

MOLECULAR DIAGNOSIS AND RISK FACTORS ANALYSIS OF HEPATOZOON CANIS INFECTION IN DOGS

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ABSTRACT

In this study we analysed risk factors and molecular diagnosis of Hepatozoonosis in the naturally infected canines of Andhra Pradesh, using thin blood smears and polymerase chain reaction. Four hundred and forty-two dog blood samples were collected and examined by microscopy and conventional PCR. Microscopic examination detected Hepatozoon canis organisms in four dogs with 0.90% case prevalence (4/442) while, 18S rRNA gene conventional PCR assay detected H. canis in twelve dogs with 2.71% case prevalence (12/442). The partial 18S rRNA gene of H. canis amplified from conventional PCR, underwent DNA sequencing. The 18S rRNA gene sequences were then analysed using BLAST, confirming their identity as H. canis through comparison with existing sequences in the GenBank database. Under risk factors analysis the living condition of the dogs (stray dogs) and tick infestation were identified as major risk factors causing higher prevalence rates in canines of Andhra Pradesh.

Keywords: Risk factors analysis, microscopy, polymerase chain reaction, *H. canis* and sequencing.

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INTRODUCTION

Understanding the aetiology and clinical presentation of canine hepatozoonosis is crucial for timely diagnosis and effective management, helping to mitigate the disease impact on affected animals. Canine hepatozoonosis is caused by *Hepatozoon canis*, characterised by fever, anaemia, and

lethargy (Baneth *et al.*, 2003). This parasitic infection primarily affects dogs, leading to a range of systemic symptoms that can impact their overall health and well-being. The transmission of *Hepatozoon canis* occurs through the ingestion of infected ticks, rather than direct tick bites (Mahdy *et al.*, 2024). Asymptomatic cases of *H. canis* with low level of parasitaemia often go unnoticed. *Hepatozoon canis* infection is prevalent in dogs across Asia, and it frequently co-occurs with other parasitic infections such as *Ehrlichiosis*, *Babesiosis*, *Leishmaniasis*

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and *Anaplasmosis* (Shaw *et al.*, 2001; Mylonakis *et al.*, 2004). According to a survey conducted by Abd Rani *et al.* (2011), *H. canis* is identified as the most prevalent tick-borne parasite in Indian dogs. Research by Baneth *et al.* (2001, 2003) indicates that *Rhipicephalus sanguineus sensu lato* (s.l.) is a potential vector responsible for transmitting hepatozoonosis in dogs within India. The widespread presence of *R. sanguineus* across tropical and semi-tropical regions contributes significantly to the prevalence of serious tick-borne diseases in dogs, as noted by Shaw *et al.* (2001) and Abd Rani *et al.* (2011). Besides, the prevalence of parasites, the identification of risk factors associated with parasitic infections is necessary to formulate guidelines for control strategies. Keeping in view of the above information, the present study was carried out.

MATERIALS AND METHODS

Whole blood samples were collected aseptically from the cephalic vein of the 442 dogs presented to the Veterinary Polyclinics and Veterinary Hospitals in Andhra Pradesh for a period of one year from July 2019 to June 2020, in EDTA vacutainers. Dogs showing clinical signs of fever, anaemia, bleeding episodes, jaundice, lameness, neurological signs, paralysis, lethargy and presence of tick infestation at the time of presentation were selected. Collaborations were established in the field with veterinarians working in the Animal Husbandry department. The dogs were categorised into two groups by age, young (\leq one year) and adult (\geq one year). Breed was classified as pure-breed and mixed-breed (mongrels) and gender as male and female. A complete data base

for each dog was developed by giving questionnaire that contained questions regarding age, sex, breed, living condition of the dog, acaricide application history, and origin/owner's address to identify risk factors associated with tick-borne parasitic infection. Thin blood smears were prepared from whole blood, air-dried and fixed in 100% methanol and later stained with Wright-Giemsa for microscopic screening (Soulsby, 1982). Blood samples were further preserved at -20°C until DNA extraction. Thin blood smears in duplicate from each blood sample were prepared on clean, grease free microslides, air dried and fixed using methanol. The fixed blood smears were stained by Giemsa stain using 1:10 dilution for 30 min. The slides were washed under running tap water and were air dried. Stained blood films were examined under x1000 objective lens for haemoparasites and photographed using Olympus CX21 digital microscope camera.

All the collected ticks were processed in 10% KOH as per the standard procedure (Soulsby, 1982). An Olympus microscope at the x100 magnification was used to identify the ticks at species level considering the morphological characters like hypostome, palpal segments, capitulum, scutum and legs (Walker *et al.*, 2014).

