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Molecular Epidemiology of *Hepatozoon canis* Infection in Dogs from Odisha, India

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Abstract

Tick-borne diseases have raised serious health concerns in dogs globally, primarily in hot and humid climate of tropics and subtropics. The molecular detection and characterization of *Hepatozoon canis* were carried out in extracted DNA from blood samples of dogs (n = 212) by real-time PCR and from vector ticks (n = 41) by conventional PCR targeting the 18S rRNA gene. The present study reported low prevalence of *Hepatozoon canis* infections in dogs (9%) while high prevalence in *Rhipicephalus* spp. ticks (33.14%) in dogs in eight coastal districts of Odisha. The PCR positive blood samples (n = 19) were analyzed by multiplex PCR for co-infection, revealing the prevalence of co-infections of *H. canis* with *A. platys* (2.5%), with both *A. platys* and *E. canis* (0.9%) as well as with *A. platys* and *B. gibsoni* (0.9%). Presence of ticks, irregular anti- tick treatment and limited outdoor activity were found to be significantly associated incidence of hepatozoonosis. Hematological values of dogs with hepatozoonosis revealed anemia, thrombocytopenia and neutrophilia, while the group with mixed infection (MI) had significantly lower (P < 0.01) mean Hb, platelets, TEC, PCV, MCV, MCH, MCHC level indicating microcytic hypochromic anemia and potential thrombocytopenia in comparison to single infection (SI). The nucleotide sequence identity study revealed 98%-100% similarity of Odisha isolate with other global isolates of *H. canis*. The Odisha isolate was closely related to the nucleotide sequences of *H. canis*, isolated from dogs of West indies, as revealed by the phylogenetic analysis.

Keywords: *Hepatozoon canis*, Epidemiology, PCR, Hematology, Transmission

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INTRODUCTION

Canine hepatozoonosis is a tick-transmitted infection that occurs in both domestic and wild canids worldwide. It is caused by protozoan parasites of the family Hepatozoidae, under the genus Hepatozoon.[1] Two *Hepatozoon* species have been incriminated in causing canine hapatozoonosis namely: *Hepatozoon canis* and *Hepatozoon americanum*. *H. canis* has a broad geographic range, being reported in Africa, parts of Asia, Europe, and the America, while *H. americanum* is confined to the United States.[2]

Unlike most other tick-borne haemoparasites, *Hepatozoon canis* infects both circulating white blood cells and various tissues, particularly haemolymphatic organs. Transmission occurs through ingestion of an infected *Rhipicephalus sanguineus* tick instead of bite of tick. It develops asexually by schizogony in a vertebrate intermediate host (dog) and sexual development by sporogony and gametogony in a hematophagous invertebrate definitive host (tick).[2]

The clinical outcome of *H. canis* infection ranges from asymptomatic cases to severe disease, with common manifestations including fever, anorexia, loss of condition, anaemia, and hyperglobulinemia, which may progress to pneumonia, glomerulonephritis or hepatitis.[3] Coexistence of multiple tick-borne organisms, belonging to genus *Babesia, Anaplasma* and *Ehrlichia* frequently occur because they share the same vector, the brown dog tick (*R. sanguineus*), which is highly prevalent in India.[4] These concurrent infections often intensify clinical severity and may reduce the effectiveness of treatment.[5]

Microscopic examination of peripheral blood smears is the conventional diagnostic approach for *H. canis*; however, its sensitivity is limited in cases of low or fluctuating parasitemia, particularly in chronically infected or carrier dogs.[6] Molecular techniques, especially nucleic acid-based assays, provide superior specificity and sensitivity. Polymerase chain reaction, sequencing as well as phylogenetic analysis are widely used for accurate detection and species identification.[7] Real-time PCR (qPCR) not only detects parasite DNA but also quantifies its load, making it valuable for distinguishing between subclinical carriers and clinically diseased animals. It performs amplification of the target gene and analysis of the products, which eliminate the need for gel electrophoresis and staining with carcinogenic dye like ethidium bromide.[8] Multiplex PCR assays are also employed globally to detect multiple pathogens and co-infections in a single tube reaction.[9] Molecular detection of *H. canis* infection by targeting highly conserved region of 18S rRNA gene has been proven ideal.[10]

The hot and humid climatic condition of Indian sub-continent, owing to its subtropical location provides a favorable condition for propagation and sustainment of tick vectors. Molecular detection of *H. canis* in dogs and ticks has previously been documented in Tamil Nadu, as well as in dogs from a few other states such as Kerala, Mizoram, and Tripura using conventional PCR, and in Punjab using qPCR.[11-15] According to the Koppen-Geiger climate classification, Odisha-an eastern coastal state of India-predominantly falls under the Tropical Savanna (Aw) type, which is characterized by distinct wet and dry seasons, high temperatures, and heavy monsoon rainfall.[16] Despite being a tick-conducive region, the molecular evidence on prevalence of this pathogen from Odisha was not attempted. Furthermore, information regarding the prevalence of *H. canis* infection, its epidemiology, risk factors, and molecular characterization in this region is scarce. The current study provides the first molecular detection of *H. canis* in Odisha and establishes its prevalence in both dogs and vector ticks. The study assessed co-infections, identified potential risk factors, examined hematological changes linked to the infection, and carried out phylogenetic analysis.

