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Co-infections of major tick-borne pathogens of dogs in Andhra Pradesh, South India

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Abstract

Implementing hemoprotozoan control strategies in dogs has become difficult because of the co-infections. A multiplex polymerase chain reaction (PCR) was carried out for simultaneous detection of the co-infections of *Babesia gibsoni, B. vogeli*, *Hepatozoon canis* and *Ehrlichia canis* from dogs (N = 442) in Andhra Pradesh, South India. The co-infection combinations were classified as (i) *B. gibsoni* + *B. vogeli* + *E. canis* + *H. canis* (BEH), (ii) *B. gibsoni* + *B. vogeli* + *E. canis* (BE), (iii) *B. gibsoni* + *B. vogeli* + *H. canis* (BH) and (iv) *E. canis* + *H. canis* (EH) groups. The parasite-specific multiplex PCR amplified 18S rRNA gene of *B. gibsoni*, *B. vogeli* and *H. canis* and *VirB9* gene of *E. canis*. The age, gender, breed, medium, living condition and region of dogs were studied as risk factors for co-infections using logistic regression model. Among the co-infections, the incidence was 1.81%, 9.28%, 0.69% and 0.90% for BEH, BE, BH and EH infections, respectively. Young age (< one year), females, mongrels, rural dogs, kennel dogs and presence of ticks were the identified risk factors for overall prevalence of tick-borne pathogens. The incidence of infection was less in rainy season, especially in dogs with a previous acaricidal treatment. The study concludes that the multiplex PCR assay could simultaneously detect natural co-infections in dogs, emphasizing the need for the assay in epidemiological studies to reveal the real pattern of pathogens and select pathogen-specific treatment protocols.

Introduction

The higher prevalence of tick-borne diseases in dogs is a major concern in tropical countries with abundant tick population. Among the tick-borne diseases, babesiosis, hepatozoonosis and ehrlichiosis are common diseases and are endemic in India.[1] Canine babesiosis caused by hemoprotozoan parasites of the genus *Babesia*, is characterized by fever, anemia, lethargy, icterus and hemoglobinuria. Canine monocytic ehrlichiosis is caused by an intracellular rickettsia, *Ehrlichia canis*, which parasitizes monocytes.[2] The disease is regarded as an emerging tick-borne zoonosis and characterised by thrombocytopenia, lymphadenopathy, ocular signs, bleeding diatheses and irreversible bone marrow destruction.[2] Canine hepatozoonosis caused by *Hepatozoon canis* is another tick-borne hemoprotozoan disease characterised by fever, anemia and lethargy.[3]

The brown dog tick, *Rhipicephalus sanguineus sensu lato (sl)*, has been reported as a competent or potential vector for babesiosis, ehrlichiosis and hepatozoonosis, enabling co-infection of parasites in the dog host.[4] Information regarding the epidemiology of canine tick-borne diseases in India is limited despite the favourable climate for ticks' growth and the large population of stray dogs.

Microscopy remains the cornerstone in diagnosing hemoparasites and is considered as the simplest diagnostic test for veterinarians. Nevertheless, their diagnosis poses a significant challenge in chronically infected and carrier dogs due to the low or often intermittent parasitemia conditions. Although the currently available serological tests are highly sensitive, they are moderately specific because of antigenic cross-reactions to other hemoparasites and normal erythrocytes.[2] Therefore, molecular diagnosis based on polymerase chain reaction (PCR) test, especially multiplex PCR, has made it possible

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to detect and identify hemoparasites simultaneously with greater sensitivity and specificity.[2] One more advantage with PCR lies in its ability to amplify more than one target sequence by including more than one pair of primers in the reaction.[5-7] The potential of multiplex PCR assay in detecting mixed pathogenic infections of *E. canis*, *Babesia gibsoni, Babesia vogeli* and *H. canis* in various combinations was demonstrated earlier.[7]

Periodic surveillance of the prevalence of co-infections of these parasites within a given area is a prerequisite for successful formulation and implementation of effective parasite control strategies. However, no studies exist on simultaneous detection of tick-borne co-infections while appraising the role of risk factors in their prevalence in tropical regions. Hence, the current study aimed at detecting the risk factors and incidence of co-infections of *B. gibsoni, B. vogeli*, *H. canis* and *E. canis* of dogs in Andhra Pradesh (AP), South India, using microscopy and multiplex PCR.

