




High Asymptomatic Cases of Babesiosis in Dogs and Comparison of Diagnostic Performance of Conventional PCR vs Blood Smears

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Abstract

Purpose Babesiosis is one of the most important globally extended and quickly spreading tick-borne infections of dogs. Diagnosis of babesiosis in Sri Lanka is based on clinical signs followed by thin blood smears which could be error-prone due to undetected early infections, absence of clinical signs or low parasitemia. The present study investigated the prevalence of babesiosis in dogs presented to the Veterinary Teaching Hospital (VTH) at the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, for treatments, vaccinations, and regular check-ups, and compared the diagnosis methods of microscopy and molecular analysis.

Methods Blood samples from dogs were collected from January to June 2019. First, Giemsa stained blood smears were prepared, and then the blood samples were subjected to PCR using genus-specific primers to amplify a 411–450 bp region in the 18S rRNA gene. Twenty samples from PCR amplified products were sequenced for species identification and phylogenetic analysis. Clinical signs of the dogs were noted down, and ticks were also collected from dogs if any.

Results Results show a very high prevalence of canine babesiosis (78.6%) among the dogs brought to the VTH. The parasite was identified microscopically and genetically as *Babesia gibsoni*. A large percentage (66.7%) of infections was asymptomatic. Out of 42 blood samples, 19 (45.2%) were microscopically positive for babesiosis while 33 (78.6%) were PCR positive, showing a significant difference in the two methods of diagnosis (chi-square test, $\chi^2 = 9.462$, $p = 0.002$). Three tick species: *Rhipicephalus haemaphysaloides*, *Haemaphysalis bispinosa*, and *Rhipicephalus sanguineus* were found attached to the dogs.

Conclusion This study shows a very high prevalence of canine babesiosis among dogs in the Kandy area. Most of these infections might go undetected if only microscopy was used to diagnose. An improved, rapid diagnostic method such as the novel, PCR-based point-of-care diagnostic method that detects very low parasitemia within 30 min is needed. Moreover, as most infected dogs did not show clinical signs, they may act as reservoirs of infection. The ability of asymptomatic dogs to spread babesiosis should be investigated.

Keywords Dogs · *Babesia gibsoni* · Asymptomatic cases · *Rhipicephalus sanguineus*

Introduction

Canine babesiosis is one of the most important tick-borne diseases of dogs worldwide. It is caused by a hemoprotozoan parasite of the genus *Babesia*. Traditionally, *Babesia* species infecting dogs have been distinguished morphologically as large forms (2.5–5 μm length) and small forms (<2.5 μm length) [1]. Examples of large forms are *Babesia canis*, *B. vogeli*, *B. rossi*, *B. caballi* and the examples of small forms: *B. gibsoni*, *B. conradae* and *B. microti*-like species [2]. Among these, *B. gibsoni* and *B. canis* are the most prevalent, with global distribution reported from Europe and other parts of the world, including Asia, the United States, Australia, and Brazil [2]. All species of canine *Babesia* are

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transmitted by ticks. Among the tick species, the vector capacity has been confirmed in *Dermacentor reticulatus*, *R. sanguineus*, *Haemaphysalis* spp., *H. longicornis*, and *Ixodes* spp. [2]. Although the occurrence of the disease is associated with the seasonal activity of tick vector, dynamics of spreading of canine babesiosis has markedly changed in the last decade due to global warming, shifting use of the landscape, and the increase of wild animal populations, spreading of vectors by wild birds and animals, and the change of habitat structure of wildlife [3]. In the tick vector, both trans-stadial and transovarial transmission occurs, although the importance of the different transmission pathways varies between the large and the small forms [4]. Moreover, rarely, babesiosis can be transmitted by blood transfusion from an infected dog donor or during dog fighting [5].

Babesiosis has a wide range of clinical signs, which mainly depend on the type of the parasite that infects dogs [6] and other conditions such as dog age, immune competence, and the phase of the disease [7, 8]. Some of the characteristic clinical features are fever, anorexia, vomiting, lethargy, pale mucous membrane, splenomegaly, hepatomegaly, mild to severe thrombo-cytopenia and regenerative anemia due to hemolysis, respiratory failure, acute renal failure, and bilirubinemia to a subtle and slowly progressing infection with no apparent clinical signs [7–10].

