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Diagnosis of Babesiosis by Conventional Microscopy and PCR

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A B S T R A C T

The present study was conducted in Veterinary Clinical Complex, College of Veterinary Science, Tirupati over a period of nine months to diagnose the *Babesia* infection. 235 dogs suspected for babesiosis were collected and subjected for diagnosis using conventional and molecular techniques. Out of 235 samples examined by conventional microscopy of Giemsa stained blood smears revealed eleven positive samples which includes *Babesia gibsoni* (10) and *Babesia canis* (1) whereas with polymerase chain reaction 20 samples showed amplicons of 671 bp and 3 samples showed amplicons of 450 bp indicative of *Babesia gibsoni* and *Babesia canis* respectively.

Introduction

Babesiosis is one of the important tick-borne haemoprotozoan disease caused by the species of genus *Babesia. Babesia* species belong to the phylum Apicomplexa, class Piroplasmea, order Piroplasmida and family Babesiidae. Canine babesiosis is a clinically significant and geographically wide spread disease of domesticated dogs and wild canids (Irwin 2009). Multiple species of *Babesia* have been documented to infect dogs with babesiosis. However, *Babesia canis* and

Babesia gibsoni are the only species found to be present in Asia. *Babesia canis* is a large form of piriform (tear drop shaped) organism with 2.5-5.0 μm in size, whereas small pleomorphic organisms of size 1.0-2.5 μm, which appears as oval or signet ring form are termed as *Babesia gibsoni* (Conrad *et al.,* 1991 and Solano-Gallego *et al.,* 2011). *Babesia spp.* are transmitted by variety of ticks including brown dog tick (*Rhipicephalus sanguineus*), and different members of *Dermacenter* genus. Transmission can also occur from dog to dog through bite (Jefferies

et al., 2007) and transplacental transmission (Fukumoto *et al.,* 2005). Clinically canine babesiosis has been found to result in a wide range of presentations from sub clinical to serious illness characterised by fever, depression, pallor, jaundice, lymphadenopathy, spleenomegaly, weakness and collapse associated with intravascular and extravascular haemolysis, hypoxic injury, systemic inflammation, thrombocytopenia. After initial acute infection, the animal may become a chronic carrier (Irwin, 2009). Therefore, the diagnosis of this disease and the detection of dogs that are carriers or that have a chronic form of this disease are very important.

Currently definitive means of diagnosis of canine babesiosis is based on demonstration of the parasite in Giemsa stained thin-film blood smears examined by microscopy. However, the detection of *Babesia* parasites is difficult in dogs with unapparent or chronic infections since the parasitemia is very low. Therefore the development of highly specific and sensitive methods is required for diagnosis of infection. It has become possible to detect infection with molecular techniques like PCR and supporting haematological and biochemical findings which can correlate the pathological process of the disease.

Along with these tests various serological methods such as complement fixation test (CFT), immunofluorescent antibody test and enzyme linked immunosorbent assay (ELISA) were used for diagnosis. polymerase chain reaction (PCR) assay present a higher sensitivity and specificity than the blood smear evaluation and may differentiate species that may not be morphologically distinguished by smear method (Boozer and Macintire, 2003). In the present study the diagnosis of babesiosis in dogs was done by examination of giemsa stained blood smear and PCR methods.

Materials and Methods

Blood samples

During the period under study (December 2018 to August 2019), a total number of 235 dogs exhibited clinical signs suggestive of haemoprotozoan infection such as pyrexia, pale mucous membranes, anorexia, lymphnode enlargement, weakness and lethargy were selected and screened for babesiosis. The dogs were subjected for routine clinical laboratory evaluation including peripheral blood smear examination and PCR. A thin blood smears from the suspected dogs were prepared and then fixed in methanol for 1 minute and stained with Giemsa (1:6) solution for 30 minutes as described by Fukumoto *et al.,* (2001). Then the slide was examined for *Babesia* organisms with light microscope under 100X magnification. Blood samples were taken into EDTA tubes and kept at -20°C for upto further confirmation by PCR assay.

