



Prevalence and molecular characterization of ticks infesting dogs in Agartala, Tripura

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ABSTRACT

Due to its ability to spread disease from one host to another, tick infestation is the most frequent issue pet owners' deal with and should be taken seriously. The goal of the current study was to ascertain the prevalence of tick infestation in dogs in Agartala as well as its molecular identity and characterisation. A total of 1776 dogs of various breeds, ages, sexes, and categories were included in the study, which was conducted from March 2022 to February 2023 over the course of a calendar year. The total prevalence of ticks was reported to be 62.12%. According to study on breed, sex, age, and category, German spitz, male dogs, dogs under 1 year of age and stray dogs had the highest frequency of tick infestation. All throughout the year, tick infestations were seen, with the monsoon season seeing the highest frequency. Based on *16S rRNA* gene amplification and sequence analysis, molecular identification revealed that all isolated ticks were members of the *Rhipicephalus sanguineus* species. Using a subset of the *16S rRNA* gene, phylogenetic analysis revealed that *R. sanguineus* (OR244468.1) from Agartala (TrRS-PB) was part of the same clade as isolates from Kerala and Assam.

Keywords: Agartala, Dogs, Molecular characterization, Prevalence, Ticks, Tripura

Dogs are considered as “Man’s Best Friend”. However, their health and well being has become a concern to their owner or attendants. Like other mammals, dogs are also prone to many parasitic diseases, viz. helminthic, protozoal and ectoparasitic of which, tick infestation is a major health concern. Ticks are ubiquitous, blood sucking arthropod parasites affecting animals and humans. Biting of ticks mainly causes anaemia besides it also transmits various pathogens to animals. *Rhipicephalus sanguineus*, commonly known as the brown dog tick is the most widespread tick of the world including India (Jena *et al.* 2021). However, ticks of genera *Haemophysalis* spp, *Dermacentor* spp, *Otobius magnini* are also prevalent in India (Sahu *et al.* 2013, Bhadesiya *et al.* 2016). Earlier, prevalence of ticks in dogs has been reported in different states of India by several workers (Bhadesiya *et al.* 2016, Anish *et al.* 2020, Jena *et al.* 2021, Devi 2022). Identification of ticks has traditionally been based on their morphological keys. However, morphological similarities among different members of tick species often create confusion. To overcome this, molecular techniques which target different genetic marker are used nowadays for identification to ticks species (Ghosh *et al.* 2020). Among

them, *16S rRNA* gene has been used by several researchers as a genetic marker for molecular characterization of ticks in recent years (Papa *et al.* 2020, Elelu *et al.* 2022).

Tripura is a north-eastern state of India surrounded by Bangladesh in its three sides and Assam and Mizoram on another side. In Agartala, the capital city of Tripura, the popularity of keeping a dog as pet is growing day by day very rapidly but no systematic study on ticks have been reported in dogs so far. Considering frequent cross boundary migration of dogs with their owners now a days, it has become more pertinent to study and characterization of the circulating pathogens including ticks in dogs. Therefore, the present study was designed to explore the prevalence as well as molecular characterization of tick infestations in dogs in Agartala.

MATERIALS AND METHODS

Experimental site: The present study was conducted in and around Agartala, Tripura (latitude 23°83' N and longitude 91°28' E) for one calendar year covering all 4 seasons (pre-monsoon, monsoon, post monsoon, winter) from March 2022 to February 2023. The laboratory work was conducted at the Department of Veterinary Parasitology, Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences and Animal Husbandry, R. K. Nagar, Tripura.

Tick collection and identification: A total of 1776 dogs of different breeds, age groups (below 1 year and above

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1 year age group), sex and different categories (stray dogs, pet dogs and working dogs) were examined for the presence of ticks. Ticks were collected from dogs which were brought to the Teaching Veterinary Clinical Complex, R. K. Nagar, Tripura, different veterinary hospitals, veterinary dispensaries, dog shelters, stray dogs in NGOs and private clinics, etc in and around Agartala city. The external body coat of dogs, mainly both the inner and outer surface of ear, inter-digital spaces, dorsal and ventral surface of neck and the tail were examined thoroughly for the presence of ticks. After collection, the ticks were gently preserved into clean glass vials containing 70% alcohol for morphological identification and in phosphate buffer saline (PBS) solution for molecular identification. Temporary and permanent slides of ticks were prepared as per the procedures specified in Soulsby (1982). The ticks were identified based on the morphology under a stereoscopic binocular microscope and compound microscope following the guidelines described by Sen and Fletcher (1962) and Soulsby (1982).