Materials and Methods

Study area and demographics. The AP is a state in the southern India and lies between 12° 41' and 19.07° N latitude and 77° and 84° 40' E longitude. The climate of AP is generally hot and humid with the mean annual temperature of 33.20 ˚C. Based on the annual average rainfall, the state is divided into six agro-ecological zones with a mean annual rainfall of 1,000 mm in Coastal Andhra and 672 mm in Rayalaseema.[8,9] A total of 442 dogs, presented to private or government hospitals in Rayalaseema (n = 160) and Coastal Andhra (n = 282) regions, AP, were examined. All the procedures were carried out in full compliance with the recommendations in the guidelines of Institute's Animal Ethics Committee of Sri Venkateswara Veterinary University, AP, India.

Collection of blood samples. The dogs (n = 442) presented to the Veterinary Polyclinics and Veterinary Hospitals in AP, South India, during the period of one year from July 2019 to June 2020 were evaluated for the co-infections. Dogs were selected on the basis of presence of tick infestation at the time of presentation and/or showing clinical signs suggestive of the hemoprotozoan and hemo-rickettsial infections, namely, fever, hemoglobinuria, anemia, bleeding episodes, jaundice, lameness, neuro-logical signs, paralysis, lethargy, etc. Whole blood samples (n = 160) were collected aseptically from the cephalic vein and loaded into Ethylene diamine tetra-acetic acid (EDTA) vacutainers (Nexamo Technoplast Pvt. Ltd., Punjab, India).

Data collection and analysis. The dogs were categorized into two groups (young as ≤ one year and adult as ≥ one year) by age. Breed was classified as pure-breed and mixed-breed (mongrels) and gender as male and female. A complete database for each dog was developed by giving a questionnaire containing questions regarding age, sex, breed, living condition of the dog, acaricidal history and origin or owner's address to identify risk factors associated with these tick-borne diseases. The presence or absence of ticks was also recorded. When present, a minimum of two ticks were collected and stored in 70.00% ethanol solution for later identification at genus level using the morphological key as per the procedure.[10]

Microscopy. Thin blood smears were prepared from whole blood, air-dried, fixed in 100% ethanol and later stained with Wright-Giemsa (M/S Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) for microscopic screening.[11] Blood samples were further preserved at – 20.00 ˚C until DNA extraction. The co-infections were classified as (i) *B. gibsoni + B. vogeli + E. canis + H. canis* (BEH), (ii) *B. gibsoni + B. vogeli + E. canis* (BE), (iii) *B. gibsoni + B. vogeli + H. canis* (BH) and (iv) *E. canis + H. canis* (EH) groups.

Genomic DNA extraction. The DNA was isolated from each blood sample (200 µL) using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. On determining concentration, samples were further diluted to 100 ng μL^{-1} and stored at – 20.00 ˚C until use.

Polymerase chain reaction protocol. The multiplex PCR protocol was performed on diluted genomic DNA using cycling conditions and primers for the amplification of 18S rRNA gene of *B. gibsoni, B. vogeli*, 18S rRNA gene of *H.n canis* and *virB9* gene of *E. canis*.[7] The PCR reaction was carried out in a final volume of 50.00 µL containing 5.00 µL genomic DNA, 25.00 µL of master mix (Genei, Bengaluru, India), 0.50 pmol of each forward and reverse primers and nuclease free distilled water (18.80 µL). A negative control with ultra-pure water in substitution of DNA was run along with the samples at every PCR setup. The oligonucleotide primers (Table 1) for PCR assay were designed as per the guidelines of Kledmanee *et al*.[7] The PCR amplicons were analyzed on 2.00% (w:v) agarose gel containing 0.50 µg mL^{-1} ethidium bromide (M/S HiMedia Laboratories Pvt. Ltd., Mumbai, India) in 1X Tris-acetate EDTA buffer and visualized on ultraviolet transilluminator (Gel doc, Bio-Rad Laboratories, Hercules, USA). The positive multiplex PCR positive products were sequenced by Sanger's method and compared for similarity with sequences available in GenBank® using the BLAST program.[12]

Statistical analysis. The frequency, prevalence of overall, single species and mixed infection were evaluated. Data were analyzed looking at the influence of location, age group, gender, breed and season. The chi-square test was used for comparison of the frequencies by SPSS Software (version 20.0; IBM Corp., Armonk, USA). The data were subjected to multicollinearity test to ascertain that there is no collinearity between the independent variables. The Glejser test for heteroscedasticity was employed to test whether the variation of the error from a regression is dependent on the independent variables (factors). A *p* less than 0.05 was considered statistically significant.