DNA extraction and PCR assay

DNA was isolated from 200 µl amounts of EDTA blood from each dog using QIAamp DNA blood mini kit (QIAGEN, Hidden, Germany) according to the manufacturer's instructions. The primer sequence used for *Babesia canis* were forward primer PIROA (5'AGGGAGCCTGAGAGACGGCTACC 3') and reverse primer PIRO-B (5' TTAAATACGAATGCCCCCAAC 3') were used to amplify an approximately at 450 bp region of the 18S rRNA gene (Foldavari *et al.,* 2005) and for *Babesia gibsoni* the primer sequence was forward primer Gibb 599 (5' CTCGGCTACTTGCCTTGTC 3') and reverse primer (5' GCCGAAACTGA AATAACGGC 3') were used to amplify an approximately at 671 bp region of 18s rRNA gene (Inokuma *et al.,* 2004). The reaction mixture components i.e., nuclease free water,

DNA template, forward and reverse primers (10pM), and master mix and their quantities were standardized for 18S rRNA gene of *Babesia canis* and *Babesia gibsoni.* The amplification of DNA was carried out in thermal cycler (Proflex PCR, applied biosystems by life technologies, Singapore). For species specific DNA of *Babesia canis* the thermocycle profile consisted of initial denaturation at 94°C for ten minutes, followed by 40 cycles of denaturation at 94°C for thirty seconds, annealing at 60°C for thirty seconds, extension at 72°C for thirty seconds and this was followed by a final extension at 72°C for five minutes.

For species specific DNA of *Babesia gibsoni* the thermocycle profile consisted of initial denaturation at 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for thirty seconds, annealing at 55°C for thirty seconds, extension at 72°C for ninety seconds and this was followed by a final extension at 72°C for five minutes. Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel prestained with ethidium bromide and viewed under Gel doc (BioRad, Dell, USA). The size and specificity of PCR products were confirmed by comparing with the 100 bp DNA ladder. Negative control with distilled water was maintained in each PCR.

Results and Discussion

Out of 235 samples screened by peripheral blood smear examination of smears by Giemsa staining, 11 samples were found positive for *Babesia spp*. among these positive samples ten were found positive for *Babesia gibsoni* and one sample was found positive for *Babesia canis*. The identification was done based on the characteristic morphology of the organisms. *Babesia gibsoni* appeared as pleomorphic oval or signet ring (Fig. 1) while *Babesia canis*

appeared as piriform shaped (tear drop) either singlet or paired form (Fig.2). PCR showed greater sensitivity in diagnosing infection than microscopy 23 samples were found positive which includes eleven microscopically positive samples Out of these 23 positive samples, 20 samples showed amplicons of 671 bp and 3 showed amplicons of 450 bp which are suggestive of *Babesia gibsoni* and *Babesia canis,* respectively (Fig.3,4).

Giemsa stained blood smear examination findings in the present study corroborates with the results of Praveen *et al.,* (2015), Vipan *et al.,* (2015) and Patra *et al.,* (2018). In the present investigation *Babesia gibsoni* was the predominant species in this region which was in agreement with the earlier observations of Das *et al.,* (2015) and Kumar *et al.,* (2009) who reported higher prevalence of *Babesia gibsoni* in their study.

Microscopic examination remains the simplest, commonly used economically feasible, most accessible rapid confirmatory diagnostic method for identification of *Babesia spp*. which is frequently used under field conditions as it is conclusive. Examination of stained peripheral blood smear was considered as excellent diagnostic tool for acute infections but not suitable to diagnose asymptomatic carriers due to low level of parasitemia (Ayoob *et al.,* 2010 and Bano and Lodh, 2018).

In the present study PCR detected more number of samples as compared to peripheral blood smear examination indicating higher sensitivity and specificity of PCR which corroborates with the findings of Mahalingaiah *et al.,* (2017) and Jain *et al.,* (2017) and they opined that subclinical / chronic status of the infection can be detected by PCR.

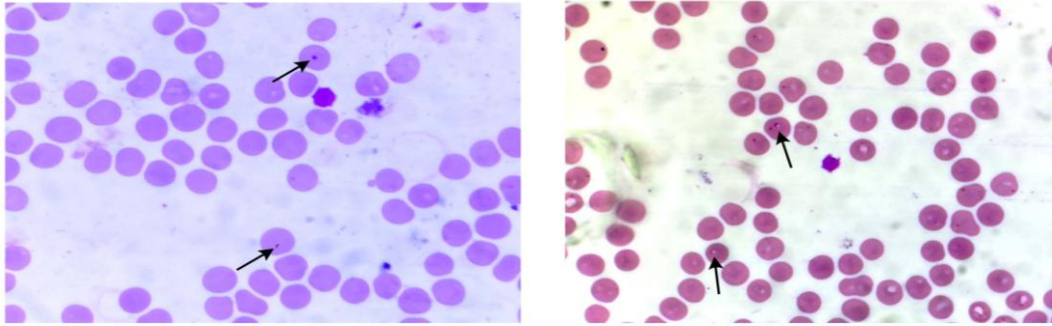**Fig.1** Intraerythrocytic pirolasms of *Babesia gibsoni*