DNA Extraction from ticks: DNA was extracted from ticks was performed using DNeasy Blood and Tissue kit (Qiagen® Kit,) following the manufacturer's recommendations with minor modifications. The isolated DNA samples were numbered as L1, L2, L3 and L4 and were stored at -20°C until further molecular detection by PCR amplification.

PCR amplification of 16S rRNA gene: The PCR amplification of the portion of 16S rRNA gene of *R. sanguineus* was carried out from tick DNA samples with the following primer pairs: Forward 5'- CCG GTC TGA ACT CAG ATC AAG T -3' and Reverse 5'- GCT CAA TGA TTT TTT AAA TTG CTG -3' (Kumar *et al.* 2011). PCR reaction was performed following the reaction composition and reaction condition as specified by Kumar *et al.* (2011) with some minor modifications for amplification of an expected 460 bp product. Briefly, the PCR reaction was carried out in a 50 μl reaction volume containing 5 μl of extracted tick DNA, 25 μl Dream Taq master mix (2x) (Thermo scientific Dream Taq green PCR MM), 2.5 μl of each forward and reverse primer and 15 μl of nuclease free water. PCR was performed with initial denaturation at 94°C for 3 min, followed by 32 cycles, each cycle consisting 1 min of denaturation at 94°C , annealing of 1 min at 50.5°C and an extension of 1 min at 74°C . Final extension was done at 72°C for 15 min. PCR products were resolved on 1.5% agarose gels and compared with 100 bp DNA ladder (Thermo scientific gel ruler 100 bp).

Nucleotide sequencing and phylogenetic analysis: The PCR amplified product from one DNA sample (L1) was sent to 1st Base Sequencing Service (Apical Scientific Sdn. Bhd., Malasia) for column purification and then bidirectional commercial DNA sequencing by the Sanger's di-deoxy nucleotide chain termination method using both forward and reverse primers on at least two independently generated PCR products. The generated sequences were compared using BLASTn algorithm with the sequences available in the National Centre for Biotechnology

Information (NCBI) database (<https://www.ncbi.nlm.nih.gov>) to identify the tick species. The accession number for the sequences was obtained from the NCBI GenBank database. Further, the sequences were used to construct a species specific phylogenetic tree based on sequence homology with the published sequences originated from different countries using the UPGMA method in Mega 7.0.21 software (Kumar *et al.* 2016).

Statistical analysis: Prevalence data was expressed as the percentage. To test the significant association between the difference groups, Chi square test was performed. The statistical analysis was done at 1% probability level using the Statistical Package for the Social Sciences (SPSS), version 25 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Prevalence of tick infestation: Out of 1776 dog examined, 1104 (62.16%) dogs were found to be infested with ticks. All the ticks were examined under microscope and identified as *R. sanguineus*, by their characteristics such as bifid coxa, festoon, Posterior anal groove, hexagonal basis capitulum, denticles on the hypostome (3+3 column), adenal plate and comma shaped spiracle, etc (Fig. 1 A-B and Supplementary Fig. 1 A-B). Sex, age, season and category wise prevalence of tick infestation in dogs has been presented in Table 1. The breed wise prevalence of tick infestation in dogs has been presented in Supplementary Table 1. Earlier, Bhadesiya *et al.* (2016) from Gujarat, Devi (2022) from Guwahati and Sarkar (2022) from Tripura also reported higher prevalence of ticks in dogs. However, the prevalence of tick's in the present study was found more in comparison to the findings of Ayodhya (2014) in Rajendranagar, Hyderabad and Krishna Murthy *et al.* (2016) in Shimoga region, Karnataka. Moreover, the present per cent prevalence was found less in comparison to the findings of Prakasan and Ramani (2007) in Kerala and Anish *et al.* (2020) in Andhra Pradesh. This difference in the prevalence of the ticks from other findings might be due to sample size, different geographical regions, different management practices as well as different environmental conditions (Odeniran *et al.* 2021).

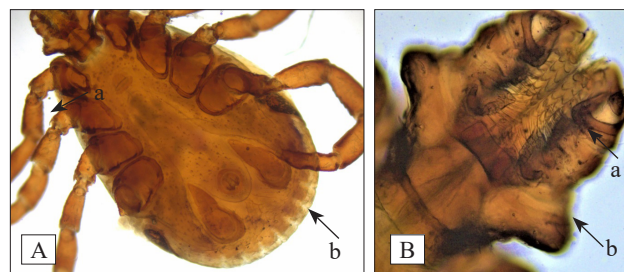


Fig. 1. A. *Rhipicephalus sanguineus*, bifid coxa (a) and festoon (b). (Mag. x10); B. *Rhipicephalus sanguineus*, denticles on the hypostome (3+3 column) (a) and hexagonal basis capitulum (b). (Mag. x10).

