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Detection of *Hepatozoan canis* in Dogs from Namakkal Region of Tamilnadu by Conventional and Molecular Approach

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A B S T R A C T

A total of 150 dogs brought to Teaching Veterinary Clinical Complex (TCCC), Veterinary College and Research Institute, Namakkal, from September, 2015 to April, 2016 were screened for the haemoparasite, *H. canis* by blood smear examination and polymerase chain reaction which revealed a positivity of 0.66 and 5.33 per cent, respectively. The PCR was found to have a high sensitivity (100.0%) and specificity (91.27%) when compared with microscopic examination. The target 18S rRNA of *H. canis* identified was found to possess a 100.0 per cent homology with the *H. canis* of dogs in Hungary, Italy, Japan, North Africa, Pakistan, Portugal and Thailand. *Rhipicephalus sanguineus* was the tick vector identified from the positive cases and the males (6.48%), cross breed dogs (10.0%) and the age group of >4 years (9.09%) were found to be highly susceptible.

Introduction

India has a wide range of climatic zones which is conducive for a diverse range of vectors and canines are continuously exposed to a spectrum of haemoparasitic diseases (Abd Rani *et al*., 2010). Canine hepatozoonosis caused by *Hepatozoon canis* was first described in India in 1904 by Bentley as leucocytozoan of the dogs (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). *H. canis* is amongst one of the most widespread canine vector borne disease

because of frequent infestation by the tick *Rhipicephalus sanguineus* (Otranto *et al*., 2011). The infection level can vary from subclinical, usually associated with low parasitemia, to severe and life-threatening disease in animals with high parasitemia, often in puppies or immunosuppressed animals and infected dogs may persistently show latent parasitism for long periods of time (Baneth *et al*., 1997).

In India, the information on the prevalence and epidemiology of hepatozoonosis in India is still under reported. Further, most cases of

hepatozoonosis in Indian subcontinent have so far been diagnosed only by traditional methods using microscopic examination of peripheral blood smears which are time consuming and does not permit reliable identification of the above parasites in clinical and asymptomatic carriers (Abd Rani *et al*., 2011). Hence, this paper presents the prevalence, molecular characterisation and the risk factors associated with the epidemiology of hepatozoonosis in dogs in Namakkal region of Tamilnadu in India.

Materials and Methods

A total of 150 dogs, irrespective of their age, sex, breed and health status presented to Teaching Veterinary Clinical Complex (TCCC), VCRI, Namakkal from September, 2015 to April, 2016 were screened. The clinical samples like blood, peripheral blood films and tick samples along with relevant epidemiological data were collected from the dogs. The blood films prepared were stained by Giemsa staining and the haemoparasites were identified based on the morphological characters (Greene *et al*., 2008). The blood samples were collected in EDTA coated vacutainers and the sample DNA was extracted as per the protocol recommended by Dneasy blood and tissue kit (Qiagen, Netherland) manufacturer. A PCR master mix of 2x (Ampliqon, Denmark) concentration was used in the reaction mixture and 100 bp DNA ladder (GeneDirex, USA) with stock solution 54µl/500µl was used in the electrophoresis. These primers were designed by using *FastPCR Software* (targeting the 18S ribosomal RNA gene - KF322145.1), custom synthesized (Eurofins and Sigma, India), with the product size 346 bp and used in this study. The sequences of the primers were as follows.

Forward:CTGACCTATCAGCTTTCGAC

Reverse:CAGCAGAACTTCAACTACGAGC

The extracted DNA was amplified using the selected primers by standardisation of PCR with following reaction mixture and cycling conditions. Reaction mixture: DNA template –3µl, master mix-13 µl, forward and reverse primer (10 pico moles, each) - 1 µl each and molecular grade water -7 µl. Cycling conditions: Initial denaturation-95°C/8min, denaturation-94°C/30sec, annealing-55°C/30sec and extension-72°C/45sec with 25 cycles; and final extension-72°C/10min. The gel was visualized under UV transilluminator and the bands of appropriate size were identified by comparison with the 100 bp ladder. The two positive amplified products were subjected to phylogenetic analysis (Eurofin) for confirmation.