MATERIALS AND METHODS

Study area

Odisha, lying on coastal region is an eastern state of India, spreading from 17° 49 to 22° 34 N Latitude and 81° 27 to 87° 29 E Longitude with a net geographical area of 1,55,708 km^2. For the current study, 8 districts viz. Khordha (20° 11' N, 85° 40' E), Cuttack (20° 28' N, 85° 54' E), Puri (19° 48' N, 85° 52' E), Ganjam (19° 22' N, 85° 06'

E), Nayagad (20° 08' N, 85° 08' E), Bhadrak (21° 06' N, 86° 50' E), Jajpur (20° 84' N, 86° 31' E) and Jagatsingpur (20° 25' N, 86° 17' E) were selected (Figure 1). These districts are located in the coastal belt of the state.

Sampling of animal

The study was carried out from February, 2024 to January, 2025. Dogs presented to Veterinary Clinical Complex (VCC), Odisha University of Agriculture and Technology, Bhubaneswar, Odisha were selected with history/ presence of tick infestation, and with or without signs of tick-borne diseases (TBD), such as fever, lymphadenopathy, generalized or limb weakness, respiratory distress, petechiae on skin and visible mucous membrane and bleeding disorder. Total of 212 canine blood samples were aseptically obtained from the cephalic or saphenous vein in EDTA vials. These samples were directly used for preparing thin blood smears and conducting hematological examinations, and subsequently stored at -80 °C for later DNA extraction. The consent for blood collection was obtained from the dog owners for the diagnostic purposes by registered professionals following the guidelines for blood collection stipulated by Committee for the Control and Supervision of Experiments on Animals (CCSEA) and the approval was acquired from Institute of Animal Ethical Committee of the University (833/IAEC, dated- 7-10-2023) for conducting the study. The details on location, sex, age, breed, tick infestation, use of acaricides, and outdoor exposure were documented and evaluated for potential risk factors.

Sampling of ticks

In this study 108 ticks were recovered from 41 infested dogs out of 212 dogs screened. They were identified by their morphology to

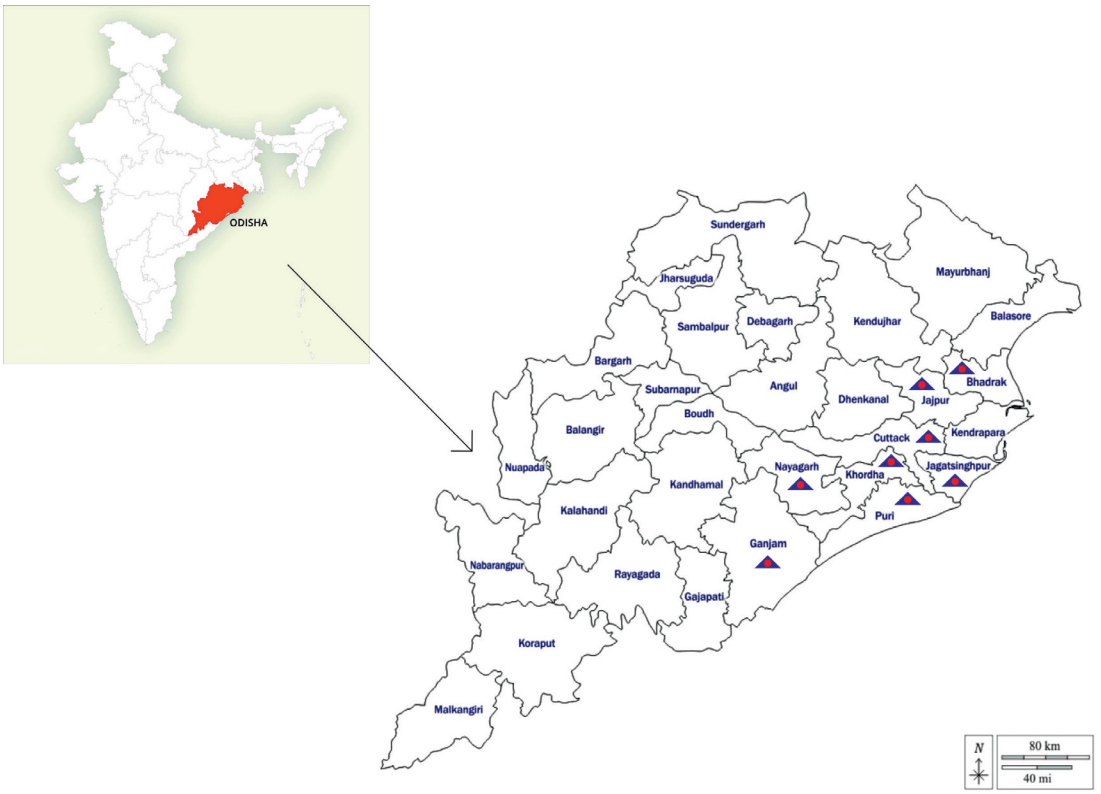

Figure 1. Map of India depicting Odisha and map of Odisha with districts included in the investigation marked by blue triangle[52,53]

the genus level[17] and were stored at -80 °C for molecular analysis. Ticks collected from a dog were considered as one pooled sample, and DNA was extracted from 41 tick pools.

Microscopy

The blood samples were examined microscopically by preparing thin smears, which were air-dried and fixed with methanol. These smears were stained using 10% Giemsa solution for 40 minutes and observed under an oil immersion objective. The presence of encapsulated, oval-shaped gamonts with a prominent central nucleus of *H. canis* within neutrophils or monocytes confirmed infection (Supplementary Figure 1).[18] Samples were considered negative if no parasites were detected after scanning a minimum of 100 oil immersion fields.[19]

Haematology

Hematological indicators include total leukocyte count (TLC), differential leukocyte count (DLC), packed cell volume (PCV), platelet count, hemoglobin (Hb), total erythrocyte count (TEC), mean corpuscular hemoglobin (MCH), mean Corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were measured using an automated Hematology analyzer (Merilyzer Celquant Vet, Meril Life Sciences, India). Ten number of apparently healthy dogs screened negative for the tick-borne infections by multiplex PCR were taken as healthy control.