R. sanguineus was the only tick species encountered in the present study. Earlier, Sarkar (2022) also reported *R. sanguineus* as the sole tick species in her study from

Table 1. Prevalence of tick infestation in dogs from Agartala, Tripura according to sex, age, category and season

Variable	Number of dogs examined	Number of dogs positive for tick infestation	Prevalence (%)	P-value
<i>Sex</i>				
Male	1002	662	66.06	<0.01*
Female	774	442	57.10	
<i>Age Group</i>				
Young (<1 year)	651	487	74.80	<0.01*
Adult (>1 year)	1125	617	54.84	
<i>Categories</i>				
Pet dogs	1076	672	62.45	>0.05 ^{NS}
Stray dogs	336	218	64.88	
Working dogs	364	214	58.79	
<i>Seasons</i>				
Pre-monsoon	473	316	66.80	<0.01*
Monsoon	642	430	66.97	
Post-monsoon	364	211	57.96	
Winter	277	147	53.06	

*, Denotes statistically significant P-value ($P < 0.01$), Chi square statistics; ^{NS}, Denotes statistically non-significant P-value ($P > 0.05$), Chi square statistics.

the same area. The present finding also correlates with that of Soundararajan *et al.* (2016) in Chennai, Tamil Nadu; Krishna Murthy *et al.* (2016) in Karnataka and Jena *et al.* (2021) in Kolkata. However, Sahu *et al.* (2013) from Odisha and Devi (2022) from Guwahati found some other species of ticks, viz *Boophilus* spp. and *Haemaphysalis* spp. along with *R. sanguineus* in their studies. This difference in the distribution of the tick species might be due to change in geographical regions as well as different environmental conditions. The present study was conducted in the urban capital area (Agartala) of the state (Tripura) and *R. sanguineus* is highly adoptable and capable of multiplication in these urban situations (so called as urban dog tick, Roopesh 2017), which justifies the prevalence of *R. sanguineus* as the only tick species in the present study.

Breed wise, prevalence of tick infestation was significantly ($P < 0.01$) higher in German Spitz (81.72%) followed by Mongrels. The higher prevalence in German Spitz might be due to the fact that they are one of the most popular and easily affordable breed in the study area which is evident from their presentation in the present study. As the breed having a long hair coat, there is every possibility that the tick attaches to the deeper part of the body between hairs and remains unnoticed to the owner. Soundararajan *et al.* (2016) also found higher prevalence in Spitz and stated that hairy breeds (Spitz, Labrador, German shepherd, Lhasa Apso, Border collie, Golden Retriever and Pomeranian) were heavily infested with ticks than the non-hairy breeds. High prevalence of tick infestation in mongrels as observed in the present study might be because they are mainly free roaming and stray which causes them in gaining and spreading the tick infestation easily. In addition, mongrels are mainly reared by the socioeconomically weaker section of people for which they are not well maintained by their owners and proper acaricidal regime is also not followed

which allows them to carry the infestation and spreading the tick for a long period of time.

Sex-wise, the prevalence of tick infestation was significantly ($P < 0.01$) higher in males (66.06%) than in female (57.10%). Previously, Sahu *et al.* (2013) in Odisha and Soundararajan *et al.* (2016) in Chennai also recorded higher prevalence of tick infestation in male dogs. The higher prevalence in male dogs might be due to the scavenging and wandering habit of male dogs during breeding season which causes them to acquire and spreading of ticks. It was also observed that certain hormonal factors are responsible for predisposing male dogs to tick infestation (Sahu *et al.* 2013). However, the present findings were contradictory to the findings of Lema *et al.* (2020) and Devi (2022) who recorded higher prevalence of ticks in female dogs.

Age-wise prevalence of tick infestation was significantly ($P < 0.01$) higher in young group (below 1 year) with 74.80 % than in adult group (above 1 year) with 54.84 %. Similar findings were also reported by Ashraf and Sajid (2015) and Krishna Murthy *et al.* (2016). The probable reasons behind higher tick prevalence observed in young dogs are their underdeveloped immunity, constant exposure to carrier mothers (Nayak *et al.* 1997) and their habit of playing and running on the grasses, chewing any objects around them from where they pick up tick infestation. However, low infestation in adult dogs might be due to their effective scratching activity and also development of resistance against tick (Sahu *et al.* 2013).

