Exploring tick-borne pathogens in community dogs in Nepal

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Abstract

Tick-borne pathogens (TBPs) in dogs are a major global health concern, with their zoonotic importance often being neglected in developing countries due to a lack of surveillance. This study aimed to highlight the incidence of six important TBPs belonging to the genera *Babesia, Theileria, Hepatozoon, Anaplasma, Ehrlichia,* and *Rickettsia* in a total of 230 community dogs from two sites: Lumbini and the Kathmandu Valley, of Nepal. A total of 75 (32.6 %) dogs were found to be infected with at least one TBP, with 11 (4.7 %) being co-infected with more than one TBP. The detection rates of TBPs were 13.9 % (n = 32) for *Ehrlichia canis,* 9.1 % (n = 21) for *Anaplasma platys,* 8.6 % (n = 20) for *Babesia vogeli*, and 6.5 % (n = 15) for *Babesia gibsoni*. None of the samples were positive for *Theileria, Hepatozoon,* or *Rickettsia*. There was a significant association between *A. platys* and *E. canis* infections, respectively, with the locations from which the samples were collected. Infections of TBPs in community dogs might be the source of infection for pet dogs or even humans in shared habitats. Further studies are

needed to determine the prevalence and diversity of TBPs in dogs in other regions of Nepal. As some of these parasites are zoonotic, concerted efforts are required to raise awareness of, and control efforts for, these tick-borne pathogens.

Keywords: *Anaplasma, Babesia,* Canine, *Ehrlichia,* Nepal

1. Introduction

Within Nepals diverse landscapes, both free-roaming community dogs and pet dogs share a unique and valued bond with humans [1,2]. Ticks are common ectoparasites and are frequently present on free-roaming dogs in Nepal [3,4]. Ticks serve as vectors for many different species of haemo-parasites responsible for canine anaplasmosis, piroplasmosis, and ehrlichiosis [4]. These tick-borne pathogens (TBPs) not only adversely affect canines but some also affect humans and therefore have a high zoonotic significance [5–7].

Canine babesiosis is widespread globally and caused by *Babesia* species, including *Babesia canis*, *Babesia gibsoni*, and *Babesia vogeli* with symptoms including fever, anemia, and jaundice [8,9]. *Babesia vogeli* stands out as the most widespread *Babesia* species worldwide [10–12]. Additionally, *Hepatozoon* species such as *Hepatozoon canis* and *Hepatozoon americanum* cause canine hepatozoonosis, which leads to clinical manifestations such as fever, muscle pain, and significant muscle damage in affected dogs [13].

The TBPs in most of the canine species representing the Anaplasmataceae family are *Ehrlichia* spp. and *Anaplasma* spp. *Ehrlichia canis* primarily causes canine ehrlichiosis, though other *Ehrlichia* species including *Ehrlichia ewingii* and *Ehrlichia chaffeensis* have also been known to infect dogs [9,14]. The clinical manifestations of canine ehrlichiosis in dogs are characterized by fever, lethargy, and bleeding disorders [9,14]. Various tick species can transmit *Ehrlichia* spp. to dogs, but the brown dog tick species complex, *Rhipicephalus*

sanguineus sensu lato, is predominantly responsible for transmission [15]. Canine anaplasmosis is primarily caused by the pathogens *Anaplasma platys* and *Anaplasma phagocytophilum.* Anaplasmosis in canines is characterized by fever, petechiae, lymphadenomegaly, and multiple organ failure, often leading to treatment failure [9,16–18].

Previous studies conducted in Nepal have reported varying prevalence rates of TBPs in dogs using different diagnostic methods. Microscopic examinations found *Ehrlichia* spp. infections in 2.7–10.7 %, *Babesia* spp. infections in 2.0–6.7 %, and *Anaplasma* spp. infections in 1.3–18.7 % of dogs in the Kathmandu Valley [19–21]. Serological testing detected *E. canis* antibodies in 11.9 % of dogs in the Kathmandu Valley [22]. A single PCR-based investigation revealed high TBPs prevalence, with *A. platys* and *H. canis* (31 %), as well as *E. canis* (13 %), *Theileria* spp. (13 %), *B. vogeli* (13 %), and *B. gibsoni* (3 %) among 70 dogs in the Kathmandu Valley [4].

Community dogs, including street and abandoned dogs in developing countries like Nepal are often infested with ticks. Previous researchers reported several tick species from dogs in Nepal such as *Rhipicephalus microplus, R. sanguineus, Haemaphysalis bispinosa, Haemaphysalis aponommoides, Ixodes nuttallianus*, and *Ixodes acutitarsus* [3,23–26]. The high prevalence of different tick species and TBPs in community dogs raises significant concerns related to zoonotic spillover, as these dogs share a common habitat near human residences and their pet dwellings. Given the lack of precise molecular data and to better understand the complexities of this issue, our study utilized molecular tools to detect and characterize the incidence of TBPs such as *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia*, *Theileria*, and *Hepatozoon* in community dogs in two different regions of Nepal: the Kathmandu Valley (mid-hills) and Lumbini (lowland Terai); which can help predict epidemiological patterns and guide effective One health measures to reduce the spread of these zoonotic pathogens.

2. Methodology

2.1 Study area

Blood samples (n = 230) were collected from dogs in two different regions of Nepal: the lowland Terai (Lumbini) (n = 110), and the mid-hills (Kathmandu Valley) (n = 120) (Figure 1). Lumbini experiences a humid subtropical summer and dry winter climate with an average temperature of 29.7 ºC (7.7 % higher than Nepals average). Annual precipitation is around 106.2 mm (4.2 in.), with 81 wet days (22.2 % of the time). The Kathmandu Valley has a subalpine climate with dry winters and cool summers. The average temperature is 18.9º C (3.1 % lower than Nepals average). Kathmandu receives an average of 345.5 mm (13.6 in.) of precipitation per year, with 205 wet days (56.2 % of the time) [27].

