Molecular detection of *Hepatozoon canis* in dogs from Kerala

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Abstract India has a wide range of agro-climatic zones which is highly conducive for a diverse range of vectors and canines are continuously exposed to the risk of spectrum of tick borne protozoan diseases. The brown dog tick, *Rhipicephalus sanguineus* is widely prevalent among dogs in Kerala and there is a high prevalence of this tick transmitted *Babesia* and *Ehrlichia* spp. infection. However, the incidence of *Hepatozoon canis* transmitted by the same tick species had not been reported in the state since 2004. Preliminary screening of client owned dogs revealed six dogs to be positive for typical gelatin capsule shaped gamonts of *H. canis* within neutrophils in blood smear by microscopic examination. A PCR assay was standardized to amplify a specific 737 bp fragment of 18S rRNA gene of *H. canis*. Phylogenetic analysis revealed closest relationship with West Indies isolate deposited at GenBank database. The present study records the molecular detection of this haemoparasite in the state, for the first time.

Keywords *Hepatozoon canis* · PCR · 18S rRNA · Phylogeny · Kerala

Introduction

The emerging pattern of climate change accompanied by increased pet travel has contributed to the propagation of vectors and escalating prevalence of vector borne diseases worldwide. The brown dog tick, *Rhipicephalus sanguineus* is widely prevalent among dogs of Kerala, the south Indian state with warm humid tropical climate and there is a high occurrence of this tick transmitted *Babesia* and *Ehrlichia* spp. infection (Augustine et al. 2017; Jain et al. 2018). However, the incidence of *Hepatozoon canis* transmitted by the same tick species is rare in Kerala and there exists only a few case reports of this infection in dogs detected by morphological study of stained blood smears (Devada et al. 1996; Smitha et al. 2003; Priya et al. 2004). Canine hepatozoonosis caused by *H. canis* was first described in India in 1904 by Bentley (Dwyer 2011). The distribution of *H. canis* is widespread in Africa, Southwestern Asia, Europe and North and South America, whereas *H. americanum* is restricted to the United States (Baneth et al. 2003). Most of the cases of hepatozoonosis in the Indian subcontinent have so far been diagnosed only by conventional methods viz., microscopic examination of peripheral blood smears which is time consuming and does not permit reliable identification of the above parasites in clinical and asymptomatic carriers (Rani et al. 2011a). However, molecular detection and characterisation of *Hepatozoon* species has been carried out based on 18S rRNA gene in several countries viz., Thailand (Kledmanee et al. 2009), Nigeria (Kamani et al. 2013), India (Rani et al. 2011b; Murugesan et al. 2017; Singla et al. 2017), Malta (Licari et al. 2017) and Mauritius (Daskalaki et al. 2018). In the context of increasing prevalence of canine vector borne pathogens in Kerala, there is an urgent need to explore the status of *H. canis* as the infection has not been reported since 2004 in the state.

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Moreover there has been no attempt to characterize this species associated with natural infections in dogs in Kerala using molecular methods. Hence, the present study was undertaken to detect the presence of *Hepatozoon* species in dogs of Kerala by using PCR and to evaluate the phylogenetic relationships of this species based on 18S rRNA gene.