Babesiosis can be primarily diagnosed using clinical signs, patient history and microscopic screening of stained peripheral blood smears [8]. These two methods have limited sensitivity and cannot identify the carriers where parasitemia is low with small numbers of peripheral blood protozoa [11]. Serological approaches are recommended to diagnose antibodies against the parasites, particularly in subclinical infections [12]. Molecular diagnosis methods are highly preferred due to their sensitivity, specificity, and reliability [8, 13]. The PCR test has the advantage in that it can detect all species of *Babesia*. Serologic or antibody titer testing in the diagnosis of babesiosis has limitations. A positive test result is dependent on an antibody response by the infected dog, which may take up to ten days to develop. Once a dog has developed antibodies to babesiosis, it may persist for years and this must be considered when performing follow-up tests [14].

In Asian countries, clinical babesiosis is often caused by *B. gibsoni* [15]. In Sri Lanka, babesiosis is considered endemic [16]. The disease incidence is mainly due to a small *Babesia* species, presumably *B. gibsoni* and occasionally by other species [17]. As early as 1953, Seneviratna studied piroplasmiasis in dogs in Ceylon (now Sri Lanka) [18]. Later, he described *B. gibsoni*, the small canine *Babesia* infections in dogs [19], pathology [20], and its treatment [21]. As clinical signs of the infection, Seneviratna [20] reported an early rise in temperature before parasites can be detected in the blood due to the parasite multiplying in

the reticuloendothelial system and fever, when present, seldom exceeds 104 °F [20]. Currently, canine babesiosis is a disease of great veterinary importance in Sri Lanka with an island-wide distribution [22]. In addition to *B. gibsoni*, *B. canis* also causes canine babesiosis in Sri Lanka [22, 23].

Seneviratna in 1953 described the tick species *Haemaphysalis bispinosa* as the principal vector of *B. gibsoni* in the enzootic areas. Globally, however, the brown dog tick, *Rhipicephalus sanguineus*, has been identified as a major vector of babesiosis [24–26]. A recent comprehensive island-wide study recorded eight tick species: *R. sanguineus*, *Rhipicephalus haemaphysaloides*, *Haemaphysalis intermedia*, *H. bispinosa*, *Haemaphysalis turturis*, *Amblyomma integrum*, *Hyalomma* spp. and *Dermacentor auratus* (Bandaranayake *et al.* Unpublished observations), *R. sanguineus* as the dominant dog tick species. Although they were recorded on dogs, their vector capacity is largely unknown.

In Sri Lanka, diagnosis of babesiosis is based on clinical signs followed by thin blood smears. This protocol could be error-prone due to undetected early infections, absence of clinical signs or low parasitemia. This study was designed to examine the prevalence of babesiosis in dogs brought to the Veterinary Teaching Hospital (VTH) at the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, for treatments, vaccinations, and regular check-ups and compare microscopy and molecular analysis methods.

Materials and Methods

Study Animals

Owned dogs that were presented to the VTH, at the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya for treatments, vaccinations, and regular check-ups were used as the study animals. Background information of the dog: age, sex, breed, skin colour, current medications, the reason for visiting the clinic, recent history of any other disease, history of tick-borne diseases, tick controlled medications, and the presence or absence of ticks were noted down after obtaining permission from the owner. The VTH was visited once a week and blood and tick samples were collected from the dogs presented to the clinic for a period of 6 months (January–June 2019) using a convenient sampling technique.

Sample Collection and Analysis

Blood samples from dogs were collected into vacutainer tubes containing an anticoagulant (EDTA) and were stored at 4 °C. Dogs were thoroughly examined for the presence of ectoparasites, and if present, they were preserved in 90% ethanol. Blood and tick samples were brought to the

laboratory. Giemsa stained thin blood smears were prepared and observed under the light microscope, and parasitemia was noted down in positive samples. Blood samples were also subjected PCR for molecular identification. Ticks were identified up to the species level using their morphology by following a morphological identification key [27].