Category-wise the prevalence of tick infestation was non-significantly ($P > 0.05$) higher in stray dogs (64.88%) followed by pet dogs (62.45%) and working dogs (58.79%). The higher prevalence of ticks in stray dogs was also reported by Krishna Murthy *et al.* (2016) and Jena *et al.* (2021). The much higher prevalence of ticks in stray dogs might be due to poor management, care and unhygienic

living condition which inputs stress to the dog (Totton *et al.* 2011). The chances of spreading the infestation among them are also high as stray dogs used to roam many places. The prevalence was found low in working pet dogs which is might be due to the fact that they are usually kept under keen observation by their owners all the time and are provided with clean shelter along with regular grooming.

Season-wise, highest prevalence of tick was observed in monsoon (66.97%) followed by pre-monsoon (66.80%), post-monsoon (57.96%) and winter (53.06%). Highest prevalence of ticks in monsoon season was also reported by Jena *et al.* (2021) and Devi (2022). The warm and humid climate in the monsoon favours the growth and multiplication of Ixodid ticks (Soulsby 1982). During monsoon and post-monsoon season, the temperature remained high and relative humidity was also ideal for the rapid multiplication of ticks which in terms might have resulted in high population of ticks. A lower temperature and dry environment during winter season probably slowed down the multiplication of ticks which resulted in low prevalence of ticks in winter.

Molecular identification of tick: The PCR amplicons of all four extracted DNA samples from ticks showed single distinct band of 460 bp when compared with the DNA ladder in agarose gel (1.5%) (Supplementary Fig. 2). On BLAST analysis of the sequence, the L1 sample showed 100% similarity at nucleotide level with the sequences of *R. sanguineus* available in the NCBI GenBank database which further confirmed the specificity. Phylogenetic analysis of 16S rRNA gene sequence of the Tripura isolates (OR244468.1) (L1) of *R. sanguineus* was carried out with additional 9 taxonomically and morphologically identical Genbank data (KC170744.1 Thailand, OL757514.1 China, KP830114.1 Cuba, MH765331.1 Madhya Pradesh India, OQ300116.1 Assam India, MG066692.1 Kerala India, MZ960040.1 Colombia, MT322611.1 Mexico, OM985246.1 USA). The phylogenetic analysis of 16S rRNA gene revealed that the tick isolate sample of *R. sanguineus* (OR244468.1) from Agartala (TrRS-PB) was present in the same clad with Assam and Kerala isolate (Fig. 2). Since the state of Tripura shares its border with Assam and the climatic condition in these two states is also somewhat similar and frequent interstate movement of people with their pets occurs, the grouping of Tripura

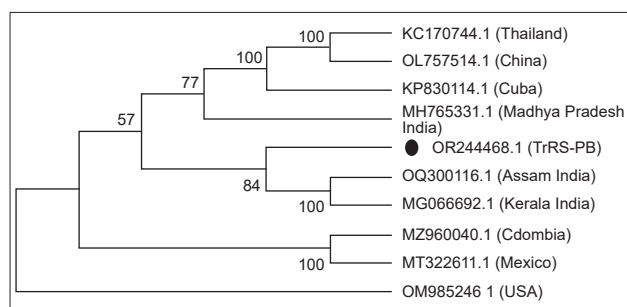


Fig 2. Phylogenetic tree constructed for *Rhipicephalus sanguineus* (TrRS-PB) from the 16S rRNA using UPGMA method in MEGA 7.0.21.

isolate with the Assam isolate in the same clad is aptly justified.

The present study reported the prevalence of ticks in dogs in and around Agartala, Tripura along with their molecular identification and characterization. A considerably high prevalence rate of tick infestation (62.16%) was observed in dogs in the study warranting treatment. *R. sanguineus* was found to be the only tick species infesting dogs by morphological and molecular identification in the study area. Breed, sex, age and category wise study showed the highest prevalence of tick infestation in German spitz breed, in male dogs, in dogs of below 1 year of age and in stray dogs, respectively. However, further studies can be carried out to assess the actual economic impact of the tick infestation in dogs in the study area. Altogether, the present study provides a baseline data on the prevalence of tick infestation in dogs in Agartala, Tripura which can be enormously helpful in formulating control strategies against tick infestation in dogs in the study area as well as in the whole state.

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