Oligonucleotide primers used for amplification genomic DNA

In the present study, 18S rRNA gene of *Hepatozoon canis* was amplified at specific loci using following primers (Kledmanee *et al.*, 2009).

Parasite/ Primer	Sequence (5'-3')	Length (bases)	Product size (bp)
<i>H. canis</i> Hep001F	CCTGGCTATACATGAGCAAAATCTCAACTT	30	737
Hep737R	CCAACTGTCCCTATCAATCATTAAGC	27	

Conventional PCR was carried out in a final reaction volume of 50 μ L. Forty-five μ L of reaction volume comprising 2x master mix, nuclease free water and forward and reverse primers which was aliquoted in each PCR tube and five μ L sample DNA was added to each tube to make the final volume. Each reaction volume contained:

PCR reaction mixture composition

Forward primer (each 0.5 pmol)	0.6 μ L
Reverse primer (each 0.5 pmol)	0.6 μ L
PCR master mix (2x)	25.0 μ L
DNA template	5.0 μ L
Nuclease free water	18.8 μ L
Total	50 μL

A negative control was run along with the samples at every PCR setup. Negative control in PCR was a mix inoculated with ultra-pure water in substitution of DNA and in equal volume.

Statistical analysis

The chi-square test was used for comparison of the frequencies by SPSS Statistics base 20. The role of risk factors viz. age group, gender, breed, living condition, and season on the frequency and type of parasitic infections were identified by binary logistic regression models using SPSS Statistics base 20. The data was subjected to multicollinearity test to ascertain that there is no collinearity between

the independent variables. To test whether the variation of the error from a regression is dependent on the independent variables (factor) heteroskedasticity Glejser test was employed. The phylogenetic analysis of the present study was done using Neighbor-Joining tree (50% boot strap consenses of *Hepatozoon canis*).

The binary logistic regression was performed on the dataset by setting the following factors as reference fulfilling the assumptions and conditions of the model. Age: adult (\geq one year); Gender: Male; Breed: Pure breed; Medium: Urban; Living condition: Stray dogs; Season: Winter; Ticks: Present; Tick control measures: Followed. (Table 2 and Fig.2)

RESULTS AND DISCUSSION

Microscopic examination of thin blood smears detected *Hepatozoon canis* infection in four dogs, yielding a case prevalence of 0.90%, whereas conventional PCR assay identified twelve *H. canis*-positive dogs, indicating a case prevalence rate of 2.71% (Table 1). The gametocytes of *H. canis* were observed in circulating neutrophils of infected dogs thin blood smears (Fig.1). All positive DNA samples underwent PCR amplification, successfully generating specific 737 base pair 18S rRNA gene amplicons for the *Hepatozoon canis* by targeting 18S rRNA gene (Fig. 3). These

amplicons were subsequently compared with sequences available in the GenBank database. Phylogenetic analysis revealed a high similarity of 99-100% with the *H. canis* 18S rRNA gene sequence deposited under **Accession No: MZ460581** (Fig. 4). So, this confirmed that the pathogen as *Hepatozoon canis*. This revealed closest association with China, South Africa, Canada, USA, Spain, Taiwan, Israel isolates deposited at Gen Bank. In this study conventional polymerase technique showed more accurate results compared to microscopy (Kappa values of *H. canis* was calculated as 0.99 and showed perfect agreement between microscopy and PCR technique as the kappa value is more than 0.81).

Binary logistic regression model for *Hepatozoon canis* infection in dogs

Under risk factors analysis the living condition of the dogs (stray dogs) and tick infestation were identified as main risk factors for *H. canis* infection (Table 2 and Fig. 2). Female dogs were 2.9 (OR=2.904; $P>0.05$) times more prone for *H. canis* infection than male animals. Mongrels were 1.6 (OR=1.591; $P>0.05$) times more likely to be infected than pure breed dogs. A notable difference in *H. canis* infection rates was observed, with pet dogs being 9.4 times less likely to be infected than stray dogs (OR=0.106; $P<0.001$). The prevalence of hepatozoonosis was 2.1 (OR=2.112; $P>0.05$) times more during summer than in winter season. Dogs without tick infestation were 11.6 (OR=0.086; $P<0.001$) times less likely to be infected with *H. canis* than dogs with tick infestation. The prevalence of hepatozoonosis was 2.9 (OR=0.345;

$P<0.05$) times more in dogs not having the previous history of tick control measures followed than in dogs with previous history of acaricide use for tick control.