2.2 Sample collection and DNA extraction

Sampling was undertaken concurrently with rabies vaccination and animal birth control campaigns organized by Animal Nepal, Lalitpur, Nepal from July to October 2022. A total of 230 whole blood samples were collected randomly from outwardly healthy community dogs from the cephalic vein and stored in ethylene diamine tetra-acetic acid (EDTA) vacutainers in an aseptic condition. Those dogs were free-roaming and often infested with ticks. They were not exposed to acaricidal treatments. DNA was extracted from 500 µl of each blood sample using DNAzol® BD (MRC, Cincinnati, OH, USA) following the manufacturers protocol and stored at −30 °C for further use. Ethical approval for this research was obtained from the Ethical Clearance Committee of the Nepal Veterinary Council (Ref No: 56/2079.80).

2.3 Conventional PCR

Screening for the family Anaplasmataceae was undertaken using an EHR PCR assay targeting the v1 hypervariable region of the 16S ribosomal RNA gene (rDNA) (approximately 345 bp)

as described previously [28]. The positive samples were further characterized by additional PCRs targeting the heat shock protein (*groEL*) and citrate synthase (*gltA*) genes.

Babesia, Theileria, and *Hepatozoon* parasites were screened by BTH nested PCR assay to amplify the majority of the 18S rDNA (1400–1600 bp) [29]. The samples which were positive for *B. gibsoni* were further characterized by amplifying the *B. gibsoni* thrombospondin-related adhesive protein gene (*BgTRAP*). The details of the primers, the annealing temperatures, and the expected amplicon sizes are provided in Table 1.

All PCR reactions were conducted in a 25 µl reaction mixture containing 12.5 µl of 2 × Gflex PCR Buffer (Mg^{2+}, dNTP plus), 0.5 µl of Tks Gflex DNA Polymerase (TaKaRa Bio Inc., Shiga, Japan), 200 nM of each primer, 1.0 µl of template DNA, and molecular grade water. The PCR condition was set as described previously [30]. The PCR products were electrophoresed in a 1.5 % agarose gel stained with Gel-Red (Biotium, Hayward, CA, USA) and visualized under UV light.

2.4 Real-time PCR

The samples were screened by a *gltA* real-time PCR for the detection of *Rickettsia* as described previously [31]. The details of the primers and probe are presented in Table 1. The reactions were performed in a 20 µl reaction mixture containing 10 µl of THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan), 300 nM of each primer, 200 nM of probe, 5.0 µl of template DNA, and molecular grade water. The reaction was performed in a C1000 thermal cycler with a CFX96 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA) at 50° C for 3 min, 95° C for 1 min, and 40 cycles of 95° C for 15 s and 60° C for 1 min. Each run included a blank, a control, and a serially diluted plasmid standards (10^6, 10^4, and 10^2 copies/reaction) as previously described [32].

2.5 Infusion cloning

Samples with mixed signals in the sequences of the second BTH-PCR products were selected for cloning to obtain single colonies for sequencing. The second PCR primers (BTH infusion second F and BTH infusion second R) were designed based on the alignment of BTH 18S rDNA sequences available in the database. The PCR products amplified with the newly designed primers were purified and cloned into the pUC19 vector (TaKaRa Bio Inc.). The infusion reaction and conditions were set as previously described [39].

2.6 Sanger sequencing

All the PCR amplicons were purified using the NucleoSpin Gel and PCR Clean-Up Kit (TaKaRa Bio Inc.) and sequenced using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) utilizing an ABI Prism 3130 x genetic analyzer (Applied Biosystems) according to the manufacturers instructions .

2.7 Phylogenetic Analysis

Sequence editing, including primer annealing site trimming, was performed using ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 7 [40]. ClustalW was used to align the obtained sequences with those of closely related organisms available in the public database. The maximum likelihood phylogenetic trees were constructed by MEGA using the General Time Reversible model for Anaplasmataceae, TN93 (Tamura-Nei 93) model for 18S rDNA of *Babesia* species, and Kimura 2 model for *BgTRAP* gene of *B. gibsoni.* Bootstrap values were generated based on 1000 replicates.

2.8 Statistical analysis

148 To examine the impact of different factors (including age, sex, and location) on the prevalence
149 of TBPs in the examined dogs, Fisher s exact test was performed using GraphPad Prism 5.0
150 software (GraphPad Software, Inc., La Jolla, CA, USA). In addition, odds ratios and
151 corresponding 95 % confidence intervals were assessed. $p < 0.05$ was designated as statistically
152 significant. Fishers exact test was also performed to test whether the co-infections occurred
153 more frequently than expected by chance.

154 **3 Results**

155 **3.1 Detection of bacterial pathogens**

156 Of the 230 blood samples tested, 53 (29 from Lumbini and 24 from the Kathmandu Valley)
157 were EHR PCR-positive for Anaplasmataceae. Nested PCRs targeting the *groEL* and *gltA*
158 genes of Anaplasmataceae detected *A. platys* and *E. canis* with infection rates of 15.4 %
159 (17/110) and 6.4 % (7/110) in Lumbini, respectively. The infection rate of *A. platys* and *E.*
160 *canis* was 3.3 % (4/120) and 20.8 % (25/120) in the Kathmandu Valley, respectively. None of
161 the examined samples were found to be positive for *Rickettsia* based on real-time PCR testing
162 (Table 2).

163 The infection rates of *A. platys* and *E. canis* were higher in young dogs (11.6 % and 14.5 %,
164 respectively) than in adult dogs (8.1 % and 13.7 %, respectively), although not at statistically
165 significant rates. Similarly, the infection rates of *A. platys* and *E. canis* were not significantly
166 different between male and females dogs (9.7 % and 16.7 % vs. 8.6 % and 11.2 %, respectively).
167 The infection rate of *A. platys* was significantly higher in Lumbini (15.5 %) than in the
168 Kathmandu Valley (3.3 %) ($p < 0.05$), while *E. canis* infections were significantly higher in the
169 Kathmandu Valley (20.8 %) than in Lumbini (6.4 %) ($p < 0.05$) (Table 4).

