International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(12): 127-129
www.biochemjournal.com
Received: 07-09-2025
Accepted: 13-10-2025

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Comparative evaluation of blood smear examination and polymerase chain reaction for the diagnosis of canine babesiosis

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i12b.6458

Abstract
Vector-borne diseases affecting dogs have worldwide distribution and continue to expand at an alarming rate. Several canine vector-transmitted illnesses including filariasis, babesiosis, ehrlichiosis, and hepatozoonosis are commonly found throughout India. This research titled "Comparative evaluation of blood smear examination and polymerase chain reaction for the diagnosis of canine babesiosis" examined 4304 dogs presented to the Veterinary Clinical Complex at the College of Veterinary Science and Animal Husbandry in Mhow (M.P.) along with private veterinary facilities throughout Indore. The research spanned twelve months from March through February. Blood specimen collection focused on dogs displaying clinical signs including elevated body temperature, deteriorated overall health, lethargy, pallid mucous membranes, and loss of appetite for initial assessment. The gathered blood specimens underwent screening to detect babesiosis through thin blood film analysis following Giemsa staining. Among fifty specimens tested using PCR, 7(14%) produced a characteristic 380 bp amplified product confirming Babesia species infection, while only 2(4%) showed positive results for intra-erythrocytic piroplasms during microscopic evaluation. Every specimen that tested positive through microscopic analysis also demonstrated positive results via PCR testing.

Keywords: Dog, canine babesiosis, polymerase chain reaction, blood microscopic examination

Introduction
Canine babesiosis is a serious and potentially life threatening tick-borne protozoan disease of canine populations, globally. The disease is caused by intraerythrocytic parasites of the genus Babesia, order Piroplasmida, phylum Apicomplexa (Irwin, 2009) [7]. Tick-borne protozoan infections with *Babesia gibsoni* and *Hepatozoon canis* are more frequent than filarial infections with *D. immitis* and *A. reconditum* (Sarma *et al*., 2019) [12]. Canine Babesia is morphologically categorised into large and small types, Babesia canis (large), *Babesia gibsoni* (small) (Schoeman and Leisewitz, 2006) [14]. Numerous ixodid ticks, such as *Haemaphysalis longicornis*, *H. leachi*, *Rhipicephalus sanguineous*, and *Dermacentor marginatus*, can spread the disease to dogs (Shaw *et al*., 2001) [15]. In the life cycle of *B. gibsoni*, two types of hosts are required, the tick and the dog. Microscopic detection of *Babesia* is still the cheapest and fastest method, although its sensitivity and specificity is limited. On the contrary diagnostic methods based on nucleic acid detection and their amplification are the most sensitive and reliable techniques. The polymerase chain reaction (PCR) for the diagnosis of *B. gibsoni* was developed in 2001 by Mosqueda *et al*., 2012 [9].

Materials and Methods
The study, conducted at the Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry in Mhow, Indore, lasted from March 2021 to February 2022. Dogs with elevated body temperatures unresponsive to antibiotics were selected for blood sample collection via cephalic vein or ear tip, using 22G needles in EDTA vials. Blood smears were prepared from fresh samples, fixed in methanol, and stained with diluted Giemsa stain before examination under oil-immersion lens for signs of babesiosis. Diagnosis was confirmed if piroplasm stages of Babesia were present in erythrocytes.

Molecular detection of canine babesiosis

Isolation of genomic DNA

DNA was isolated from blood samples of 50 animals using GeneiPure blood genomic DNA purification kit (Genei, laboratories private limited) according to the manufacturer's instructions. In brief, DNA was extracted from the blood sample of suspected animals by GeneiPure blood genomic DNA purification kit.

Checking of the amplified product

The amplified products were examined using horizontal submarine agarose gel electrophoresis. A 2% w/v agarose (SIGMA-ALDRICH) gel was produced in 1X TBE. After cooling it to 60°C, ethidium bromide (10 mg/ml) @ 5 µl per 100 ml of agarose solution was added to a final concentration of 0.5 µg/ml and was stirred gently. The agarose solution was put in to the sealed casting tray. The gel was made to a thickness of roughly 4 mm. The agarose gel was allowed to set completely at 4°C temperature and then the comb was gently removed. After removing the adhesive tape, the gel casting platform was immersed in the electrophoresis tank filled with 1X TBE buffer. A 100 bp ladder (Genei) was used as a marker in a different lane after 5 µl of PCR product was combined with 1 µl of 6x gel loading dye (xylene cyanol and bromophenol blue). The electrophoresis was done at 80 volt for 1 hr. Gel Doc (Genesnap, Syngene) was used to record the amplified products in the gel after they were viewed under a UV trans-illuminator.