DNA extraction from ticks and blood

Each pooled tick sample was preserved by freezing and then finely pulverized with liquid nitrogen using a sterilized mortar and pestle for DNA extraction.[20] Genomic DNA was isolated from both whole blood samples of dogs and ticks with the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines with minor modifications. The step involving PBS addition was omitted, and the DNA was finally eluted in a final volume of 50 µl. The purity and concentration of the extracts were determined using a nano-spectrophotometer, and the samples were stored at -80 °C for future analysis.

Polymerase chain reaction (PCR) assays

The primers and cycling conditions used in different types of PCR assays are mentioned in Tables 1 and 2 respectively.

Quantitative Real-time PCR for analysis of blood sample

A real-time singleplex quantitative real-time PCR (qPCR) was standardized by use of a previously described primer pair targeting the 18S rRNA gene of *H. canis* that produced an amplicon of 106 bp.[14] The reaction volume was set by adding 0.5 µl of each primer (10 µM), 10 µl of Quantinova SYB-green PCR master Mix (2X) (Qiagen, Germany) and a 1 µl eluted DNA. Final 20 µl reaction mixture was made by adding nuclease free water. A two-step quantitative real-time PCR protocol comprising of hot start and amplification followed by melt or dissociation was performed in real-time PCR system (G8830A, AriaMx system, Agilent, USA).

Singleplex conventional PCR for analysis of tick sample

A conventional singleplex PCR was standardized using a previously described primer pair targeting the 18S RNA gene of *H. canis* which produced an amplicon of 737 bp.[21] A PCR reaction mixture comprising of 20 µl volume was standardized containing 0.5 µl of each primer (10 µM), 10 µl of Fast cycling PCR super Mix (2X) (Qiagen, Germany), 1 µl eluted DNA and 8 µl nuclease free water (NFW).

Multiplex conventional PCR

A multiplex PCR (mPCR) was also performed for detection of co-infections using the eluted DNA from *H. canis* positive blood samples detected by quantitative real-time PCR with previously used set of oligonucleotide primers specific for *H. canis,*[21] *A. platys*, *B. gibsoni, E. canis*[22] and *B. vogeli*[23] respectively.

A 50 µl volume of PCR reaction mixture was standardized containing 0.5 µl of each primer (5 pairs each 10 µM), 25 µl of multiplex master Mix (2X) (Qiagen, Germany), 10 µl NFW and a 5 µl template DNA.

The amplifications of conventional PCR were performed using the Thermal Cycler

(T100TM Thermal Cycler, Bio-Rad, USA). PCR products were subjected to agarose gel (1.5%) horizontal electrophoresis using ethidium bromide dye (Himedia, India) in Tris-acetate-EDTA (TAE) buffer at 85V for 60 min along with 100 bp DNA ladder and documented using Ultraviolet gel documentation system (Gel Doc TM EZ Imager, Bio-Rad, Hercules, CA, USA).

Sequencing and Phylogenetic analysis

The positive samples of *H. canis*, which showed a distinct bright band in multiplex PCR, were purified from gels using the QIAquick® Gel Extraction Kit (Qiagen, GmbH, Germany) as per the manufacturer's instructions and subsequently sequenced by Eurofins Laboratories, Bangalore. The obtained sequences were aligned using MEGA version 12, and one representative sequence was submitted to the GenBank database under the accession number PV613156. The partial 18S rRNA gene sequence was compared with previously reported sequences available in GenBank. Multiple sequence alignment was performed using the MUSCLE algorithm, and similarity with homologous sequences was assessed using nucleotide (nBLAST) Basic Local Alignment Search Tool. Sequence identity analysis was carried out with the Clustal V method, while phylogenetic relationships were inferred from the nucleotide alignments by applying the maximum-likelihood method with 500 bootstrap replications in MEGA 12.[24]

Statistical analysis

The statistical evaluation was conducted using SPSS version 22 statistical windows software program. The link between multiple risk factors with prevalence of Hepatozoonosis along with