170 The positive samples in the EHR-PCR were subjected to nested PCR targeting of the *groEL*
171 and *gltA* genes. Sequence analysis of the amplified products of *groEL* and *gltA* PCRs identified

two haplotypes of *A. platys* (with frequency of 90.5 % and 9.5 %) which are closely related to each other and a single haplotype of *E. canis.* The partial sequences of the *groEL* gene of *E. canis* showed 99.9 % (1053/1054 bp) similarity to *E. canis* strain (MG953295) reported from ticks in South Africa. The partial *groEL* gene sequences of *A. platys* from this study (LC831036-LC831056) showed (99.8–99.9 %) (1217/1219 bp-1219/1220 bp) nucleotide identity with *A. platys* (CP046391) reported from dogs in Saint Kitts and Nevis. The *gltA* sequence of *A. platys* (LC831057-LC831077) showed 99.8–100 % (543/544 bp-544/544 bp) sequence identity with previously reported *A. platys* isolate (KR011928) in a tick from China. Similarly, the *gltA* sequences of *E. canis* were 100 % (592/592 bp) identical to a *E. canis* (LC545960) sequence reported from a dog in Myanmar.

3.2 Detection of protozoan pathogens

A total of 26 dogs (12 from Lumbini and 14 from the Kathmandu Valley) were positive for BTH PCR. The infection rates for *B. vogeli* and *B. gibsoni* were 7.3 % (8/110) and 7.3 % (8/110), respectively in Lumbini, whereas, the infection rates for *B. vogeli* and *B. gibsoni* were 10.0 % (12/120) and 5.8 % (7/120), respectively in the Kathmandu Valley. All examined samples were negative for *Theileria* spp. and *Hepatozoon* spp. (Table 2).

The infection rate of *Babesia* spp. was higher in adult dogs (19/161, 11.8 %) than in young dogs (7/69, 10.1 %), although this difference was not statistically significant. Similarly, there was no significant association between the infection rate of *Babesia* spp. and the sex of dogs and sampling location (Table 4).

The 18S rDNA sequences were successfully amplified to near full length from all positive blood samples whereas the samples with mixed signals were selected for cloning. In this way, the presence of *B. vogeli* and *B. gibsoni* was further confirmed by cloning and sequencing of

195 the 18S rDNA. Sequence analysis of BTH PCR products showed a single haplotype each of *B.*
196 *vogeli* and *B. gibsoni*. Three haplotype of *B. gibsoni* were obtained based on *BgTRAP* gene
197 (with frequencies of 60 %, 20 %, and 20 %). Sequence similarity search in BLAST revealed
198 that the *B. vogeli* sequence obtained in this study (LC830822) had 99.9 % (1399/1400 bp)
199 nucleotide similarity with *B. vogeli* (MK881089) reported from a dog in China. Similarly, the
200 *B. gibsoni* sequences (LC830842) showed 100 % (1419/1419 bp) nucleotide similarity with *B.*
201 *gibsoni* (MN134515) reported from a dog in India.

202 **3.3 Detection of mixed infections**

203 Overall *E. canis* was the most frequently detected TBP in dogs at a prevalence of 13.9 %
204 (32/230), which was followed by *A. platys* 9.1 % (21/230), *B. vogeli* 8.6 % (20/230) and *B.*
205 *gibsoni* 6.5 % (15/230) (Table 2). The overall rate of infection with at least one TBP was 32.6 %
206 (75/230). Single infections occurred in 27.8 % (64/230) of the dogs, while co-infection with
207 more than one TPB occurred in 4.7 % (11/230) of the dogs examined (Table 3).
208 There was no significant association between *E. canis* and *B. gibsoni/B. vogeli* infections ($p >$
209 0.05) (Supplementary Tables 1 & 2), whereas there was a positive association between the
210 occurrence of *B. vogeli* and *B. gibsoni* infections ($p = 0.001$) (Supplementary Table 3).

211 **3.4 Phylogenetic analysis**

212 Phylogenetic analysis, based on 18S rDNA, showed that the partial sequence of *B. vogeli*
213 (LC830822) obtained in this study was clustered with *B. vogeli* from Japan, China, Egypt, and
214 Taiwan (Fig. 4). The sequence of *B. gibsoni* (LC830842) was found together with *B. gibsoni*
215 from India, Korea, Germany, Japan, and China (Fig. 4). Additionally, the sequence of *A. platys*
216 and *E. canis* obtained in this study was clustered with that of *A. platys* and *E. canis* from other
217 Asian countries (Fig. 2, Fig. 3). The phylogenetic tree of the *BgTRAP* gene showed that *B.*

gibsoni in Nepal shared a common genetic cluster with those from India and Bangladesh, which formed a different genetic group to that of East Asia (Fig. 5).

4 Discussion

TBPs pose a widespread challenge to One Health [41]. Dogs serve as reservoir hosts for a variety of zoonotic TBPs. However, there is limited information available on the epidemiology of tick-borne diseases affecting dogs in Nepal, with most of the work based on microscopic examination of blood smears [19–21]. Only one molecular study had been carried out for the detection of TBPs in dogs of Kathmandu. This study was conducted to expand knowledge of the geographical range of TBPs in dogs in two different climatic zones i.e., lowland Terai (Lumbini) and mid-hill (Kathmandu Valley).

In the Kathmandu Valley, *E. canis* was the most prevalent pathogen with an infection rate of 20.8 % (25/120), which was higher than previous reported in microscopic studies by Phuyal et al. [19] and Bhatta et al. [20] with rates of 8.0 % and 10.6 % respectively, but lower than those reported by Díaz-Regañón et al. [4] with a rate of 27.1 % in clinically symptomatic dogs using PCR. *Ehrlichia canis* has also been reported commonly from other Asian countries, with varying detection rates including 16.1 %, 1.3 %, and 39.2 % in India, China, and Thailand, respectively [42–44]. The variation in detection rate could be due to differences in the protocol used, dog husbandry practices, climatic conditions, and tick vectors [45,46]. The *gltA/groEL* gene sequences of *E. canis* of this study clustered in one clade together with isolates from other Asian countries indicating a high degree of genetic relatedness among the strains in the regional geographic context. It suggests a close evolutionary relationship and potential cross-border dissemination of *E. canis,* likely driven by shared ecological factors, vector distributions, and host movement across the region.