Prior research by Allen *et al.* (2008), Li *et al.* (2008), and Singh *et al.* (2017) utilized nested PCR for the molecular identification of *H. canis* by amplifying the 18S rRNA gene Kaur *et al.* (2020) innovated by creating and evaluating a multiplex PCR assay. This assay was specifically developed for the simultaneous detection of *B. vogeli*, *E. canis*, and *H. canis* within canine samples. This novel assay was subsequently benchmarked against the established singleplex polymerase chain reaction method. Following tissue merogony in the transmission cycle, *Hepatozoon canis* parasites can persist in the bloodstream of infected dogs for extended durations, leading to a state of latent parasitism. According to Baneth and Weigler (1997), when these latently infected dogs experience concurrent infections, it may trigger the reactivation of tissue schizonts. This reactivation can subsequently result in an increase in parasitemia and the manifestation of more pronounced clinical symptoms. Gavazza *et al.*, (2003) stated that immunosuppression caused by a concurrent infection may predispose dogs to *H. Canis* infection. The severity of *H. canis* infection in canines spans a wide range, from asymptomatic cases with minimal parasitaemia to life-threatening conditions, especially in puppies or canines with compromised immune systems (Hervas *et al.*, 1995). Diagnosing *Hepatozoon canis* infection in symptomatic dogs is often straightforward and highly

successful. A common diagnostic approach involves the microscopic examination of Romanowsky-stained blood films, which allow the direct visualization of infected leukocytes containing the parasite (Ibrahim *et al.*, 1989; Baneth and Weigler, 1997). Alternatively, PCR testing can be utilized to detect the presence of parasite DNA in peripheral blood samples, offering another reliable method for diagnosis. In the present study only four (0.90%) dogs were found to be positive for hepatozoonosis in direct parasitological examination while molecular methods revealed a 2.71% infection rate of *H. canis*. This disparity highlights the superior detection capability of molecular methods over traditional microscopy for *H. canis*. The findings imply that while the disease may not be widespread, accurate diagnostic tools are crucial for its identification. Therefore, relying solely on microscopy might lead to underestimation of *H. canis* prevalence, underscoring the importance of employing more sensitive techniques like PCR for comprehensive epidemiological studies and effective disease management. Vincent-Johnson *et al.* (1997) reported that the presence of EDTA anticoagulant in blood samples can lead to the rapid departure of gametocytes from leukocytes. This phenomenon may subsequently complicate the identification of gametocytes when examining blood smears. Consequently, the initial processing of blood samples significantly impacts the ability to identify gametocytes, necessitating careful consideration of anticoagulant choices for precise diagnostic outcomes. The low prevalence of Hepatozoonosis in the studied area also suggests that other hemoparasites

might be more prevalent, warranting further investigation. However, a study conducted by Sreedevi *et al.* (2020) over five years in Krishna and neighboring districts of Andhra Pradesh revealed that *Hepatozoon canis* was the least common hemoparasite among dogs, with a prevalence of 1.52% when observed through microscopy. This finding provides insight into the epidemiological landscape of canine parasitic infections in the region.

Numerous studies spanning several years have highlighted the diagnostic utility of PCR for *H. canis*. Researchers have widely adopted PCR for its high sensitivity in detecting the parasite. For example, a study by Inokuma *et al.* (2002) demonstrated the use of PCR for analyzing the 18S rRNA gene sequence of *Hepatozoon* in Japanese dogs. Subsequently, Criado-Fornelio *et al.* (2003) emphasized that DNA detection of *H. canis* primarily relies on PCR targeting the 18S ribosomal RNA marker. Further research by Karagenc *et al.* (2006) and Gotsch *et al.* (2009) continued to validate PCR's role in diagnosing *H. canis*. Later, Abd Rani *et al.* (2011) found PCR to be more sensitive than microscopic examination for detecting hemoparasites. By 2014, Rojas *et al.* also utilized PCR in canine leishmaniasis diagnosis, noting its superior sensitivity and specificity compared to other methods. More recently, Singh *et al.* (2017) conducted molecular detection of *H. canis* in dogs from Iran using PCR methods. These examples collectively underscore the global acceptance and proven sensitivity of PCR as a diagnostic technique for *H. canis*. In India, canine hepatozoonosis prevalence was reported at 13.78% in Punjab by Singh *et al.*

