in the present study not only confirmed the existence of *B. canis* and *B. gibsoni* but also, conforming to the other reports (Birkenheuer et al. 2003; Garcia de Sa et al. 2006), proved to be superior to the blood smear evaluation, which did not provide definite diagnostic information in regards to *B. canis* infection. The results are in agreement with Martin et al. (2006) who opined it as a promising tool with 100 % specificity and sensitivity. On the basis of present PCR results, it could be mentioned that highest number (45.94 %) of positive cases having mixed infection of *B. canis* and *B. gibsoni*. This is important for the purpose of obtaining maximum response to therapy in clinical cases as observed by Shaw et al. (2001) and Walter et al. (2002) who reported drug regimen against the two species were different and treatment against one species might be unsuccessful due to usual presence of mixed infection. Molecular analysis in the present study provided powerful information over blood smears examination, which did not provide definite diagnostic information on *B. canis* compared to that of *B. gibsoni*. It could be presumed that large numbers of dogs of India might be suffering from canine babesiosis which remained undiagnosed or under reported and molecular diagnosis using PCR could unveil its actual prevalence.

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