Anaplasma platys is the causative agent of canine anaplasmosis and is transmitted by *R. sanguineus* complex ticks [47–49]. *Anaplasma platys* was detected in 9.1 % of the dogs, which is higher than previous reports of 1.3 % in Nepal [20], 8.2 % in Bangladesh [50], 3.0 % in India [51], and 6.0 % in China [52]. However, the detection rate is lower than those in the previous studies: 31.4 % in Nepal [4] and 16.3 % in India [53]. In the phylogenetic trees based on the *groEL* and *gltA* genes, the detected *A. platys* were clustered in one clade, suggesting that a single genotype of *A. platys* is prevalent in Nepalese dogs.

Babesia vogeli and B. gibsoni are the causative agents of canine babesiosis and are reported to be transmitted by *R. sanguineus* and *Rhipicephalus haemaphysaloides* [54,55] The prevalence of *B. vogeli* in this study was 8.6 % of the sampled dogs, which is slightly lower than the previous report from Nepal (12.9 %) [4]. The prevalence of *B. vogeli* in other Southeast Asian countries ranged from 3.0 % to 10.0 % [42,44,51,56,57]. Phylogenetic analysis showed that *B. vogeli* has no geographic clustering within Southeast Asia since 18S rDNA sequences of *B. vogeli* from Nepal, China, Egypt, Taiwan, and Japan were clustered in a single clade. This suggests that *B. vogeli* in dogs in Nepal are highly similar to those present in other countries, due to the broad geographic range and adaptability of its tick vectors of the genus *Rhipicephalus*.

The infection rate of *B. gibsoni* was 6.5 % in this study, which was lower than those reported from India (28.6 %) [58], Japan (8.8 %) [59], China (9.2 %) [60], Bangladesh (30 %) [38], and Pakistan (46.8 %) [61]. Phylogenetic analyses showed that *B. gibsoni* sequences geographically cluster with those previously reported from India, Bangladesh, Korea, Japan, and China, possibly due to the distribution of its tick vectors, *Haemaphysalis* spp., as well as potential differences in the mobility and migration patterns of its canine hosts in the region.

Consistent with the previous reports by Bhatta et al. [20] and Phuyal et al. [19], our study did not detect *Theileria* spp. and *Hepatozoon* spp. in the tested dogs. This contrasts with the findings of Díaz-Regañón et al. [4], who detected these pathogens in the dogs in the Kathmandu Valley. The most probable reason for this discrepancy is that 65 % (46/70) of the dogs enrolled in the study by Díaz-Regañón et al. [4] showed clinical sings. On the other hand, our study included only outwardly healthy dogs, which may have lower pathogen load compared to those exhibiting clinical signs.

In this study, 32.6 % (75/230) of the dogs were positive for at least one TBP, with an overall prevalence of *A. platys* of 9.1 %, *Babesia* spp. of 11.3 %, and *E. canis* of 13.9 %. The results of the present study vary from the previous studies of dogs in Nepal, with the overall prevalence of TBPs of 12.0 %-27.3 % by microscopic examination [19–21] and 81.4 % by PCR [4]. Primarily, the variations in prevalence rates might be attributed to differing diagnostic techniques. For example, the sensitivity of PCR is always higher than that of microscopic visualization of smears at very low detectable levels. A lack of diagnostic expertise and the greater chances of human error can lead to false negatives, especially when there are very low numbers of circulating blood pathogens. The sampling from pet dogs with good health care in addition to their small sample size (50–150) could be the reasons for the lower prevalence rates of TBPs in dogs in previous microscopic studies [19,20]. Canine TBPs were also reported from other Southeast Asian countries, with infection rates ranging from 52.3 % in India [51], 43.1 % in Thailand [62], 4.4 % in China [44], and 52.9 % in Bangladesh [50]. The difference in infection rate could be due to the sample size, sample selection criteria, geographical difference, and sampling time [57].

In this study, there was a significant difference in the prevalence rate of *A. platys* and *E. canis* from the two sampling sites. *Ehrlichia canis* (20.8 %) was most prevalent in the Kathmandu Valley, followed by *B. vogeli* (10.0 %), *B. gibsoni* (5.8 %), and *A. platys* (3.3 %), while *A.*

platys (15.4 %) was most prevalent in Lumbini, followed by *B. gibsoni* (7.3 %), *B. vogeli* (7.3 %), and *E. canis* (6.4 %). There was a significant association between *B. vogeli* and *B. gibsoni* infections, whereas there was no significant association between *E. canis* and *B. gibsoni/B. vogeli* infections (Supplementary Tables 1–3). This could be attributed to shared tick vectors, potential pathogen interactions that facilitate or inhibit co-infections, differences in host immune responses, varying ecological niches, and sample size limitations. These findings suggest that while *Babesia* species may co-occur due to similar transmission dynamics, *E. canis* may function under different ecological conditions, warranting further research to clarify these relationships.

Individuals with multiple TBP infections may have more complicated health problems and might act as the source of infection to other susceptible host [63,64]. In the present study, 11 (4.7 %) dogs had co-infection of *B. gibsoni* and *B. vogeli*, which was the most common co-infection among TBPs detected in the current study. According to Kopparthi et al. [65] mixed infections of *B. gibsoni*, *B. vogeli*, and *E. canis* were reported in dogs in India. Similarly, Sarma et al. [51] observed co-infection with *B. gibsoni* and *H. canis* in 34 % of dogs in India. Such mixed infections could be responsible for the variable pathogenicity of these parasites in infected dogs [66,67]. This signifies that whenever a dog manifests severe or atypical symptoms with co-infections, a comprehensive diagnostic approach is essential [65,68].

In our study, the infection rate of TBPs was not significantly affected by the age and sex of the dogs. The dogs were free-roaming community dogs without proper care or health inspections. There was a relatively equal risk of TBP infections across age and sex groups, consistent with previous studies in Nepal and India [4,19,51]. Community dogs can act as sentinels and reservoirs for various zoonotic pathogens, potentially transmitting infectious agents to humans, livestock, and pets. Early diagnosis, treatment, and vector control are crucial to managing the

transmission of TBPs within the dog population and to other species. While our study provides invaluable insights into the epidemiology of key TBPs circulating in community dogs in two ecological zones of Nepal, future studies identifying responsible tick vectors and their associated pathogens are required to fill gap in our understanding of the epidemiological profiles of canine tick-borne diseases in Nepal.