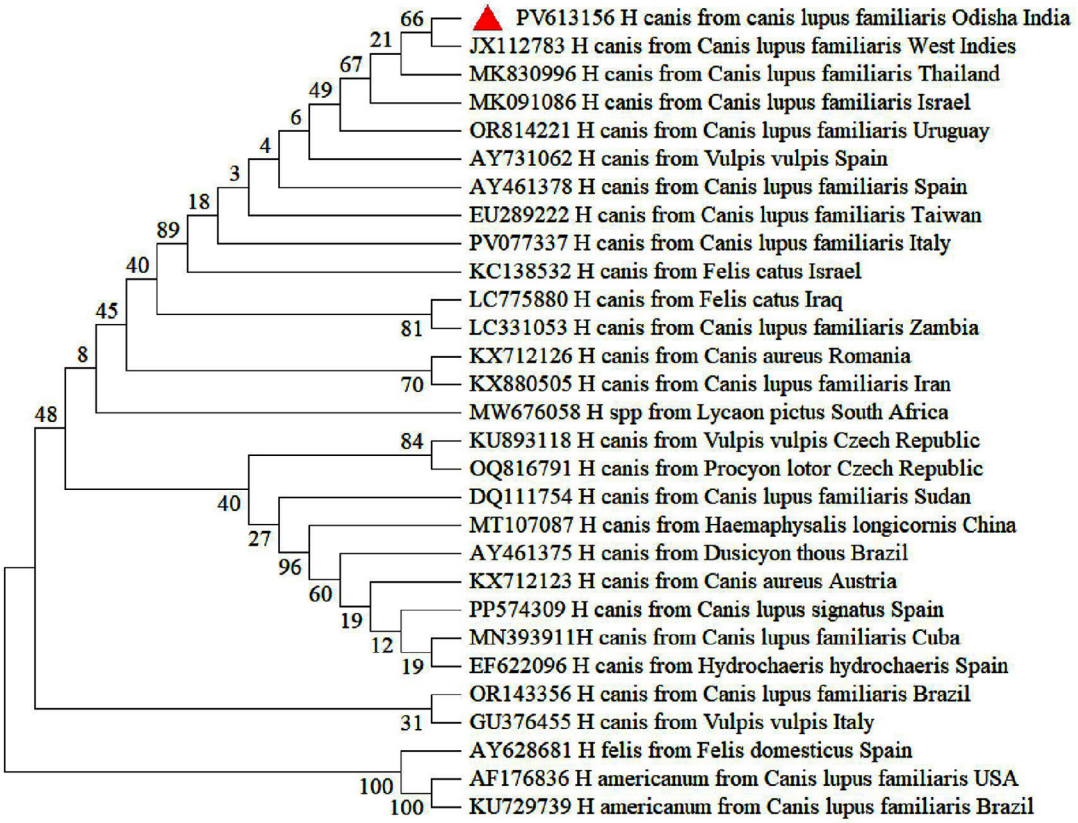

Figure 2. Phylogenetic analysis of *H. canis* based on partial sequence of 18S rRNA gene. Evolutionary analysis was conducted on 500 bootstrap replications using maximum likelihood method. Sequences are presented by GenBank accession number, pathogen species, host and country of origin

other tick-borne haemoparasitic co-infections was determined by the Chi-square test and probability of error was accepted up to 5% ($p < 0.05$). The Chi-square value (χ^2), degrees of freedom and p-values (≤0.05) were evaluated to determine the strength of association between variables. Analyzed risk factors included locality (Khordha, Cuttack, Puri, Ganjam, Nayagad, Bhadrak, Jajpur and Jagatsingpur), sex (male or female), age groups (<1, 1-5, or >5 years), breeds (Non-descriept, Golden Retriever, Labrador, Spitz and others), tick infestation, anti-tick treatments (nil, within 2 months or beyond 2 months) and outdoor activity (nil, limited, feral). One-way analysis of variance (ANOVA), by Duncan's multiple comparison tests with post hoc analysis was applied for comparison among groups for hematology.

RESULTS

Among the 212 dogs screened from 8 selected districts, total 04 (1.4%) dogs found to be microscopically positive for *H. canis* infection, However, 19 (9%) dogs are found to be positive by the SYBR Green based real-time PCR analysis that exposed the Tm values of 74.0 °C and the C_T value of 35 was set as the cut-off for positivity (supplementary Figure 2). The significant difference ($\chi^2 = 41.41$, df = 1, p = 0) between prevalence of both these method depicts higher sensitivity of the real-time PCR assay. Out of 19 qPCR positive samples, Multiplex PCR assay revealed 16 positive cases (7.5%), among which 11 dogs found to have mixed infection while 05 dogs were infected with single pathogen. *H. canis* was found to be the most commonly co-infected with *A. platys* in 06 (2.8%) cases. Co-infection of *H. canis, E. canis, A. platys* and *H. canis, A. platys, B. gibsoni* was recorded to be 02 (0.9%) in both cases, whereas co-infection of *H. canis, B. gibsoni, B. vogeli* and *A. platys* was 01 (0.5%) leading to an overall 57.89% concurrent infection of *H. canis* with other tick-borne pathogens (TBPs) (Table 3 and supplementary Figure 3). Though there was no

Table 1. The primer sequences used in real-time, singleplex conventional and multiplex PCR assay

No.	Parasite	Sequence	Size	Target gene	Reference
1	*H. canis*	HEP F 5'TCA ACT TTA TTAGAA GAG GCG CAT T 3' HEP R 5'TTT TCA CTT TGC GAT TTG CTA AGT T 3'	106 bp	18S ribosomal RNA gene	Thomas et al.[15]
2	*H. canis*	HEP F 5'CCTGGCTATACATGAGCAAAATCTCAACTT 3' HEP R 5'CCAACTGTCCCTATCAATCATTAAAGC 3'	737 bp	18S ribosomal RNA gene	Kledmanee et al.[21]
3	*B. vogeli*	BCV F 5'GTGAACCTTATCACTTAAAGG 3' BCV R 5'CAACTCCTCCACGCAATCG 3'	602 bp	18S ribosomal RNA gene	Kaur et al.[23]
4	*B. gibsoni*	BAG F 5'TGGGGTTTTCCCCTTTTTAC 3' BAG R 5'TTC AGC CTT GCG ACC ATA C 3'	489 bp	18S ribosomal RNA gene	Sathish et al.[22]
5	*A. platys*	AP F 5'GCCTTATGGGTCACCAGAGA 3' AP R 5'TTCAACTTCACGCCTCATTG 3'	292 bp	Heat shock protein GroEL gene	Sathish et al.[22]
6.	*E. canis*	EC F 5'CAGCCACACTGGAACTGAGA 3' EC R 5'GAGTGCCCAGCATTACCTGT 3'	817 bp	16S ribosomal RNA gene	Chen et al.[51]