Comparisons of diagnostic method

A minimum of 50 blood sample was collected for the comparison between two diagnostic methods (blood smear examinations and PCR). The blood sample was collected from those dogs who showed the high rise of temperature and not responding to the antibiotic therapy.

Results and Discussion

The present analysis was attempted to explore clinical occurance of canine babesiosis in arround Indore Madhya Pradesh in India linked with epidemiological consequences. A total of 4304 dogs that were brought to the Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Mhow (M.P.) and private veterinary clinics around Indore were examined for signs of anorexia, weakness, pale mucous membranes, high increase temptarure, and poor general health.

1. Molecular detection of canine babesiosis

Polymerase chain reaction was carried out with genus specific primer pair OFP GTCTTGTAATTGGAATGATGGTGAC and ORP ATGCCCCCAACCGTTCCTATTA (Augustine *et al*., 2017) [2] to amplify a 380 bp fragment of the 18S ribosomal RNA gene of *Babesia* spp. for this purpose, 50 blood samples were collected randomly from suspected dogs and the DNA was isolated for further use.

Out of the fifty samples submitted to PCR, 7(14%) revealed a particular 380 bp amplicon indicating Babesia spp. infection, but only 2(4%) were positive for intra-erythrocytic piroplasms following microscopic analysis. All of the samples positive by microscopic examination were likewise positive by PCR. When compared to microscopic inspection, PCR revealed much higher effectiveness of detection of Babesia spp. Low positive was discovered in the current investigation using traditional diagnostic techniques, such as Giemsa-stained blood smear testing, compared to PCR, which may be because there were more chronic cases. In these cases, the amount of parasitemia was quite low, so these cases were not found by microscopic inspection.

Similar findings have also been reported by Dumanli *et al*. (2005) [4], Sasaki *et al*. (2007) [13], Singh *et al.* (2014) [16], Gabrielli *et al.* (2015) [5], Jain *et al*. (2017) [8], Ahmad *et al*. (2018) [1], Ma *et al*. (2021) and Ranatunga *et al*. (2022) [11].

Table 1: Comparison between PCR and blood smear examination as a diagnostic tool

Diagnostic Method	Incidence (%)
PCR	7(14%)
Blood smear examination	2(4%)

2. Blood smear examination

In the present study, examination of Giemsa's stained blood smears under oil immersion lens revealed intra-erythrocytic piroplasms of *Babesia* spp. only in fifty four out of four thousand three hundred four cases examined. Mostly, the piroplasms were appeared as pyriform shape with indistinct internal structures.

Similar findings have been reported by Singh *et al.* (2014) [16], Vipan *et al*. (2015) [17], Augustine *et al*. (2017) [2] and Preena *et al.* (2021) [10]. Abnormalities in erythrocytes structure including anisocytosis, poikilocytosis, basophilic stippling and presence of reticulocytes observed by Das *et al*. (2015) [3].

The abnormality in erythrocytes shape is mainly due to toxic action of parasite in the erythrocytes, erythrocyte oxidation, and immune-mediated process (Homer *et al*., 2000) [6].

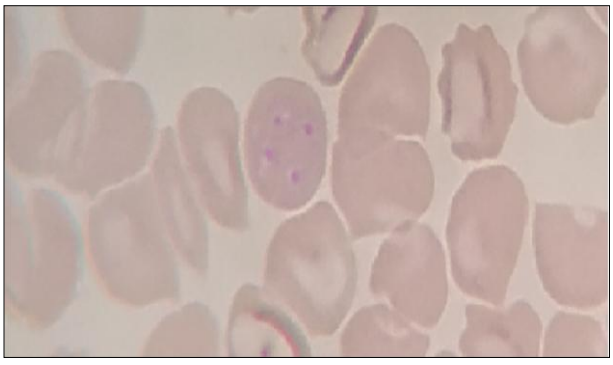

Fig 1: Giemsa stained dog blood smear showing babesiosis infection merozoites) in RBC

Conclusion

The present study highlights the continued clinical occurrence of canine babesiosis in and around Indore, Madhya Pradesh, and emphasizes the limitations of conventional diagnostic methods in detecting chronic or low-grade infections. Among the suspected cases, PCR proved to be significantly more sensitive than microscopic examination, detecting *Babesia* spp. in 14% of samples compared to 4% by smear evaluation. The findings underscore the importance of incorporating molecular diagnostic tools, particularly PCR, for accurate detection and timely management of canine babesiosis. Early and reliable diagnosis is crucial not only for effective therapeutic intervention but also for understanding the epidemiological

dynamics of the disease in the region. The study further suggests that reliance solely on microscopy may lead to underdiagnosis, especially in chronic cases with low parasitemia, and therefore recommends PCR as a valuable adjunct in routine diagnostic protocols.

Acknowledgements
The Department of Veterinary Medicine at MHOW, Indore (M.P.) is thanked by the authors for helping to carry out the study.

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