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Prevalence, Molecular Identification and Phylogenic Analysis of *Hepatozoon canis* from Naturally Infected Dogs in Andhra Pradesh, South India

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ABSTRACT

Background: The present study is carried out for the detection of *Hepatozoon canis* organism from naturally infected dogs in Andhra Pradesh, South India.

Methods: A total of 97 dogs blood samples were analyzed by microscopy and conventional PCR and the amplicons of *H. canis* were sequenced and compared with the sequences deposited at GenBank database.

Result: Blood smear examination could detect infection with *Hepatozoon canis* pathogens in two dogs with 2.06 prevalence, while conventional PCR assay revealed that 9 dogs were positive for *H. canis* with 9.28 prevalence rate. All the positive samples were further subjected to conventional PCR and amplified 18S rRNA gene of *H. canis* (737 bp), without any non-specific amplification. DNA sequences of the conventional PCR amplicons were subjected to Boot strap analysis and confirmed as *H. canis*. Of 97 dogs examined, 8.71% of dogs were infested by ticks and were identified as *Rhipicephalus sanguineus*. The conventional PCR assay could detect more natural infections of *Hepatozoon* spp. in dogs emphasizing the need of the assay in epidemiological studies.

Key words: 18SrRNA gene, Conventional PCR assay, DNA sequences, *Hepatozoon canis,* Microscopy, Tick infestation.

INTRODUCTION

Hepatozoonosis in dogs was first described in 1905 by Bentley as leucocytozoan of canines responsible for causing the disease. *H. canis* is widely distributed in Africa, South western Asia, South and North America whereas *H. americanum* is restricted to USA (Baneth *et al*., 2003). There are scanty reports on molecular detection of *Hepatozoon canis* transmitted through brown dog tick in India however few studies from Kerala have reported the infection by morphological studies of stained thin peripheral blood smears (Priya *et al*., 2004; Lakshmanan *et al.,* 2018). However, molecular studies for detection of *Hepatozoon canis* has been carried out in Nigeria (Kamani *et al*., 2013), in Thailand (Kledmanee *et al*., 2009) and in India (Murugesan *et al*., 2017 and Lakshmanan *et al*., 2018). In Andhra Pradesh no attempt was carried out on molecular characterization of *Hepatozoon canis* associated with natural infections in dogs. Hence, the present study was undertaken by amplifying 18S rRNA gene of *Hepatozoon canis* using polymerase chain reaction and for better understanding of phylogenetic relationships.

MATERIALS AND METHODS

The whole blood collected from the dogs of 23 Veterinary hospitals of Andhra Pradesh were subjected to examination at Tirupati Parasitology Laboratory for 8 months period (From May 2021 to December 2021). Immediately thin blood smears were prepared and stained with Giemsa stain. The Giemsa-stained blood smears were screened microscopically under 1000x microscope and haemoparasites were identified based on the morphological characters (Greene *et al.,* 2008). Remaining blood was stored at -20°C for further DNA extraction. DNA was isolated from each blood sample (200 µL aliquots of EDTA blood, stored at -20°C) using a QIAamp DNA Blood Mini Kit (Quiagen ® Kit, Germany), according to the manufacturer's instructions with slight modifications. Polymerase chain reaction was performed in a 25 µl of final reaction volume containing Taq DNA polymerase, DNA template, nuclease free water and primers. In the present study 18S rRNA gene of *H. canis* was amplified using the following primers designed for identification.

Hepatozoon FP 14094: ATACATGAGCAAAATCTCAACTT and Hepatozoon R14095: CCAACTGTCCCTATCAATCATTAA.

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Cycling conditions were carried out with initial denaturation at 94°C for 5 min followed by 34 cycles of denaturation (94°C, 30s), annealing (60-67°C, 30s) extension (72°C, 30s) and a final extension (72°C,30s) (Kledmanee *et al*., 2009). All the amplified products were subjected to electrophoresis using 2% agarose gel (Himedia Low EEO) containing 0.5 µg/ml ethidium bromide in 1x TAE buffer and visualized on UV transilluminator. The horizontal gel electrophoresis was carried out at 100 volts for 55 min. The amplified PCR products were verified by comparison with 100 bp ladder (Genei Merck, Bengaluru, India). and the image of the gel was captured by gel doc system (Bio-Rad). The amplicons were sequenced and compared with the sequences deposited at GenBank database. To validate the molecular identification the randomly selected DNA amplicons of 18s rRNA gene of *H. canis* were subjected to Boot srap analysis. The phylogenic analysis was carried out on partial sequencing of 18 srRNA gene of *H. canis* and the strains revealed closet association with other *H. canis* species from out side of India.