Table 2. Cycling conditions for different type of PCR

No	Type of PCR	Cycling Condition
1	Real-time PCR	95 °C/2 min, 40 cycles of (95 °C/5 s, 60 °C/10 s), 95 °C/30 s, 65 °C/30 s, 95 °C/30 s
2	Singleplex conventional PCR	95 °C/2 min, 40 cycles of (95 °C/5 s, 60 °C/5 s, 68 °C/10 s), 72 °C/3 min
3	Multiplex conventional PCR	95 °C/15 min, 35 cycles of (94 °C/30 s, 60 °C/90 s, 72 °C/90 s), 72 °C/10 min

Table 3. Prevalence of *H. canis* infection and various co-infections in dogs with respect to locality

District	Total no. of dogs	No of tick infested dogs	Microscopy	PCR Assay				
				HC	HC+AP	HC+AP+EC	HC+BG+AP	HC+AP+BV+BG
Khordha	65	10 (15.4)	2 (3.1)	6 (9.2)	1 (1.5)	0	2 (3.1)	0
Cuttack	32	5 (15.6)	1 (3.1)	4 (12.5)	1 (3.1)	0	0	1 (3.1)
Puri	21	4 (19)	0	1 (4.8)	0	0	0	0
Ganjam	22	4 (18.2)	0	2 (9.1)	1 (4.5)	1 (4.5)	0	0
Nayagad	21	8 (38.1)	0	2 (9.5)	1 (4.8)	1 (4.8)	0	0
Bhadrak	15	2 (13.3)	0	1 (6.7)	0	0	0	0
Jajpur	19	2 (10.5)	0	0	0	0	0	0
Jagatsingpur	17	6 (35.3)	1 (5.9)	3 (17.6)	2 (11.8)	0	0	0
TOTAL	212	41 (19.3)	4 (1.9)	19 (9)	6 (2.8)	2 (0.9)	2 (0.9)	1 (0.5)
Pearson Chi-Square value		9.757 (0.203)	4.113 (0.767)	4.498 (0.721)	7.461 (0.382)	7.941 (0.338)	4.566 (0.713)	5.68 (0.581)

HC: *Hepatozoon canis*, HC+AP: *Hepatozoon canis* with *Anaplasma platys*; HC+AP+EC: *Hepatozoon canis* with *Anaplasma platys* and *Ehrlichia canis*. HC+BG+AP: *Hepatozoon canis* with *Babesia gibsoni* and *Anaplasma platys*. HC+AP+BV+BG: *Hepatozoon canis* with *Anaplasma platys*, *Babesia vogeli* and *Babesia gibsoni*. The figures in parenthesis for different districts represented in percent

Table 4. Risk factor analysis for *H. canis* infection

Risk factors	Parameters	No. of Dogs	No. of positives	Incidence	χ^2 value	Degree of freedom	p-value
Season	Summer	72	7	9.7	0.108	2	0.947
	Rainy	90	8	8.9			
	Winter	50	4	8			
Breed	Non-descriept	88	7	8	2.06	4	0.725
	Golden retriever	20	2	10			
	Labrador	48	4	8.3			
	Spitz	34	5	14.7			
	Other	22	1	4.5			
Sex	Female	103	9	8.7	0.012	1	0.911
	Male	109	10	9.2			
Age	<1 year	38	5	13.2	1.731	2	0.421
	1-5 year	132	12	12			
	>5 year	42	2	2			
Tick infestation	Yes	41	10	24.4	14.829	1	0.001*
	No	171	9	5.3			
Antitick treatment	Nil	32	9	28.1	17.719	2	0*
	Within 2 months	22	0	0			
	Beyond 2 months	158	10	6.2			
Outside activity	Nil	88	0	0	22.543	2	0*
	Limited	50	12	24			
	Feral	74	7	9.5			

*p-value is significant at ≤0.05

Table 5. Haematological changes in *H. canis* infected dogs

Parameters	PCR +ve for *H. canis* (n = 8)	*H. canis* with Co-infections (n = 11)	Control (n = 10)	p-value
Haemoglobin (g%)	9.69 ± 0.29^{b}	6.92 ± 0.48^{a}	13.83 ± 0.93^{c}	0*
TLCs (Thousands/µl)	15.03 ± 1.12	12.03 ± 1.78	11.01 ± 0.42	0.125
Neutrophil (%)	73.5 ± 1.03^{b}	68.63 ± 4.09^{b}	60.1 ± 1.29^{a}	0.012*
Lymphocyte (%)	23.12 ± 1.12^{a}	28.18 ± 4.19^{ab}	33.2 ± 2.22^{b}	0.110
Monocyte (%)	1.37 ± 0.18	1.27 ± 0.19	1.7 ± 0.15	0.218
Eosinophil (%)	1 ± 0	1 ± 0	1 ± 0	1
Basophil (%)	1 ± 0	0.9 ± 0.09	1 ± 0	0.457
Platelets (Thousands/µl)	118 ± 8.34^{b}	75 ± 5.68^{a}	328.5 ± 9.81^{c}	0*
Packed Cell Volume (%)	28.08 ± 1.1^{b}	20.81 ± 1.49^{a}	40.9 ± 0.97^{c}	0*
TEC (Millions/µl)	4.97 ± 0.39^{b}	3.64 ± 1.14^{a}	6.78 ± 0.19^{c}	0*
Mean Cell Volume (fl)	66.01 ± 0.51^{b}	63.79 ± 0.77^{a}	69.4 ± 0.37^{c}	0*
Mean Cell Hemoglobin (pg)	23.01 ± 0.21^{b}	21.56 ± 0.32^{a}	23.37 ± 0.41^{b}	0.001*
Mean Cell Hemoglobin Concentration (g/dl)	31.08 ± 0.24^{b}	29.79 ± 0.22^{a}	32.36 ± 0.55^{c}	0*

*p-value is significant at ≤0.05

Values (mean ± S.E.) that have no common superscript (small letter in a row) vary significantly at $p \leq 0.05$.