5 Conclusions

To our knowledge, this study represents the first molecular investigation of TBPs in community dogs in two different climatic zones of Nepal. Our study identified *B. vogeli, B. gibsoni, A. platys,* and *E. canis* in the dog populations. The detection of both single and mixed infections highlights the need for modifications of diagnostic and treatment protocols for TBPs among dogs. Accurate diagnosis of TBPs in ticks and infected hosts is crucial for the prevention and control of these infections at the community level through effective veterinary care and vector control. Furthermore, the detection of TBPs of zoonotic potentials warrants a comprehensive One Health approach for future research to elucidate the role of community dogs in zoonotic spillover of TBPs to humans.

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CRediT authorship contribution statement

Gita Sadaula Pandey: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Chet Raj**

Pathak: Resources, Writing – review & editing, **Sunil Thapa**: Resources, Investigation, **Amir Sadaula:** Investigation, Writing – review & editing, **Prajwol Manandhar**: Investigation, Writing – review & editing, **Abdelbaset Eweda Abdelbaset**: Investigation, Formal analysis, Writing – review & editing, **Yongjin Qiu:** Investigation, Software, Writing – review & editing**, Mackenzie L. Kwak:** Writing – review & editing, **Naoki Hayashi:** Formal analysis, Software, Writing – review & editing**, Nariaki Nonaka**: Supervision, Writing – review & editing. **Ryo Nakao**: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interests

The authors declare that there is no conflict of interest.

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Data availability:

The sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) under the accession numbers LC830967-LC830998 for the *gltA* gene of *E. canis,* LC831057-LC831077 for the *gltA* gene of *A. platys*, and LC831004-LC831035 for the *groEL* gene of *E. canis,* LC831036- LC831056 for the *groEL* gene of *A. platys*, LC830822-LC830841 for 18S rDNA of *B. vogeli*, LC830842-LC830856 for 18S rDNA of *B. gibsoni*, LC830999-LC831003 for *BgTRAP* gene of *B. gibsoni*.

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- Studies on tick-borne pathogens (TBPs) in dogs in Nepal are limited.
- Community dogs were molecularly examined for piroplasma and rickettsial infections.
- 32.6% (75/230) of the dogs were infected with at least one TBP.
- Co-infections with multiple TBPs were found in 4.7% of the dogs.
- *Ehrlichia canis, Anaplasma platys, Babesia vogeli* and *Babesia gibsoni* were detected.

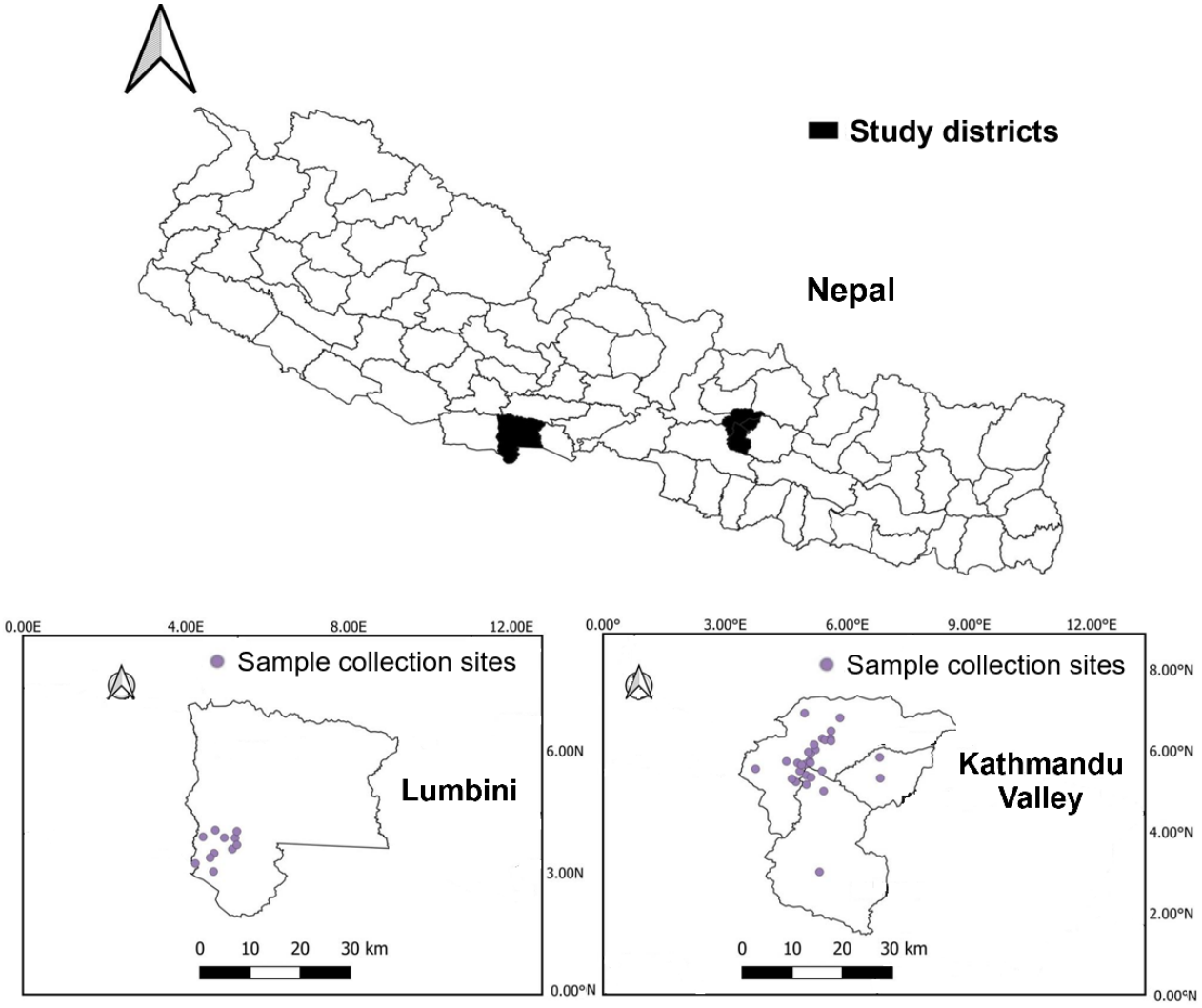

Figure 1: Map of Nepal showing the sampling locations where tick-borne pathogens in community dogs were screened.