Genomic DNA Extraction and PCR Gene Amplification

Genomic DNA was extracted using DNeasy[®] Blood and Tissue Kit (Qiagen, Hilden, Germany). The extracted DNA was subjected to PCR using a genus-specific primer pair of *BJ1* (5-GTCTTGTAATTGGAATGATGG-3) and *BN2* (5-TAGTTTATGGTTAGGACTACG-3), which amplify 411–450 bp region in 18 s rRNA gene (small subunit rRNA) of *Babesia* species [28]. The PCR was performed in a thermal cycler (Eppendorf Mastercycler[®] gradient), and each PCR reaction mixture (30 µl) included 15 µl of Go Taq master mix, 0.5 µl of each forward and reverse primers, 3 µl of template DNA, and adequate nuclease-free water. The amplification process was conducted with the following program: initial denaturation at 94 °C for 10 min. This was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The final extension was at 72 °C for 5 min [28]. The amplified PCR products were separated using ethidium bromide-stained agarose gel (2%) electrophoresis and visualized under UV illumination.

DNA Sequencing

Twenty PCR amplified products were sequenced for species identification and phylogenetic analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequencing was conducted using the Genetic analyzer 3500 series (Applied Bio Systems[®]). The obtained sequences were used in the NCBI Nucleotide Blast Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), to search for a series of reference sequences. The sequences obtained for the marker were then aligned with the recovered reference sequences using Mega 7.0 software via the Clustal W algorithm [29]. Any inaccuracies of the automated sequences were manually inspected. Then, the forward and reverse end noises were trimmed, and consensus sequences were created. Next, ambiguous regions were eradicated, and all sequences were aligned using Clustal W algorithm [29].

Phylogenetic Analysis

Phylogenetic analysis was carried out based on 411–450 bp of the 18S rRNA sequence located between the primers *BJ1* and *BN2* of the *Babesia* parasites. The analyses included 20

sequences from Sri Lankan isolates. For comparison, the corresponding 18S rRNA sequences of *Babesia* spp. were included in the alignment, and *Plasmodium falciparum* was included as the out-group. The Unweighted Pair Group Method using Arithmetic averages (UPGMA) tree was created using MEGA 7.0 software. The analysis was carried out using 1000 iterations.

Statistical Analysis

The association between the prevalence of babesiosis and the host demographic factors like age, sex, breed, and skin color were determined using univariate analysis of odd ratios and 95% confidence intervals using a chi-square (χ^2) test and Cramer's V coefficient (CVC) for independence. The difference between the two diagnosis protocols: microscopy and PCR was compared using a chi-square test. Statistical analyses were performed using Minitab 17.0 software (Minitab Inc. USA).

Ethical Clearance

The study protocols received ethical approval from the Ethical Review Committee of the Postgraduate Institute of Science (PGIS), University of Peradeniya, Sri Lanka.

Results

Study Animals

A total of 42 dogs that were brought to the VTH from eight districts were sampled. Most of them were from the Kandy district (28), while few visited from other districts: Nuwara Eliya (4), Kurunegala (3), Kegalle (2), Colombo (2), Matale (1), Gampaha (1), and Polonnaruwa (1). Among these 19 dogs (45.2%) were males, and 23 (54.8%) were females, comprising 20 (47.6%) puppies and 22 (52.4%) adults (Table 1). There were 27 pedigreed and 15 non-pedigreed dogs. Of the purebred dogs, eight were German shepherds, five were Labrador, four were Rottweilers, three were Golden retrievers, and seven belonged to other groups, including Dalmatian, Pomeranian, Boxer, and Terriers, all of which were locally bred. The non-pedigreed dogs include crossbred and mongrels. The history of dogs indicated tick infestations in 12 (28.6%) dogs while nine dogs (21.4%) were infected with ticks during the visit to the hospital. The owners reported that they had used locally available external ectoparasite control medications to eliminate the ticks from the dogs.