Table 6. Prevalence (in %) of Hepatozoon canis in ticks related to the host infection status

H. canis	Positive ticks		Negative ticks		Statistics	
	Rhipicephalus spp.	*Haemaphysalis* spp.	*Rhipicephalus* spp.	*Haemaphysalis* spp.	Pearson Chi-Square value	p-value
Positive Dogs	08 (19.5%	0	01 (2.4%)	01 (2.4%)	42.65	0*
Negative Dogs	06 (14.63%)	0	23 (56.09%)	02 (4.87%)		
Total	14 (34.14%)	0	24 (58.53%)	03 (7.3%)		

*p-value is significant at ≤0.05

significant risk of hepatozoonosis with respect to location, however Jagatsinghpur district revealed the highest prevalence of infection (17.6%) and zero prevalence was recorded from Jajpur district. Among 08 cases of single infection, only 03 dogs revealed clinical signs of intermittent fever, lethargy and emaciation, whereas among 11 cases of mixed infection, 08 dogs revealed signs such as inappetence, lymphadenopathy, tachypnea, fever, pale mucous membranes, hind limb weakness, rhinitis and bloody diarrhea.

The analysis risk factors associated with prevalence of hepatozoonosis (Table 4) revealed no significant risk of infection associated with breed predisposition, age and sex of dogs. However, highest incidence of hepatozoonosis was found in summer season (9.7%), spitz breed (14.7%), male dogs (9.2%) and younger dogs of <1 year age group (13.2%).

Tick infestation, anti-tick treatment and outdoor activity of dogs revealed highly significant association ($p < 0.01$) with risk of *H. canis* infection. Out of 212 screened dogs, 41 dogs found to be currently ticks infected, whereas, 171 dogs had history of tick infestation. Highest incidence of infection was recorded in dogs with presence of tick (24.4%) rather than previously infested dogs (5.4%). Likewise, dogs without any anti-tick treatment were found to have highest incidence of infection (28.1%), but not a single infection was found in dogs that had undergone anti-tick treatment within 2 months of screening and in dogs that are strictly domesticated without any outdoor activity. Surprisingly, domesticated dogs with limited outdoor activity are found to have significantly higher incidence of hepatozoonosis (24%) surpassing the feral dogs (9.5%).

The hematological parameters of PCR positive dogs for *H. canis* (both single infection as well as mixed infection) and healthy control group were depicted in Table 5. Hematological values of single and mixed infection groups revealed significantly lower level of mean Hb, platelets, TEC, MCV, MCH, MCHC, PCV level and higher relative neutrophil count with normal TLC level, compared to the healthy control group. Amongst the infected group, dogs with mixed infection were found to have a significantly ($P < 0.01$) lower mean Hb, platelets, TEC, PCV, MCV, MCH, MCHC level than

the groups with single infection while leucocyte parameters remained unaltered.

The identification of 108 ticks collected from 41 tick-harbouring host revealed that 38 dogs were infested with *Rhipicephalus* species, and only 3 dogs were infested with *Haemaphysalis* spp., while no mixed tick infestation were detected during screening. A total 101 number of *Rhipicephalus* and 7 *Haemaphysalis* ticks were collected from 41 infested dogs. Among the 41 pooled samples, the incidence of *protozoa* DNA in *Rhipicephalus* spp. was 34.14% (14/41) (supplementary Figure 4), while the *Haemaphysalis* spp. were found negative. Out of 14 positive ticks, 8 *Rhipicephalus* spp. ticks were derived from positive dogs (19.5%), while 6 (14.63%) were derived from uninfected dogs. However, in 01 (2.4%) case, *Rhipicephalus* ticks collected from *H. canis* positive dogs had no evidence of *H. canis* DNA (Table 6).

The phylogenetic analysis using a 737 bp fragment of the partial 18S rRNA gene of *H. canis* retrieved from the NCBI record revealed that nucleotide sequence from Odisha isolate was clustered in one major clade with several mini subclades and was closely related to the sequences of *H. canis*, isolated from dogs of West Indies, Thailand and Israel (GenBank: JX112783, MK830996 and MK091086 respectively) with exclusion of the clade containing other species of *Hepatozoon* including *H. felis* and *H. americanum* (Figure 2). The nucleotide sequence identity study revealed 98%-100% similarity with other global isolates of *H. canis*, included in this study with the highest divergence of 1.7% between *H. canis* isolates from Sudan (DQ111754) and Odisha (PV613156) (Figure 3).

DISCUSSION

This researchprovides the first molecular confirmation of *Hepatozoon canis* from dogs and vector ticks in Odisha along with a systematic epidemiological approach and evaluation of hematological alterations in affected dogs with hepatozoonosis and concurrent infections (mostly *A. platys* infections).