RESULTS AND DISCUSSION

The microscopic examination of thin blood smears revealed typical gelatin capsule shaped gamonts inside neutrophils indicating *Hepatozoon canis* infection (Fig 1). Out of 97 samples examined, only two (2.06%) samples were found positive (Table 1). Out of 97 samples screened subjected to PCR analysis, nine samples - showed amplification of 737 bp DNA fragment specific to *H. canis* with prevalence rate of 9.28% (Table and Fig 2). The phylogenetic analysis revealed 100% similarity with 18S rRNA gene of *Hepatozoon canis* deposited at the GenBank database (Accession No:MZ460581) (Fig 3) and confirmed the pathogen as *Hepatozoon canis*. In the present study, PCR assay has shown more reliability as shown in Table1.

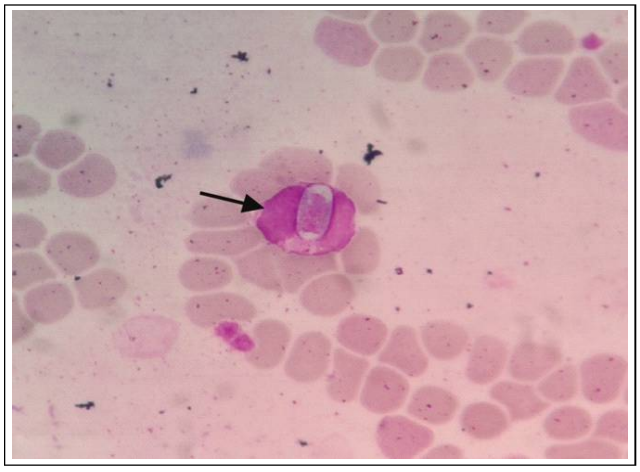


Fig 1: Blood smear of dog showing gametocyte of *Hepatozoon canis* in neutrophil.

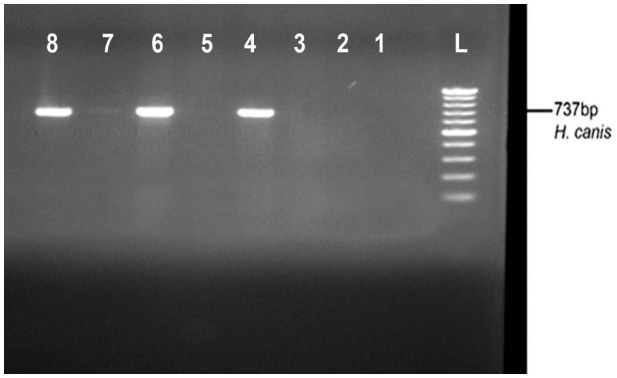
The identification of *Hepatozoon canis* in the present study was done by detecting gametocytes in the blood smear of affected dogs and by amplifying a variable region of the 18S rRNA gene using nested PCR which was in accordance with the studies of (Allen *et al*., 2008; Li *et al*., 2008; Singh *et al*., 2017) and Mohanapriya *et al*. (2020). Identification of *H. canis* is based on clinical manifestations and through PCR assay (Lakshmanan *et al*., 2018). Abd Rani *et al*. (2011) reported that *H. canis* (30%) was the most common haemoparasite infecting dogs in India followed by *E. canis* (20.6%) and *B. gibsoni* (0.2%) based on Conventional PCR assay. *Hepatozoon canis* is transmitted by oral ingestion of infected ticks containing mature sporozoites (Baneth *et al*., 2007). *Hepatozoon canis* deserves attention on account of its pathogenicity in natural hosts (Baneth *et al*., 2003). The prevalence of *H. canis* is low compared to other canine haemoparasites in India. (Singh *et al*. 2012; Bhattacharjee and Sarmah, 2013). A Low prevalence of *H. canis* may be due to low detection limit of light microscopy and in contrast, higher prevalence of 30% was recorded by PCR studies in Namakkal, Tamil Nadu, India (Murugesan *et al*., 2017). So, PCR assay is most efficient tool for the detection of *Hepatozoon canis* infections in chronic cases which is in accordance with the studies of Otranto *et al*., (2011). In the present study, the sequence of 18S rRNA of *H. canis* was found to have complete homology with isolates of Chaina, Sudan, South Africa, Spain, Israil and Taiwan however, no published isolates are available for comparison with previous isolates in India, Whereas Miranda *et al*., (2014) reported a similar finding with 99.0 per cent homology. In accordance with the present results, many researchers observed low sensitivity of microscopy, while evaluating the efficacy of the molecular method and routine blood smears in the detection of tick-borne pathogens in dogs (Singh *et al*., 2014; Jain *et al*., 2018; Rucksaken *et al*., 2019).