Fig.2 Intraerythrocytic pirolasms of *Babesia canis*
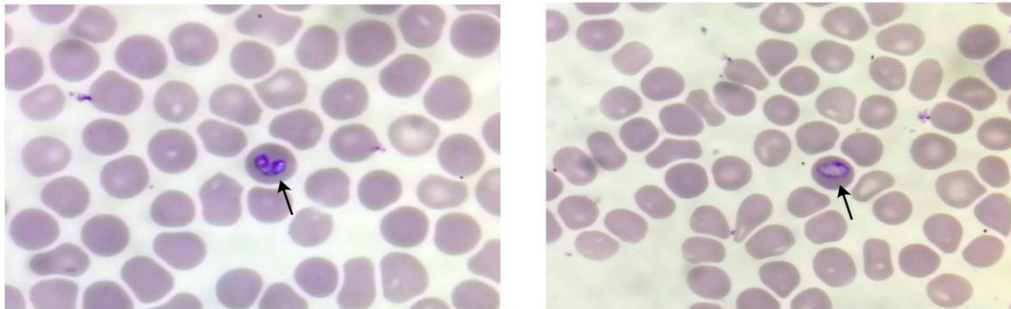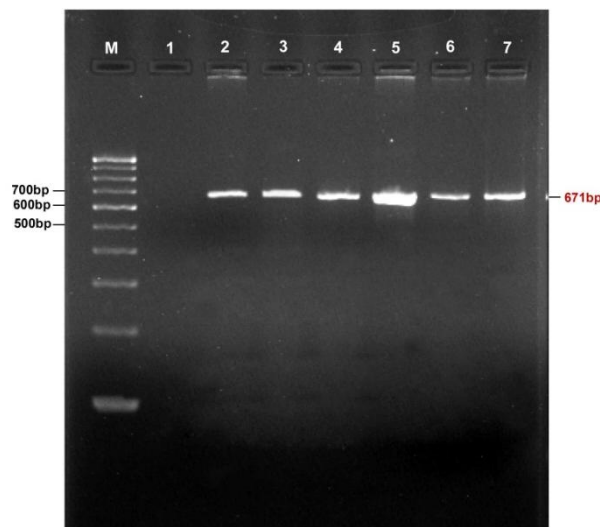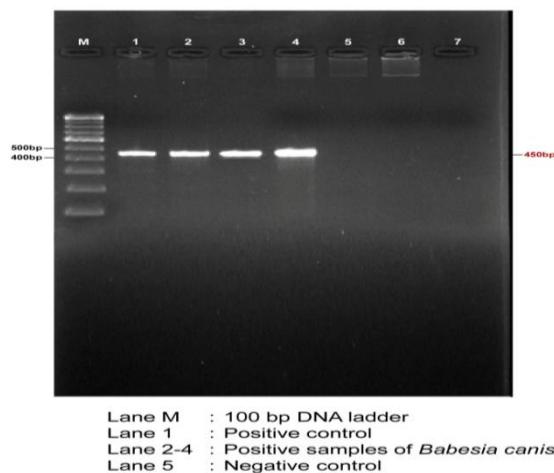

Fig.3 *Babesia gibsoni* positive samples by PCR

Lane M   : 100 bp DNA ladder
Lane 1   : Negative control
Lane 2-7 : Positive samples of *Babesia gibsoni*

Fig.4 *Babesia canis* positive samples by PCR

Lane M   : 100 bp DNA ladder
Lane 1   : Positive control
Lane 2-4 : Positive samples of *Babesia canis*
Lane 5   : Negative control

PCR is the most sensitive and specific test available for diagnosis of *Babesia* infection. This methodology targets parasitic DNA rather than anti-babesial antibodies, and is therefore a reliable diagnostic tool in per acute, acute, and chronic infections. It is capable of diagnosing babesial infections with small volume of blood sample having extremely low parasitemia. PCR is commonly used in clinical diagnosis, identification of new strains, and to differentiate alike and genetically distinct *Babesia spp.* in epidemiologic studies as endorsed by Ayoob *et al.,* (2010) and Inokuma *et al.,* (2004).

Other diagnostic methods available for detection of *Babesia spp.* were flow cytometry, indirect fluorescent antibody test (IFAT), enzyme linked immuno sorbent assay (ELISA) etc., although these are effective for detection of antibodies to *Babesia spp.*, cross-reactivity occurs between *Babesia spp., Toxoplasma spp., and Neospora spp.* infections and they fail to differentiate current and past infections (Ayoob *et al.,* 2010 and Dantas-Torres and Figueredo, 2006).

In conclusion, hence proper control measures for ectoparasitic should be followed in dogs. Utility of multiple diagnostic tests is suggested for confirmatory detection and epidemiological disease investigations of canine babesiosis in dogs. Molecular techniques like PCR were highly sensitive method for diagnosis of babesiosis infection in dogs as it detects low infection or chronic stage of infection when compared to conventional microscopy.

Acknowledgements

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