Table 1. The primer sequence of different species-specific parasites used in the current study.

Parasite	Primer	Sequence (5'-3')	Length (bases)	Product size (bp)
E. canis	Ehr1401F	CCATAAGCATAGCTGATAACCCTGTTACAA	30	380
	Ehr1780R	TGGATAATAAAACCGTACTATGTATGCTAG	30	
***Babesia* spp.**	Ba103F	CCAATCCTGACACAGGGAGGTAGTGACA	28	619
	Ba721R	CCCCAGAACCCAAAGACTTTGATTTCTCTCAAG	33	
H. canis	Hep001F	CCTGGCTATACATGAGCAAAATCTCAACTT	30	737
	Hep737R	CCAACTGTCCCTATCAATCATTAAAGC	27	
B. gibsoni	Gib599F	CTCGGCTACTTGCCTTGTC	19	671
	Gib1270R	GCCGAAACTGAAATAACGGC	20	
B. vogeli	BAB1F	GTGAACCTTATCACTTAAAGG	21	546
	BAB4R	CAACTCCTCCACGCAATCG	19	

Results

Demographics. Age of the animals ranged from three months to 15 years. A total of 172 dogs were less than one year and 270 were above one year; while, the males to female ratio was 1.125:1 (234 *vs*. 208). The majority (79.60%) of animals were pure breed (n = 352). Of the sampled dogs, 276 were urban and 166 were rural dogs. Of 442, 397 (89.81%) dogs were found to be infected with tick infestation and all ticks (n = 908) were identified as *R. sanguineus s.l*.

Microscopy and multiplex PCR. The microscopic images (1,000× magnification) of *E. canis*, *H. canis* and *B. gibsoni* and B. *vogeli* are presented in Figures 1A, 1B, and 1C, respectively. Subjecting to PCR revealed the presence of tick-borne pathogens yielding 380 bp (*E. canis*), 737 bp (*H. canis*) and 619 bp (*B. gibsoni* and *B. vogeli*) as shown in Figure 1D. The microscopic examination of Giemsa-stained peripheral thin blood smears revealed the presence of co-infections in nine dogs (2.00%). Whereas, the multiplex PCR assay revealed 12.67% (n = 56) prevalence of co-infections with the highest incidence of BE combination (9.28%; n = 41). Forty-seven samples identified as negatives (false-negatives) by Giemsa-staining were found positive with at least one co-infection by multiplex PCR. In comparison with microscopy, the sensitivity of PCR test was 100% to detect the incidence of co-infections in dogs.

Incidence of co-infections. The incidence of single infections of *E. canis*, *H. canis*, *B. gibsoni* and *B. vogeli* was 30.77%, while; that of co-infections was 12.67%. Within the co-infections, the incidence was 1.81%, 9.28%, 0.69% and 0.90% for BEH, BE, BH and EH infections, respectively. The prevalence of co-infections of major tick-borne pathogens is presented in Tables 2 and 3, respectively.