During the present study, the correlation between incidence of *Hepatozoon canis* DNA with the presence of ticks (*R. sanguineus*) was not noticed in dogs which is in contrary with previous studies of *Hepatozoon canis* infection in dogs to be positively correlated with the presence of

Table 1: Prevalence of *Hepatozoon canis* in Microscopy and conventional PCR assay.

Total no. of samples collected	Total no. of samples processed	No. of Samples found positive under microscopy	No. of samples found positive under PCR	Grand total of all positive samples under (microscopy and PCR)
97	97	2(2.06%)	9(9.28%)	11(5.67)

ticks (O' Dwyer *et al*.,2001, Forlano *et al*.,2005, Aktas *et al*.,2013). In this study out of 97 dogs only eight (8.71) dogs showed presence of ticks. Most of the sampled dogs did not show tick infestation which may be due to grooming activity by their owners. Hence, good care and management of pets by their owners may be the reason for lesser ticks' infestation in the sampled dogs. Microscopy could be able to identify only 2.02 per cent prevalence of hepatozoonosis in the present study area indicating lesser reliability than PCR. *Hepatozoon canis* was the least prevalent (1.52%) haemoparasites in dogs in Krishna and nearby districts andhra Pradesh during last five years of study using



L: 100bp DNA ladder; 4th lane is positive control and 1and 2 nd lanes are negative control and 6and 8 lanes are showing amplicons of 18S rRNA gene of *H. canis.*

Fig 2: PCR product of *Hepatozoon canis.*

microscopy (Sreedevi *et al*., 2020). Molecular technique (PCR) for *H. canis* has been utilized in various studies, worldwide and it has been shown to be a sensitive diagnostic technique (Inokuma *et al*., 2002; Rojas *et al*., 2014). In India, Singh *et al*. (2017) reported 13.78 per cent prevalence of canine hepatozoonosis from Punjab. Further molecular evidence of *H. canis* (6.1%), either as single or as concurrent infection, by the multiplex PCR assay indicated that the prevalence of canine hepatozoonosis is low in Andhra Pradesh state (Kopparthi *et al*. 2023).

The prevalence of hepatozoonosis in India, estimated either by conventional microscopic examination (Bhattacharjee and Sarmah, 2013; Gautam Patra *et al*., 2020; Sreedevi *et al*., 2020) or molecular studies (Laummaunwai *et al*., 2014; as in the present study), was comparatively low than other tick-borne infections. The reality that *H. canis* was less frequently found than *Babesia* spp. and *E. canis* in dogs from Andhra Pradesh might be correlated to the hypothesis that the local tick vector populations more frequently harbour some specific pathogens than others (Latrofa *et al*., 2014). Hepatozoonosis was even absent in certain regions of world (semiarid region of Brazil) though the tick vector *Rhipicephalus sanguineus* exist in that region (Rotondono *et al*., 2015). However, *H. canis* was the predominant species than other tick-borne pathogens identified in dogs from north, north-east and central parts of India (Abd Rani *et al*., 2011), Angola (Cordoso *et al*., 2016) and from Tamil Nadu, India (Manoj *et al*., 2020). Some haemoprotozoan diseases like babesiosis in dogs is endemic in tropical

Fig 3: Phylogenic analysis of *Hepatozoon canis*.

countries like India because of presence of tick vectors as well as reservoir stray dog population round the year (Kopparthi *et al*., 2021).

CONCLUSION

The study successfully detected the presence of *Hepatozoon canis* in naturally infected dogs in Andhra Pradesh, South India, utilizing both microscopy and conventional PCR methods. The results indicated a low prevalence of 2.06% through blood smear examination, while PCR analysis showed a higher prevalence of 9.28%. The amplification of the 18S rRNA gene verified the identity of the pathogen, affirming the effectiveness of the conventional PCR in revealing more natural infections of *Hepatozoon* species. Additionally, it was noted that approximately 8.71% of the dogs were infested with ticks, specifically *Rhipicephalus sanguineus*, highlighting the importance of veterinary surveillance and the need for such assays in epidemiological studies to understand and combat tick-borne infections in canines.

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Conflict of interest

The authors declare that they have no conflict of interest.

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