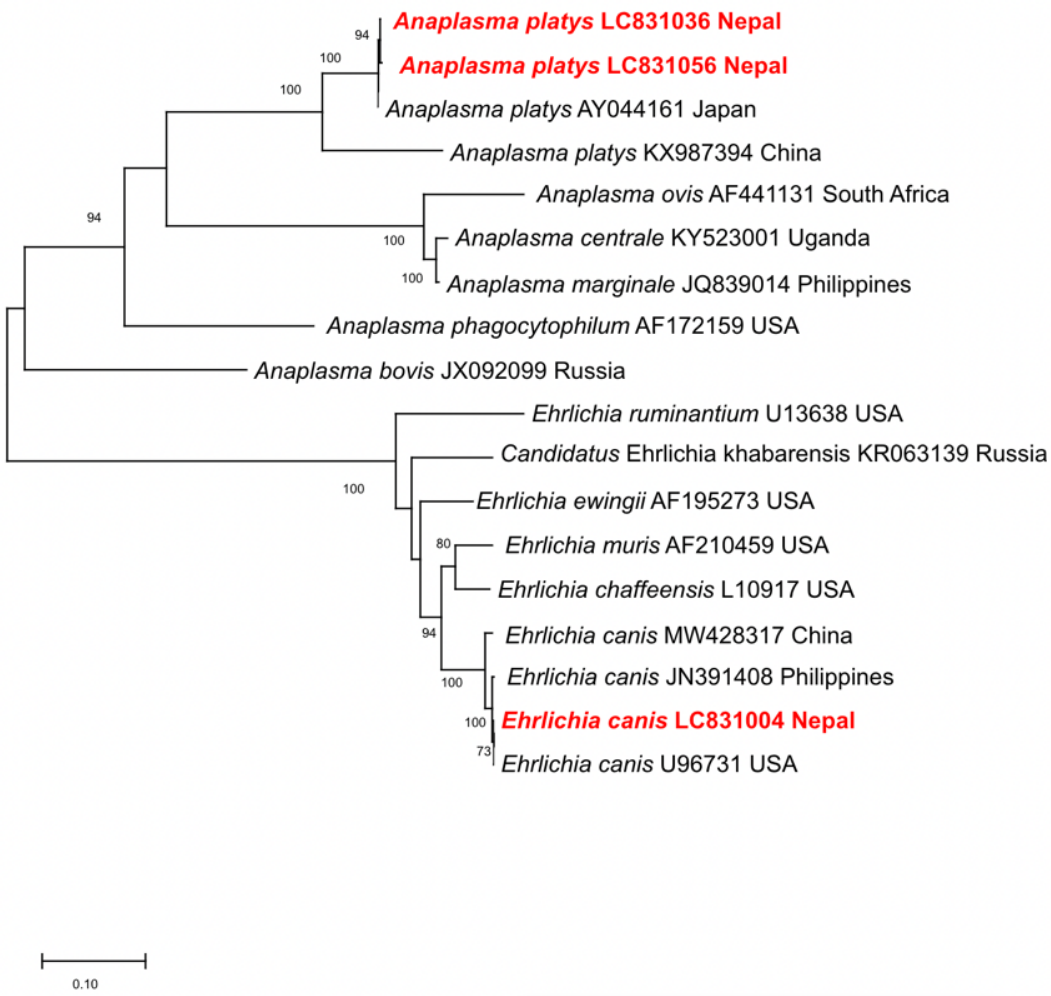

Fig. 2: Maximum likelihood phylogenetic tree based on the *groEL* gene of *Anaplasma platys* and *Ehrlichia canis* detected in dogs. Bootstrap values >70 from 1000 replicates is shown above the branch.