In the present study, only 1.4% dogs were microscopically positive, whereas prevalence of hepatozoonosis detected by real-time PCR and conventional multiplex PCR were 9% and 7.5% respectively depicting their higher sensitivity. Though, detection of capsular gamonts in blood

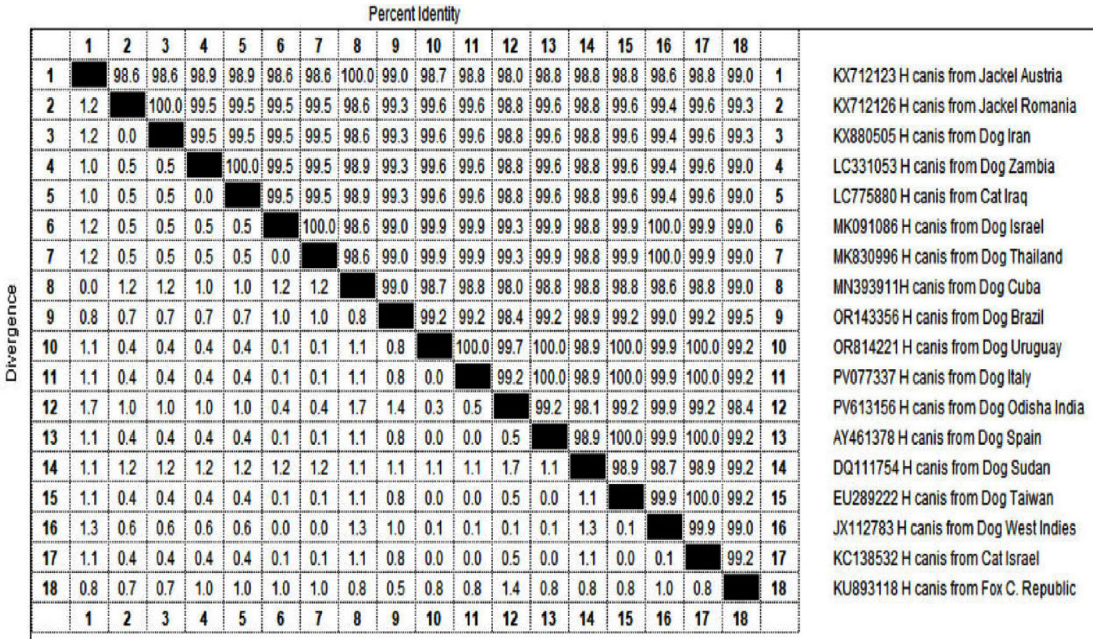

Figure 3. Nucleotide difference estimation (lower left triangle) and percentage identity (upper right triangle) for *H. canis* isolate from Odisha, India

films or microschizonts with a distinct "wheel-spoke" shape in bone marrow, spleen or lymph node tissue sections through microscopy are labeled as the 'gold-standard' test for diagnosis of hepatozoonosis, still it lacks reliability due to its inability to detect infections and co-infections in carrier animals as well as during subclinical and latent infection in diseased animal.[25,26] On the other hand, PCR assays have been proven to be highly efficient in diagnostic performance owing to their higher specificity and sensitivity in diagnosis of ongoing infection.[27] Multiplex PCR assays provide the benefit of detecting multiple parasitic DNA targets simultaneously within a single reaction tube.[8] However, real-time PCR is considered more promising than conventional PCR and sequencing techniques for diagnosing and monitoring various diseases. This is because real-time PCR tracks amplicon formation during the reaction, enabling highly sensitive and precise quantification of a particular DNA in samples.[28] The detection of *H. canis* infection in dogs (9%) through molecular methods found herein, Odisha targeting 18S r RNA gene was less than that of global reports recorded in Asia (11.4%-42.9%) Europe (44.67%-57.8%) North America (47.5%), South America (53.3%),[29] However the state average also falls below the prevalence rates of other states of India such as 43.8% in Maharastra, 24% in Ladakh, 25.4% in Punjab and 38% from North eastern states, but higher than those of 6.63% in Tamil Nadu, 4% in Kerala.[12-15] Such variation in prevalence may be attributed to factors such as difference in sample size and, sampling strategy (dogs with presence or history of tick infestation).

Due to the continuous progress of molecular diagnostic testing, co-infections of canine tick-borne diseases have been found to be a common global phenomenon[30] and this study firmly suggests the same for the dogs of this geographical location. *H. canis* is often found to be co-infected with other TBPs as the *Rhipicephalus* ticks harbour and transmits multiple pathogens to healthy dogs upon exposure.[31,32] The highest rate of coinfection mostly with *A. platys* (5.1%) found herein, has been also previously reported in Tamil Nadu as 8.3%,[13] however, *H. canis* was reported to be most commonly coinfected with *B. gibsoni* in northeast India and Punjab (33% and 4.7%)[16,17] and in Delhi, Mumbai and Haryana with *E. canis* (28%, 36.4% and 7.5%)[4,32] Consistent with the other studies, some dogs revealed the clinical sign of canine hepatozoonosis, however, most mono-infected cases were found to be asymptomatic and clinical signs are more prominent in case of mixed infections.[11,33]

Recent evaluations of risk factors linked to the prevalence of hepatozoonosis showed no significant differences based on season, age, sex, or breed, which is consistent with our observations.[5,11,25,34] However, significantly higher prevalence were depicted in non-descript breeds[14]; terrier breeds,[11] and although the highest risk is during summer when tick activity is greatest,[34] infections may also be detected in colder months, most likely due to lingering infections from earlier exposures.[35]