Results and Discussion

The screening of 150 Giemsa stained peripheral blood smears for haemoparasitic infections revealed a prevalence of 0.66 per cent for *H. canis* and the gamonts of *H. canis* were identified in the neutrophils (Figure 1). A similar low prevalence of 1.08, 1.41, 2.3 and 4.8 per cent but higher than that observed in this study was recorded for *H. canis* in India (Singh *et al*., 2012; Bhattacharjee *et al*.; Senthil Kumar *et al*., 2009). The reason for the low prevalence recorded in this study could be attributed to the low detection limit of light microscopy which is approximately 0.001 per cent parasitaemia (Matsuu *et al*., 2005), though blood smear examination has been the simplest, reasonably sensitive test during acute and clinically significant infections. In contrast, PCR revealed a prevalence of 5.33 per cent for *H. canis* amplified at 346 bp targeting 18S rRNA gene (Figure 2). A similar low prevalence was recorded for *H. canis* in Turkey by PCR (Aydin *et al*., 2015), however, a high prevalence of 30.0 per cent was recorded in India. This could be attributed to the high sensitivity of PCR which is the most efficient

tool in the detection of subclinical and in apparent infections with a very low level of parasitaemia that can go undetected by the conventional microscopic examination. In this study, the PCR was found to have a high sensitivity (100.0%) and specificity (95.30%) in the detection of *H. canis* and this is in agreement with similar findings of Otranto *et al.*, (2011).

Table.1 Comparison of homology of the *H. canis* from positive cases with published sequencing results

S. No	Haemoparasite with target gene	GenBank accession number	Haemoparasites with target gene	Place of isolation
1.	*H. canis* (Targeted: (**KF322 145.1**))	KU535870.1	*H. canis* isolate, 18S rRNA gene	Pakistan
		KU765200.1	*H. canis* isolate, 18S rRNA gene	Thailand
		KX069244.1	*H. canis* isolate, 18S rRNA gene	Hungary
		KU961967.1	*H. canis* isolate, 18S rRNA gene	Italy
		LC018210.1	*H. canis* isolate, 18S rRNA gene	Portugal
		KP715301.1	*H. canis* fox isolate, 18S rRNA gene	Italy
		KX082907.1	*H. canis* clone, 18S rRNA gene	Isreal
		KJ499481.1	*H. canis* isolate, 18S rRNA gene	North Africa
		KU765202.1	*H. canis* isolate, 18S rRNA gene	Japan

Table.2 Age, sex, breed and season-specific prevalence of *H. canis*

Epidemiological determinants		Total (n=150)	No. of positives (n=8)	Per cent positives
Age	<1 year	34	2	5.88
	1-2 years	40	1	2.5
	2-4 years	32	1	3.12
	>4years	44	4	9.09
P value				0.532^{NS}
Sex	Male	108	7	6.48
	Female	42	1	2.38
P value				0.315^{NS}
Breed	Pure	106	4	3.77
	Cross	10	1	10.0
	ND	34	3	8.82
P value				0.414^{NS}
Season	Southwest monsoon	12	5	41.66
	Northeast monsoon	82	1	1.21
	Winter	41	2	4.87
	Summer	15	-	-
P value				0.0001**

[NS] Non-significant, * Significant at 95.0 per cent level, ** Significant at 99.0 per cent level

Figure.1 A Giemsa stained blood smear revealing the gamont of *H. canis* in a neutrophil (x100)
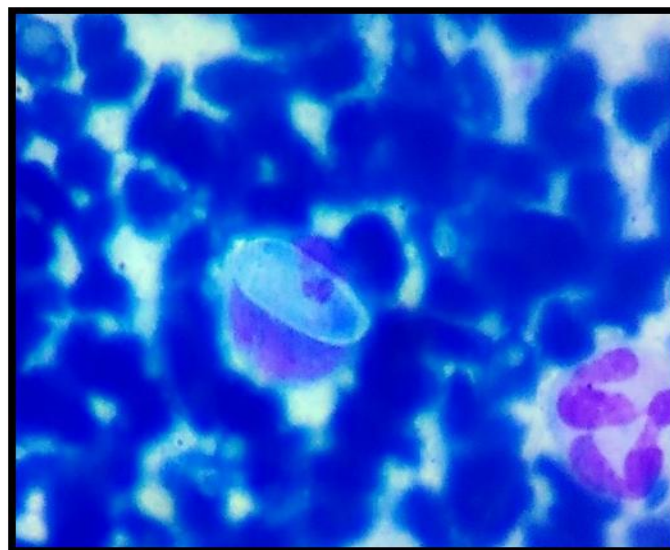
Figure.2 PCR amplified products of 18S rRNA gene of *H. canis* in 1.5 per cent agarose   gel showing bands at 346 bp
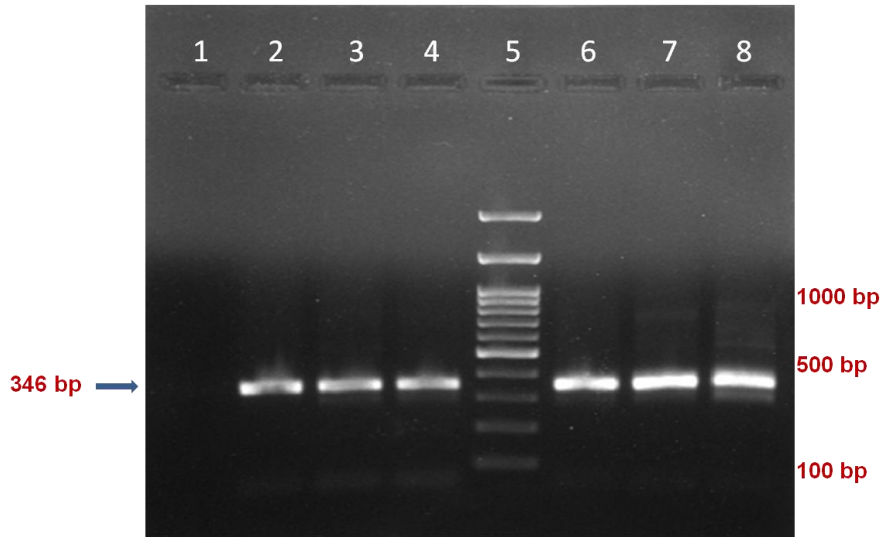
Lane 1 – Negative control, lane 2- Positive control, lane 5 - Ladder and lane 3,4,6,7 and 8- Test samples .