Table 1 Background information and history of the dogs sampled at the Veterinary Teaching Hospital

Variable	Number of dogs	Percentage (%)
Sex		
Male	19	45.2
Female	23	54.8
Age		
Puppies (< 1 yr)	20	47.6
Adults (> 1 yr)	22	52.4
Breed		
Pedigreed	27	64.3
Non-pedigreed	15	35.7
Current medications for <i>Babesia</i>		
Yes	14	33.3
No	28	66.7
Tick infestation		
At the time of visit	9	21.4
Past infestations	12	28.6
Not reported	21	50.0
District		
Kandy	28	66.7
Other	14	33.3
Microscopy		
Positive symptomatic	6	14.3
Asymptomatic	13	30.9
Negative	23	54.8
PCR		
Positive symptomatic	11	26.2
Asymptomatic	22	52.4
Negative	9	21.4

Microscopic Examination of Blood Smears

Of the 42 dogs, 19 (45.2%) were microscopically positive for *Babesia* sp. in the Giemsa stained blood smears. The level of parasitemia ranged from 0.5 to 30.4% of invaded erythrocytes.

PCR-Based Identification of the Parasite

Among the 42 dogs, 33 (78.6%) were positive for *Babesia* DNA with a fragment at 411–450 bp amplicons in gel electrophoresis. All microscopically positive samples were PCR positive. In addition, 14 microscopically negative samples were PCR positive for *Babesia* DNA.

Sequence and Phylogenetic Analysis

The consensus sequences of the Sri Lankan isolates identified in the present study were submitted to Genbank

under the accession numbers MN988978–MN988997. The sequencing data obtained from the primers *BJ1* and *BN2*, which amplify a region of the 18S rRNA gene, are used to construct the phylogenetic tree (Fig. 1).

Statistical Analysis

Of the infected dogs, only 33.3% showed clinical signs while 66.7% were asymptomatic.

Statistical analysis revealed no relationship between the prevalence of babesiosis and any of the host demographic factors tested, including age, sex, breed, and skin colour. However, a strong association between the microscopical results (45.2%) and the molecular results (78.6%) was observed with a significantly higher number of cases shown positive in the molecular analysis than microscopy ($\chi^2 = 9.462$, $p = 0.002$). Moreover, no dog breed predilection showed a difference in the prevalence of asymptomatic infection between mongrels (45.5%) and purebred dogs (54.5%; Fisher's Exact test, $p = 0.05425$).