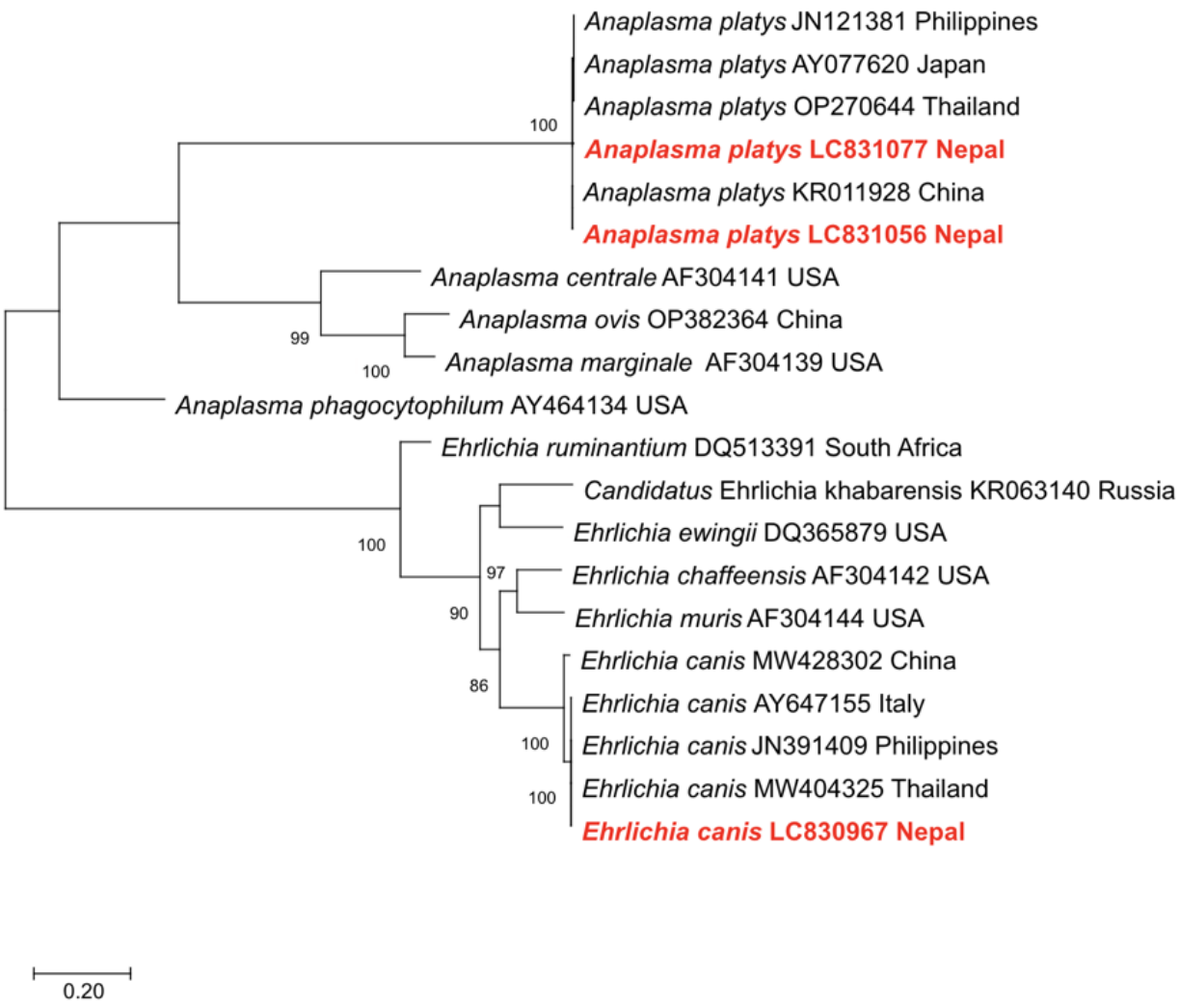

Fig. 3: Maximum likelihood phylogenetic tree based on the *gltA* gene of *Anaplasma platys* and *Ehrlichia canis* detected in dogs using the General Time Reversible model. The bootstrap values >70 from 1000 replicates is shown above the branch.

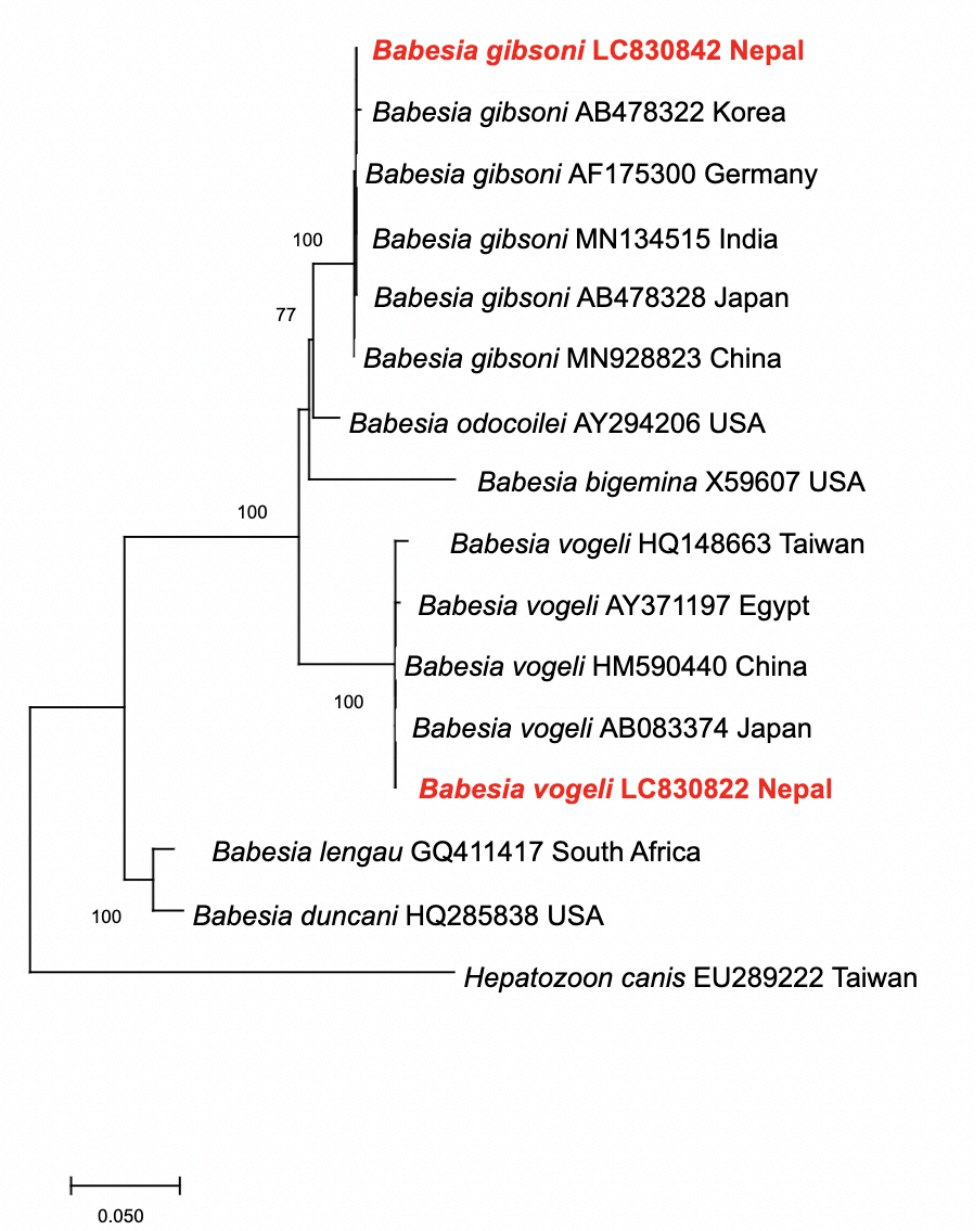

Fig. 4: Maximum likelihood phylogenetic tree based on the 18S rRNA gene of *Babesia* spp. detected in dogs. The tree was rooted with *Hepatozoon canis* and bootstrap values >70 from 1000 replicates are shown above the branch.