Figure.3 Phylogenetic relationship of 18S rRNA gene of *H. canis* with other published isolates (GenBank) and its homology and divergence

Nucleotide Substitutions (x100)
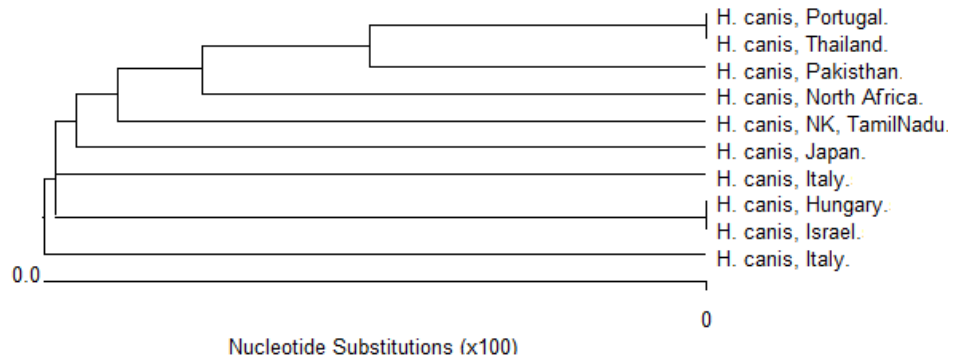
Percent Identity

	1	2	3	4	5	6	7	8	9	10		
1		99.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	1	H. canis, Hungary.
2	0.0		99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	2	H. canis, Israel.
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	3	H. canis, Italia.
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	4	H. canis, Italy.
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	5	H. canis, Japan.
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	6	H. canis, NK, TamilNadu.
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	7	H. canis, North Africa.
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	8	H. canis, Pakisthan.
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	9	H. canis, Portugal.
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		10	H. canis, Thailand.
	1	2	3	4	5	6	7	8	9	10		

Percent divergence

In the present study, the sequence of 18S rRNA of *H. canis* was found to have a complete homology (100.0%) with isolates of Hungary, Italy, Japan, North Africa, Pakistan, Portugal and Thailand; however no published isolates are available for comparison with previous reports in India (Table 1 and Figure 3), where as Miranda *et al.,* (2014) reported a similar finding with 99.0 per cent homology.

In the epidemiology, the tick vector involved in the transmission was found to be *H. canis* is *R. sanguineus*. A high prevalence of *H. canis* (9.09%) was detected in the age group of above 4 years old (Aktas *et al*., 2013), however the parasite was reported in all age groups from pups younger than 3 months of age to old dogs in the previous study. Among the sex, males (6.48%) were found to be highly susceptible (Gavazza *et al*., 2003; Tarun Kumar *et al*., 2012), however, no gender predilection for *H. canis* had been reported by Greene. Cross breeds (10.0%) were found to be highly susceptible, where as more incidences of *H. canis* were recorded in pure breeds (Tarun Kumar *et al*. 2003) and no breed predilection in previous studies. A high incidence of *H. canis* was recorded in south-west monsoon (41.66%) and this could be due to the prevailing high temperature and relative humidity which might have favoured the increased activity of tick vectors. However, there was no statistically significant difference found to exist among the different ages, sex and breeds of positive cases, and different seasons (Table 2).

In this study, the PCR was found to be a highly sensitive and useful tool in the detection of subclinical and asymptomatic carrier status of *H. canis* in dogs when compared to the conventional microscopic examination and routine screening by PCR would help in the identification of this parasite in carriers and thereby institution of suitable treatment for the control of disease.

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