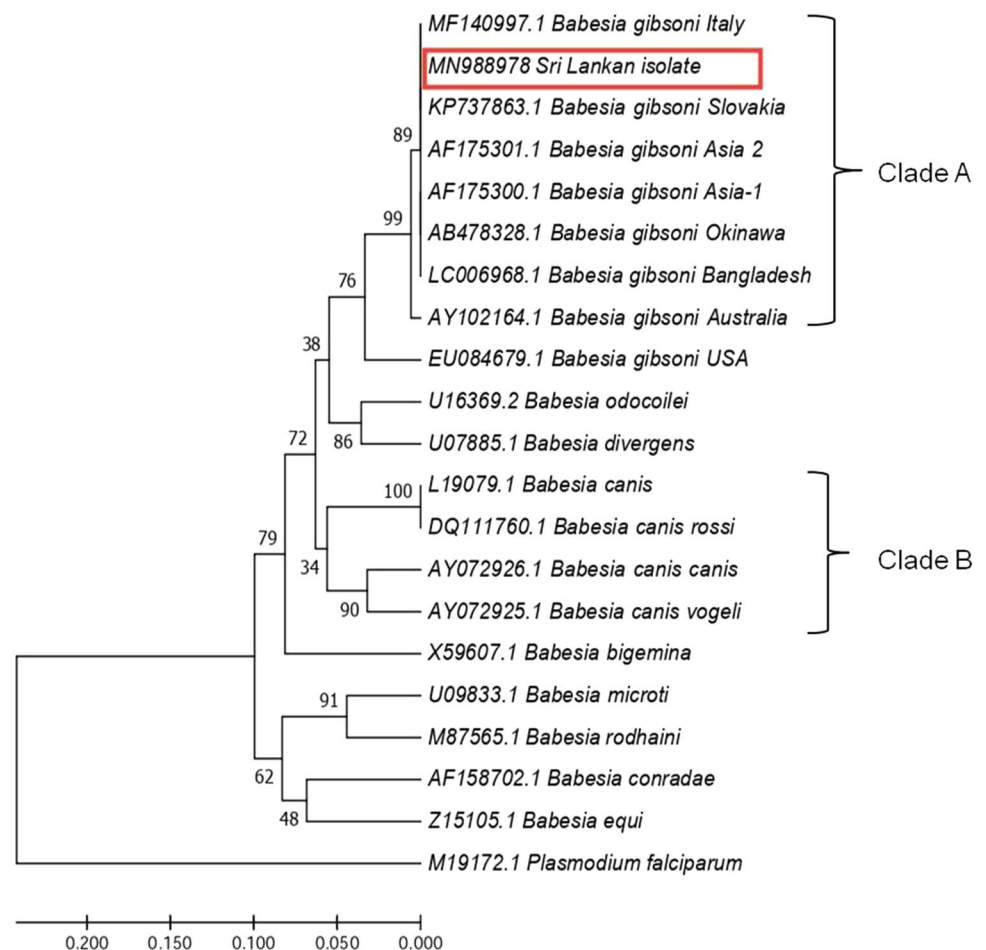
Tick Identification

Seventeen ticks belonging to three species were collected from nine dogs (21.4%) including males (29.4%), and females (11.8%) of *R. haemaphysaloides*, and males (11.8%), and females (17.6%) of *H. bispinosa*, a nymph belong to the *R. haemaphysaloides* (5.9%), nymphs of *R. sanguineus* (11.8%) and larvae of *H. bispinosa* (11.8%). Each dog had only one species of tick.

Discussion

This study reports a high prevalence of canine babesiosis (78.6%) among the dogs brought to the VTH with a very high percentage of asymptomatic cases (66.7%). The prevalence of canine babesiosis reported in recent studies carried out in Sri Lanka is much lower: Weerathunga *et al.* [23] reported 15.0% infection of *B. gibsoni* in three Divisional Secretariat Divisions (Rambewa, Tirappane, and Galenbidunuwewa) in the Anuradhapura district and a similar percentage of 13.8% prevalence of *B. gibsoni* was reported in an island-wide survey of free-roaming, privately owned dogs and working dogs of Sri Lanka Air Force [22]. Nevertheless, these two studies have used only microscopy for diagnosis. A study using molecular evidence from Kerala, South India reported 47.33% of canine babesiosis due to *B. gibsoni* [30]. Another study conducted in stray dog population in Assam, India also reported *B. gibsoni* as the highest prevalent infection with an infection rate of 47.16% in hospital dogs and 47.72% in stray dogs [31]. Yet, these are not as high as the prevalence reported in the present study.

Fig. 1 The phylogenetic tree constructed using *Babesia* genus-specific primers that amplify the 18S rRNA gene and the evolutionary history was inferred using the UPGMA method. An optimal tree with the sum of branch length = 1.13965637 is shown. The gene sequences of *Babesia* sp. obtained from the dogs naturally infected with *Babesia* and sequences of several species obtained from Genbank. The identity and Genbank accession numbers were used to indicate each sample. The tree was constructed with a bootstrap analysis of 1000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7



Only, *B. gibsoni* infections were recorded although previous studies from Sri Lanka reported the presence of both *B. gibsoni* and *B. canis* in dogs in single as well as mixed infections [17,20,22,23,]. The geographic distribution of the two species in the island could be different. Weerathunga *et al.* [23] sampled the dogs only from the Anuradhapura district where as Jaythilake *et al.* [22] carried out an island-wide survey of hemoparasites in dogs reported *B. canis* only from Colombo District. Moreover, the prevalence of *B. canis* is very low (1.25% in [23] and 0.6% in [22]) compared to that of *B. gibsoni* infection. Absence of *B. canis* in the current study can only be confirmed after analyzing a large sample or it could be because it has a restricted geographic distribution.