(2017). Worldwide, varied prevalence rates of hepatozoonosis in dogs ranging from 3.3 to 82.0 per cent (Tababar *et al.*, 2009; Rojas *et al.*, 2014; Cardoso *et al.*, 2016) have been reported. Hepatozoonosis in India generally shows a lower prevalence compared to other tick-borne infections. This trend is observed through various diagnostic approaches, including conventional microscopic examination, as noted by Bhattacharjee and Sarmah (2013), Gautam Patra *et al.* (2020), and Sreedevi *et al.* (2020), and molecular methods, as demonstrated by Laummaunwai *et al.* (2014) and in the current study. This lower prevalence of *H. canis* may suggest unique epidemiological factors at play within the Indian subcontinent that differentiate the spread and impact of hepatozoonosis from other tick-borne pathogens. Such factors could include environmental conditions, host immunity, or specific characteristics of the tick vector populations in the region. Further research into these contributing elements could provide valuable insights into the regional dynamics of hepatozoonosis, potentially informing more targeted control and prevention strategies. Understanding these differences is crucial for a comprehensive approach to managing tick-borne diseases in India. The comparatively lower prevalence of *Hepatozoon canis* infections in the canine population of Andhra Pradesh, when contrasted with *Babesia* species and

Ehrlichia canis, may be attributable to specific characteristics of local tick vector populations that harbour pathogens (Latrofa *et al.*, 2014). This observation aligns with similar findings in other regions, where the absence of hepatozoonosis has been noted. For instance, despite the documented presence of the primary tick vector, *Rhipicephalus sanguineus*, hepatozoonosis has surprisingly not been detected in certain semi-arid areas of Brazil (Rotondono *et al.*, 2015). These geographical variations highlight the complex interplay of factors influencing disease prevalence, including vector competence, host susceptibility, and environmental conditions. Such regional discrepancies underscore the need for localized epidemiological studies to fully comprehend the transmission dynamics of *Hepatozoon canis*. Understanding these specific factors can significantly improve our ability to implement effective prevention and control strategies tailored to different geographical and ecological contexts. Conversely, studies have indicated that *H. canis* is the leading tick-borne pathogen affecting dogs in various regions, including northern, northeastern, and central India, as well as Angola and Tamil Nadu, India (Abd Rani *et al.*, 2011; Cardoso *et al.*, 2011; Manoj *et al.*, 2020). This highlights a contrasting epidemiological pattern compared to other areas.

Table.1. Showing the prevalence of *Hepatozoon canis* in Microscopy and conventional PCR assay

Type of infection	Parasite	Total no. of samples processed	No. of Samples found positive under microscopy	No. of samples found positive under conventional PCR
Single infections	<i>H. canis</i>	442	4(0.90)	12 (2.71)

P<0.01 (X² = 13.517; P=0.000236; df=1); Figures in parenthesis indicate percent

Table.2. Risk factor analysis for *H. canis* infection in dogs by logistic regression model

Risk factor	Chi square value	Odds ratio	95% CI		Wald P Value
			Lower	Upper	
Age					
≥ one year	0.619	1			0.420
≤ one year		0.619	0.191	2.006	
Gender					
Male	3.446	1			0.063
Females		2.904	0.897	9.404	
Breed					
Pure breed	0.610	1			0.438
Mongrels		1.591	0.487	5.194	
Medium					
Urban	3.446	1			0.063
Rural		2.904	0.897	9.404	
Living condition					
Stray dogs		1			
Kennel dogs	0.598	1.808	0.397	8.231	0.439
Pet dogs	18.375	0.106	0.031	0.362	<0.001
Season					
Winter		1			
Summer	0.928	2.112	0.446	9.989	0.335
Rainy	0.086	0.764	0.125	4.659	0.769
Ticks					
Present	27.311	1			<0.001
Absent		0.086	0.028	0.267	
Tick control measures					
Followed	4.025	1			0.045
Not followed		0.345	0.117	1.020	

Logistic regression model *P<0.05, **P<0.01, ***P<0.001

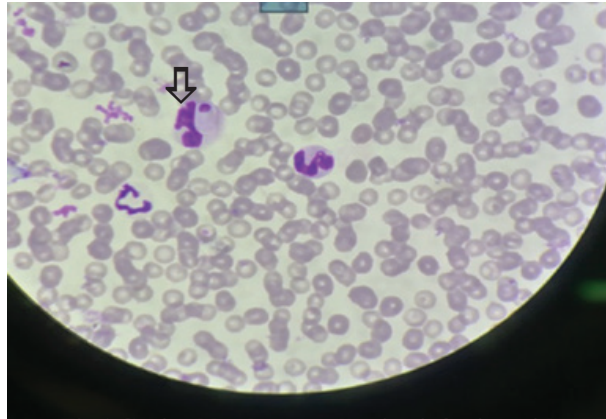


Fig.1.Canine blood smear showing infection with *Hepatozoon canis* in the monocyte and neutrophils.