Materials and methods

Preliminary screening of client owned dogs ($n = 150$) presented to University Veterinary Hospital, Mannuthy during 2017–2018, with complaints of pyrexia and anaemia was performed by light microscopic examination ($1000 \times$ magnification) of Giemsa's stained blood smear of dogs upon pre-informed owner's consent. Whole blood samples were collected under aseptic conditions adhering mandates of institutional ethical committee guidelines for disease diagnosis. The nucleic acid extraction was performed using commercial kit (Qiagen, Germany). The quality of DNA in the final elutes was estimated using nanospectrophotometer (Nano drop 2000C, ThermoScientific, USA). Gradient PCR was performed in a 25 μ L reaction volume containing 2.5 μ L of buffer ($10 \times$) without $MgCl_2$, dNTP (200 μ M each), $MgCl_2$ (2.0 mM), *Taq* DNA polymerase (1.5 U), 25 pmol each of primers (Forward: HEP-F5'-CCT GGC TAT ACA TGA GCA AAA TCT CAA CTT-3' and Reverse: HEP-R-5'CCA ACT GTC CCT ATC AAT CAT TAA AGC-3') targeting a partial region of 18S rRNA gene (Kledmanee et al. 2009) and 5.0 μ L of template DNA. A no template control (NTC) was included in each run. Cycling conditions with initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation (94 °C, 30 s), annealing (60–67 °C, 30 s) extension (72 °C, 30 s) and a final extension at 72 °C for 5 min was adopted. The amplified PCR products were subjected to electrophoresis in 1.5% agarose gel (Hoefer, USA) and visualized under Gel-documentation system (Bio-Rad Laboratories, USA). The amplicons were bidirectionally sequenced at Agri Genom Labs Pvt Ltd, Cochin. The sequences were aligned by EMBOSS (www.bioinformatics.nl/cgi-bin/merger) and BLAST analysis was carried out at NCBI BLAST (www.blast.ncbi.nlm.nih.gov/blast) to analyze their similarity with other sequences deposited at GenBank database. Phylogenetic analysis was done using DAMBE (5.5.24) adopting Maximum likelihood method with 1000 bootstrapping.

Results

Microscopic examination of blood smears identified six dogs to be positive for typical gelatin capsule shaped gamonts of *H. canis* inside neutrophils in the blood smear. A specific amplicon of 737 bp was obtained at an annealing temperature of 65 °C with known positive control (Fig. 1). All the six samples were PCR positive. However, positive PCR signals were not obtained with blood smear negative samples. The amplicons were sequenced and compared with the published sequences deposited at GenBank database. Phylogenetic analysis revealed 99–100% similarity with 18S rRNA gene of *H. canis* deposited at the Genbank (Accession No. MG 543849). This additionally confirmed the identity of this intra-leukocytic tick transmitted pathogen in dogs in Kerala to be *H. canis*. To ascertain the phylogenetic relationship, the evolutionary tree derived from maximum likelihood method after 1000 bootstrapping with *Sarcocystis sarctosi* as outgroup showed that Kerala isolate shared the closest relationship with West Indies isolate (Fig. 2). The clade containing the isolates from Thailand and India was the farthest.

Discussion

The present study is the first report on molecular detection of *H. canis* in naturally infected dogs from Kerala. The prevalence of *H. canis* in India is low compared to other canine haemoparasites (Singh et al. 2012; Bhattacharjee and Sarmah 2013; Senthil Kumar et al. 2009). The reason for the low prevalence could be attributed to the lower sensitivity by light microscopy which can detect approximately 0.001 per cent parasitaemia (Matsui et al. 2005), though blood smear examination has been the simplest, reasonably sensitive test during acute and clinically significant infections. Though, *Babesia gibsoni* was the most prevalent canine haemoparasite in Kerala since a few years (Augustine et al. 2017; Jain et al. 2017), the occurrence of *H. canis* deserves attention on account of its pathogenicity

Fig. 1 Gel showing amplification of 737 bp DNA fragment specific for *H. canis*. M, 100 bp ladder; L1, known positive control; L2–L7, positive samples; L8, NTC

Fig. 2 Phylogenetic tree of 18S rRNA gene of *H. canis*

in natural hosts (Baneth et al. 2003). The disease was reported in the state after a long gap of more than a decade. Increasing pet travel and tourism could also contribute to disease spread between countries. The close evolutionary relationship with West Indies isolate throws light on this aspect. Phylogenetic evaluation revealed that the previously reported Indian isolate from Asiatic lion (JN 584477) was the farthest in evolutionary tree, which could be due to the strain variations in species affecting felines. Vector distribution pattern and epidemiology of canine vector borne disease in Kerala is likely to be influenced by the rapidly changing climate in the state and more emphasis need to be given for control of such diseases. Besides, the findings of the study also stressed need to include *H. canis* also in the list of differential diagnosis of tick borne pathogens among canines in Kerala.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interest.

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