Presence of ticks rather than history of tick infestation was significantly related to *H. canis* infection similar to previous reports[5,12] as the ticks, acts as putative vector for *H. canis* infection and ingestion of infected ticks may lead to active and ongoing infection depending upon the immune status of the dogs.[36] Applying proper tick control strategies, including the use of suitable acaricides and routine grooming, helps lower the risk of occurrence in dogs and absence of treatment significantly increases the risk of infection.[37] Although strict in-house domestication was found to have significant role in minimizing the haemoparasitic infections due to minimal tick exposure, still dogs with limited outdoor activity unexpectedly revealed higher prevalence than feral dogs. This might be due to the fact that outdoor activity of pet dogs near vegetation during dusk and dawn increases the chance of tick exposure.[38] Although feral dogs have greater exposure to tick vectors and more frequent contact, their immune system appears to be better adapted to hemoparasitic infections than that of owned dogs. The notably higher expression of the granzyme B gene in stray dogs, compared with owned dogs, contributes to the commencement of apoptotic pathways within their immune defense.[39] Some authors report that common haematological alterations in *H. canis* infected dogs with or without concurrent infection; have been anaemia followed by thrombocytopenia that corroborates with our finding.[36,40] In most cases, the condition presents as

normochromic, normocytic regenerative anemia[26]; however, reduced hemoglobin concentration and diminished red cell staining suggestive of microcytic hypochromic anaemia, observed in this study, have also been described previously.[34,41] The leukocyte count in dogs infected with *H. canis* is generally normal or elevated.[3,36] The anemia and neutrophilia recorded here are likely associated with necrosis and inflammatory changes induced in different organs like liver, spleen and lungs.[42] Thrombocytopenia in *H. canis* infection may result from enhanced sequestration of platelets within the liver or spleen, or from reduced platelet production due to bone marrow hypoplasia.[43]

In the present study, coinfection of *H. canis* mostly with *A. platys* revealed a significant drop in mean Hb, platelets, TEC, PCV, MCV, MCH, MCHC level than single infected dogs suggestive of potential pathogen induced red cell destruction, loss of red cell morphology and poor hemoglobinization. Though hematological alterations in *H. canis* and *A. platys* coinfected dogs were not previously reported, however, anemia and cyclic episodes of thrombocytopenia were reported to be a consistent finding of *A. platys* infection[44,45] hence the coinfection with *H. canis* might have exacerbated the condition.

Similar to earlier finding, *Rhipicephalus* spp. ticks were found to be the most predominant vector, parasitizing the canine population of Odisha, followed by *Haemaphysalis* spp.[46] Although the *Rhipicephalus* species (*R. sanguineus*) ticks are recognized as the most prominent ticks for *H. canis*, with prevalence of 42.5% in Tamil Nadu, India[11] and the primary route of infection is through the ingestion of ticks that contain mature oocysts, still other tick species, namely *Haemaphysalis longicornis* and *Haemaphysalis flava* were reported to carry and spread *H. canis* infections in Japan and *Amblyomma ovale* tick in Brazil.[47,48] However, only *Rhipicephalus* spp. was found to harbour the pathogen at a prevalence of 34.14% in our study, and *Haemaphysalis* species were negative for *H. canis* DNA.

Although in 19.5% instances, the *H. canis* DNA was found in both ticks and their host, still 14.63% ticks were found positive for the pathogen though collected from uninfected dogs. It may be explained by the fact that there is no evidence that *Hepatozoon* spp. are transmitted through a blood meal or by salivary secretion from the final tick vector to the vertebrate host. The only confirmed route of infection is through ingestion of an infected tick. Alternatively, since the tick is a three-host species, it may acquire *H. canis* from other dogs during its larval or nymphal stages.[36] Moreover, 2.4% *Rhipicephalus* spp. ticks found negative though collected from infected dogs as an attachment time of at least 48 hr is necessary to acquire infection.[49] Vertical transmission of *H. canis* infection during early gestation has also been recorded in dogs.[50] On the basis of evolutionary tree, the similarity among the geographically distinct global isolates and *H. canis* Odisha isolates indicate a global spread of the pathogen and its vectors.

CONCLUSION

The maiden molecular report of *H. canis* in host and ticks with a lower prevalence rate than other parts of India and abroad may indicate a low circulation of this pathogen in Odisha. Globalization, increased travel of pets, importation of dogs from endemic areas and climate change may be responsible factor for vector distribution of pathogen and introduction of this vector-borne pathogen in this particular geographical area. The vector role of *Rhipicephalus* spp. ticks were confirmed in this study. The dogs positive for *H. canis* mostly found to be concurrently infected with *A. platys.* In dogs with anaemia and thrombocytopenia having presence/history of tick infestation, *H. canis* infection and/or co-infection should taken into consideration for differential diagnosis of tick-borne pathogens in this area. The evolutionary pattern observed herein may be further investigated by targeting other genes.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.20.1.19

Additional file: Figure S1-S4.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

MD and SB collected resources. RCP acquired funding. SKS, RCP and SB supervised the study. MP and SRM applied methodology. CM, MD and DKK performed analysis. MP and GRJ performed data curation. MP performed investigation. SRM performed validation. MP wrote the manuscirpt. MD reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

This study was approved by the Institutional Animal Ethical Committee (Regd. No. 433/CPCSEA), College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar vide reference no 833/IEAC dated 7.10.2023.

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