Out of the infected dogs, two-thirds did not show any clinical signs. This means most dogs could act as reservoirs of infections being asymptomatic. Although there was no breed predilection showing a difference in the prevalence of asymptomatic infection between mongrels and purebred dogs, it is anticipated that better natural resistance against haemoparasites in mongrels compared to owned dogs or pure breeds of dogs [32]. In general, mongrels are more robust. The asymptomatic dogs are

sub-clinically infected and may provide a continuous source of infection for other dogs, especially pedigree dogs, who are highly susceptible to infectious diseases due to inbreeding. The absence of clinical signs indicates that these dogs may be chronically or subclinically infected with these haemoparasites or as Dantas-Torres and Otranto [33] point out, they may be having clinical-pathological abnormalities and organ dysfunctions. Chronic infections may not pose an immediate threat to the animals; these dogs remain possible reservoirs for infections. In addition, stressful conditions, concurrent illnesses, and parturition may induce clinical signs in chronically infected animals [34]. The presence of asymptomatic cases should alert veterinarians on the significance of screening potential blood donors during a blood transfusion. Recently, Neelawala *et al.* [17] observed recurrence of following acute infection of babesiosis after anti-babesial treatment in 11.8% of dogs brought to the VTH. Furthermore, it could be possible that the host displays infection tolerance traits, which reduces the infection's damage. The mechanisms and research on tolerance phenomenon in canine babesiosis have relevance for disease control measures targeting natural reservoirs.

Phylogenetic analysis shows that the clade A exclusively consisted of *B. gibsoni* reported in Asia (including Sri Lanka, Malaysia, Bangladesh, India, China, Japan) and European countries of Australia, Italy, and Slovakia it is independent of the others. There is no intra-specific variation among different isolates of *B. gibsoni* in clade A and no divergence/evolutionary deviations were reported in the case of *B. gibsoni* compared to *B. canis*. Recent studies have been shown that the genetic sequences isolated from the dogs reported in Alabama, Oklahoma, North Carolina, Missouri, and Indiana were identical to the sequences isolated from dogs in Sri Lanka, Japan, and Malaysia [35, 36]. But the sequence of the USA isolate of *B. gibsoni* (EU084679) was clustered as a separate group from clade A. This phylogenetic tree shows that all the *B. gibsoni* were originated from a common ancestor (79,100). The deviation of the USA isolates from the others might be due to variations in their geographical distribution, seasonal variations, and the distribution of the tick vectors when compared to others.

The host demographic factors: age, sex, breed, and skin color did not have a predilection for prevalence of the babesiosis. However, we cannot generalize these finding, uncritically without a large sample size. Silva [37] points out that certain breed like Rottweilers, Labradors, Boxers and cross bred dogs of any age are more susceptible for babesiosis than other breeds. Young adults of German Shepherds, Doberman and Pomeranians also show higher susceptibility.

A significant difference in the two diagnosis methods was observed where 33.4% was clinically undetected when only the microscopy was used. A study carried out in Kerala south India, 71 (47.33%) were found to be PCR positive for *B. gibsoni*, while only 40 were blood smear positive [30] and similar finding have been reported in other studies of Bell and Ranford-Cartwright [38] and Sasaki *et al.* [39]. Microscopy has revealed only 45.2% were infected dogs while molecular analysis showed 78.6%. Even in-clinic serology tests do not differentiate active infection from prior exposure; therefore, molecular techniques have become the preferred method for detection of most canine vector-borne haemoparasites. Liu *et al.* [14] report a QubeMDx PCR system which enables a rapid, sensitive and reliable diagnosis of *B. gibsoni* near the dog patient. Within 30 min, this diagnostic assay can detect parasitemia as low as 0.002% in the dog blood providing a reliable point-of-care test to assist in the diagnosis of *B. gibsoni*.

Conclusions

The prevalence of canine babesiosis was high among the dogs brought to the VTH. *Babesia gibsoni* was the common parasite among dogs in and around the Kandy

District. Presence of high asymptomatic cases (66.7%) and a significantly high number of microscopy undetected cases provide important information to veterinarians and highlight importance of having improved diagnostic method to control the disease.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11686-022-00549-x>.

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Author contributions RASR: Sample collection and analysis, data analysis and writing of the manuscript. AD: sample collection, editing the manuscript; SDDSS: supervision of lab work and editing the manuscript; RSR: study conception and design, supervision, editing the manuscript.

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Data availability Informed.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interests.

Ethical approval The study protocols received ethical approval from the Ethical Review Committee of the Postgraduate Institute of Science (PGIS), University of Peradeniya, Sri Lanka.

Consent to participate Verbal informed consent was obtained from the dog owners.

Consent for publication Verbal informed consent was obtained from the dog owners.

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