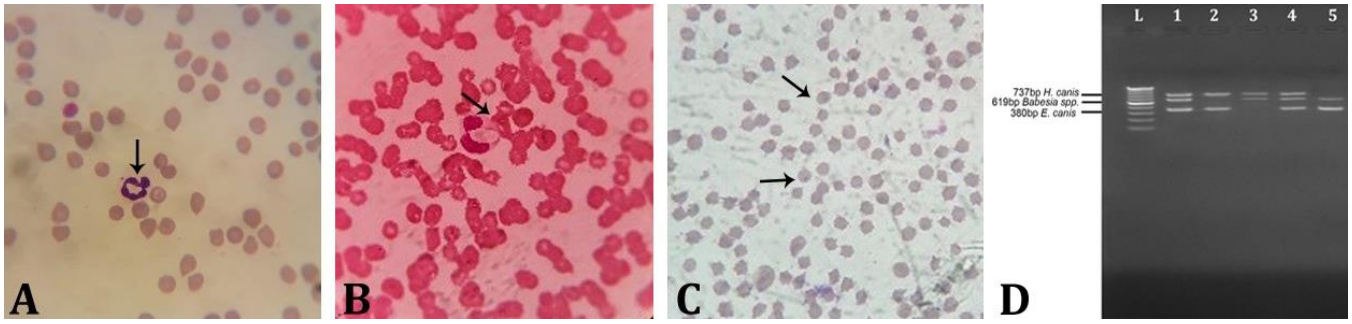

Fig. 1. Photomicrographs of **A)** *E. canis*; **B)** *H. canis*; **C)** *Babesia* spp. indicated by black arrows. (Giemsa staining; 1,000×). **D)** Agarose gel electrophoresis of amplicons of hemoparasites. L: 100bp DNA ladder; Lanes 1, and 4: Co-infection with three spp.; Lane 2: Co-infection with *E. canis* and *H. canis*; Lane 3: Co-infection with *E. canis* and *B. gibsoni* and *B. vogeli*; Lane 5: Co-infection with *Babesia* spp. and *E. canis*.

Table 2. The prevalence (%) of co-infections of major tick-borne pathogens in dogs by the host-attributes.

Co-infection combinations	Total (n = 442)	Age		Gender		Breed		Medium	
		< 1 year (n = 172)	≥ 1 year (n = 270)	Female (n = 208)	Male (n = 234)	Pure breed (n = 352)	Mongrels (n = 90)	Urban (n = 276)	Rural (n = 166)
Single infections[1]	136(30.77)	71(41.27)	65(24.07)	67(32.21)	69(29.49)	93(26.42)	43(47.78)	96(34.78)	40(24.10)
Co-infections									
B. gibsoni + B. vogeli + E. canis + H. canis	8(1.81)	2(1.16)	6(2.22)	3(1.44)	5(2.14)	5(1.42)	3 (3.34)	6(2.17)	2(1.20)
B. gibsoni + B. vogeli + E. canis	41(9.28)	18(10.47)	23(8.51)	22(10.58)*	19(8.12)	27(7.73)	14(15.56)*	24(8.70)	17(10.24)
B. gibsoni + B. vogeli + H. canis	3(0.69)	1(0.58)	2(0.74)	1(0.48)	2(0.85)	2(0.57)	1(1.11)	1(0.36)	2(1.20)
E. canis + H. canis	4(0.90)	2(1.16)	2(0.74)	1(0.48)	3(1.28)	3(0.85)	1(1.11)	2(0.72)	2(1.20)
Total co-infections	56(12.67)	23(13.37)	33(12.22)	29(13.94)	29(12.39)	37(10.51)	19(21.11)	33(12.00)	23(13.86)

[1] Single infections include the individual infections of *E. canis*, *H. canis*, *B. gibsoni*, and *B. vogeli*

* indicate significant difference at $p < 0.05$.

Table 3. The prevalence (%) of co-infections of major tick-borne pathogens in dogs by living condition and region.

Co-infections combinations	Total (n = 442)	Living condition			Region	
		Kennel dogs (n = 36)	Pet dogs (n = 335)	Stray dogs (n = 71)	Rayalaseema (n =160)	Costal Andhra (n = 282)
Single infections[1]	136(30.77)	25(69.44)***	80(23.88)	31(43.66)*	44(27.50)	92(32.62)
Co-infections						
B. gibsoni + B. vogeli + E. canis + H. canis	8(1.81)	2(5.56)**	4(1.19)	2(2.82)	2(1.25)	6(2.13)
B. gibsoni + B. vogeli + E. canis	41 (9.28)	5(13.89)*	28(8.36)	8(11.27)	10(6.25)	31(10.99)
B. gibsoni + B. vogeli + H. canis	3 (0.68)	1(2.78)	0 (0.00)	2(2.82)*	0 (0.00)	3(1.06)
E. canis + H. canis	4(0.90)	1(2.78)	1(0.30)	2(2.82)*	0 (0.00)	4(1.42)
Total co-infections	56(12.67)	9(25.00)**	33(9.85)	14(19.72)*	12(7.50)	44(15.60)

[1] Single infections include the individual infections of *E. canis*, *H. canis*, *B. gibsoni*, and *B. vogeli*.
*, **, and *** indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively.