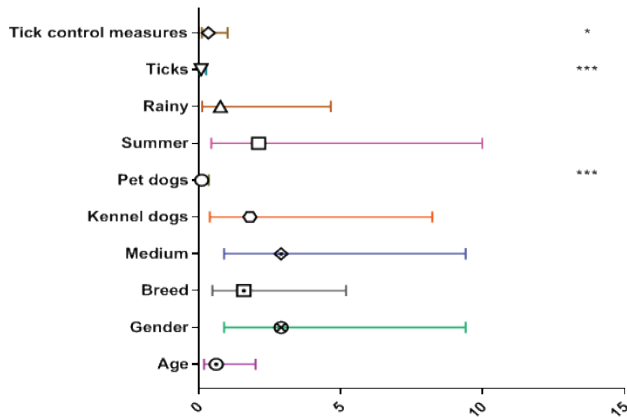


Fig.2. Risk factors with confidence intervals for *Hepatozoon canis*

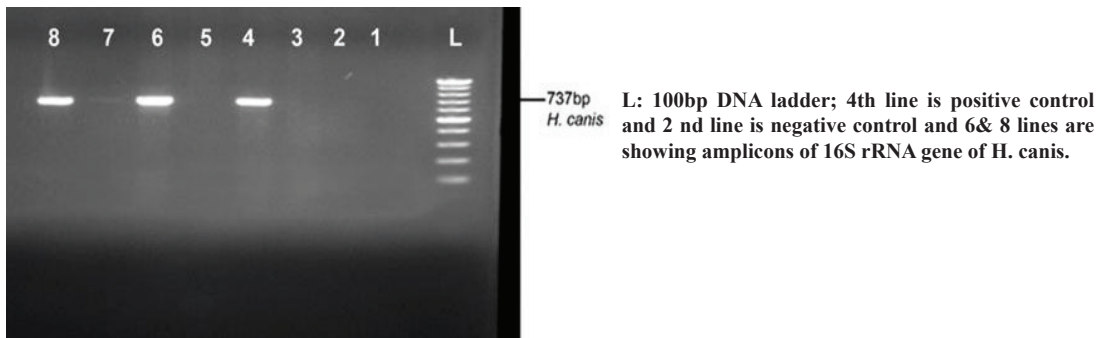


Fig.3. Showing the Conventional PCR product of *H. canis*

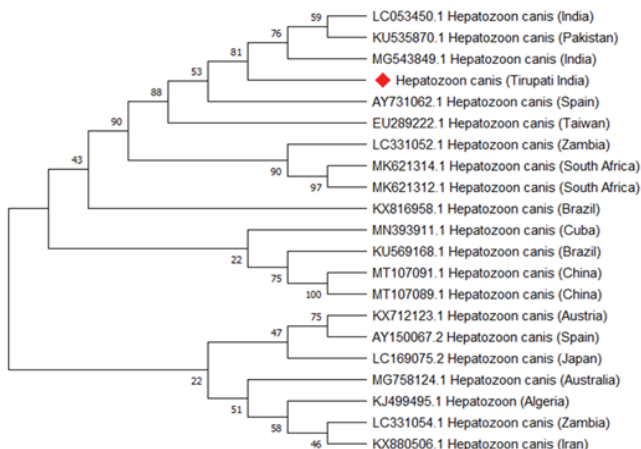


Fig.4. Neighbor-Joining tree (50%bootstrap consensus) using 18S rRNA gene sequences of *Hepatozoon canis*

CONCLUSION

The research indicates that *Hepatozoon canis* infection is present in dogs of Andhra Pradesh, South India. Out of 442 dog blood samples, microscopic examination identified *H. canis* in four dogs, showing a case prevalence of 0.90%. In contrast, conventional PCR detected *H. canis* in twelve dogs, resulting in a case prevalence of 2.71%. Hence, molecular methods demonstrated high sensitivity for detection of *H. canis* infection than traditional microscopy. Furthermore, the study identified significant risk factors associated with *H. canis* infection. Both the living condition of the dogs (specifically stray dogs) and the presence of tick infestation were found to be major risk factors for acquiring *H. canis* infection. These observations emphasize the critical role of molecular diagnostics in precisely determining the prevalence of *H. canis*. Furthermore, they

highlight the significance of managing stray dog populations and implementing effective tick control measures as key strategies for mitigating *H. canis* infections in the region.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest for this manuscript.

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