The total co-infection percent was influenced by living condition with a higher incidence in kennel dogs followed by stray dogs. The chi-square test revealed that the kennel dogs were more prone ($p < 0.01$) for BEH co-infection; while, the incidence of BH and EH combinations was higher ($p < 0.05$) in stray dogs. Further, the prevalence of BE co-infections was higher ($p < 0.05$) in females, mongrels and kennel dogs. The information provided by owners suggested that 369 of the 442 dogs (83.48%) have received acaricidal treatment for tick control. Pet dogs were received acaricidal treatment once or twice a year with cypermethrin or macrocyclic lactones. After organizing the groups by last known acaricidal treatment, significant difference in parasite prevalence was observed between dogs assumed to be followed treatment and not-followed except for canine babesiosis.

Discussion

Microscopy revealed a six-fold discrepancy in the pathogen quantification compared to multiplex PCR. The microscopy method reported a false negative rate of 83.92%. Only nine out of 56 dogs were correctly diagnosed with any of the mixed co-infections of BEH, BE, BH or EH. Rucksaken *et al*. compared the conventional PCR with routine blood smear detection test and found a false negative rate of 36.73%.[13] The higher false negative rate in this study could be due to the low extent of blood parasitic load with few infected cells, hampering the microscopic examination. Based on the PCR, 56 dogs out of 442 (12.67%) were found infected with at least one tick-borne pathogen. Likewise, Kordick *et al*. reported a higher incidence rate of co-infections using PCR assay in a kennel of North Carolina, United States.[4] The multiplex PCR assay revealed higher sensitivity to detect these tick-borne pathogens during natural infections and offered the advantage of detecting more than one parasite DNA in a single reaction.

The discrepancy between two tests is a common phenomenon observed earlier by several researchers.[13] The microscopy method requires highly experienced personnel for accurate diagnosis of co-infections and hence, the misdiagnosis is a common problem with microscopy. Moreover, co-infections may cause more complex disease conditions; therefore, importance should be given to such co-infections through simultaneous detection of pathogens by multiplex PCR for the selection of appropriate treatment protocol.[14]

The high prevalence rate obtained in the current study might be due to the fact that the study was carried out on dogs referred to the Veterinary Clinics presenting clinical manifestations consistent with these tick-borne diseases. The rate of co-infections in dogs in AP was lower (12.70%) than in other parts of India, which evidenced a range of 19.50% to 39.00%.[1] Yet, the target parasites covered in the current study were different from those studies. The occurrence of co-infections of babesiosis, ehrlichiosis and hepatozoonosis could be attributed to the existence of *R. sanguineus s.l.*, which is a common tick vector for these pathogens.[15] The *R. sanguineus s.l.* was the exclusive species found on dogs in the study area. In concurrence with the earlier reports, the co-infection with *Babesia* species was a frequent phenomenon in the study area.[16]

Kennel and stray dogs are more prone for tick-borne pathogens; presumably due to the higher extent of tick infestation compared to the pet dogs.[17] The higher prevalence in female population might be due to hormonal disturbances, weakening their immune system. The acaricidal treatment or deworming protocols might have lessened the tick infestation; thus, decreasing the incidence of tick-borne diseases. Apart from the diagnostic challenges, co-infections may modulate disease severity through synergistic effects. Consequently, summarizing different types of co-infections could ease the diagnostic challenges raised during exposure to multiple pathogens and pave the way for effective treatment.

The multiplex PCR could simultaneously detect natural co-infections of ehrlichiosis, hepatozoonosis and babesiosis in dogs and could be used for epidemiological mapping of various tick-borne pathogens. The study concludes that, apart from the routinely used conventional methods, PCR-based assays should be employed to identify causative agents of tick-borne diseases during their early phase, clarify the true spectrum of diseases and evaluate the responses to treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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