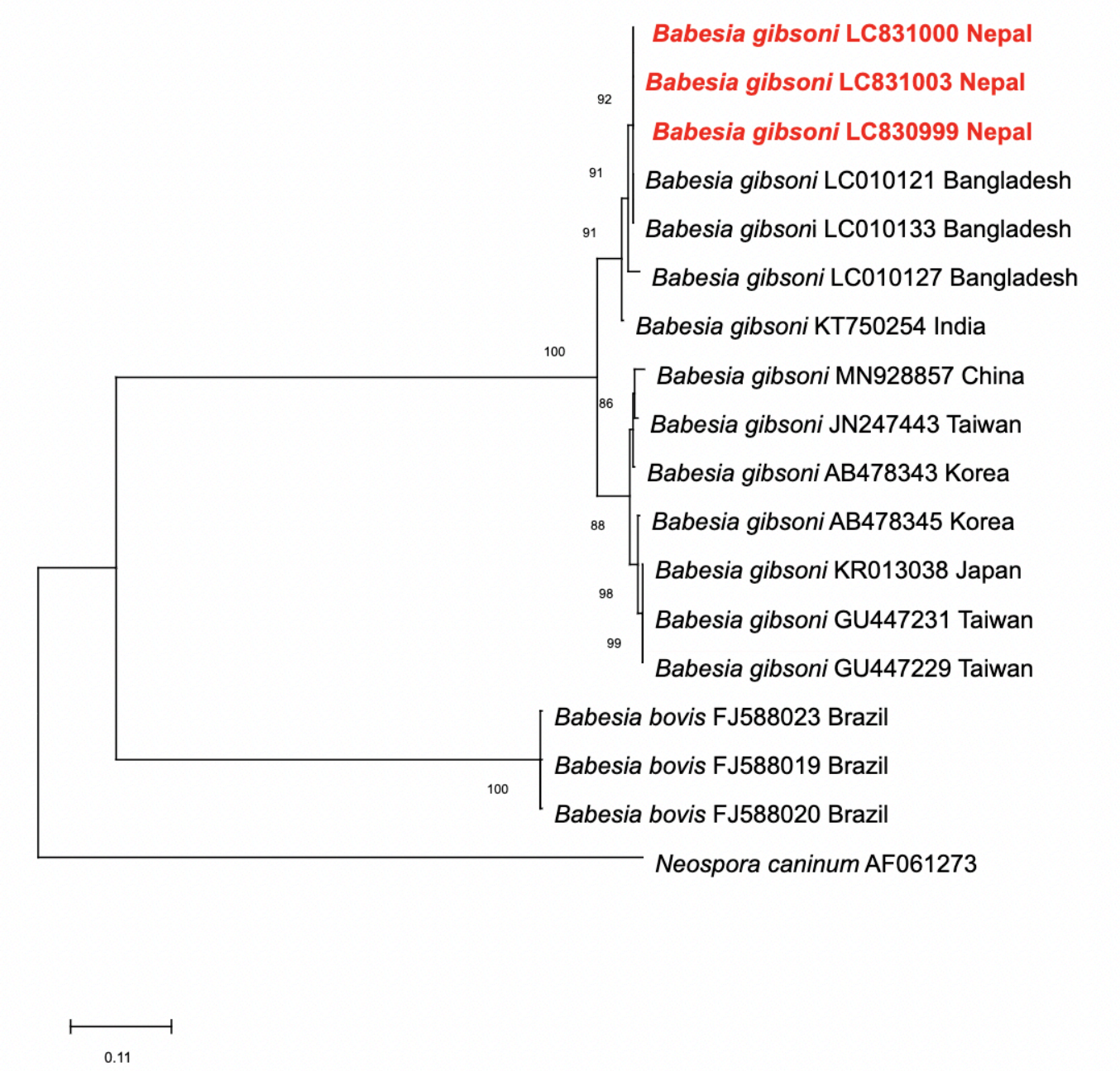

Fig. 5: Maximum likelihood phylogenetic tree based on the *BgTRAP* gene of *Babesia gibsoni* detected in dogs using the Kimura 2-parameter. The tree was rooted with *Neospora caninum* and bootstrap values >70 from 1000 replicates are shown above the branch.

Table 1: The list of primers and probe used in this study.

Primer name	Sequence (5'-3')	Target organism(s)	Target gene	Purpose	Product size (bp)	Reference
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	*Babesia, Theileria* & *Hepatozoon*	18S rDNA	1st PCR	1400–1600	[29]
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	*Babesia, Theileria* & *Hepatozoon*	18S rDNA	2nd PCR	1400–1600	[29]
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
EHR16SD	GGTACCYACAGAAGAAGTCC/	Anaplasmataceae	16S rDNA	PCR	345	[28]
EHR16SR	TAGCACTCATCGTTTACAGC					
HS1-F	CGYCAGTGGGCTGGTAATGAA	Anaplasmataceae	*groEL*	1st PCR	1300	[33]
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*Anaplasma*	*groEL*	2nd PCR	1256	[34]
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*Ehrlichia*	*groEL*	2nd PCR	1100	[35]
groEL_rev2	GCCGACTTTTAGTACAGCAA					
F4b	CCAGGCTTTATGTCAACTGC	Anaplasmataceae	*gltA*	1st PCR	800	[36]
R1b	CGATGACCAAAACCCAT					
EHR-CS136F	TTYATGTCYACTGCTGCKTG	*Anaplasma* & *Ehrlichia*	*gltA*	2nd PCR	650	[36]
EHR-CS778R	GCNCCMCCATGMGCTGG					
gltA84F	GACCTACGATCCGGGATTCA	*Anaplasma*	*gltA*	PCR	580	[37]
gltA69R	CCGCACGGTCGCTGTT					
BgTRAPtF1	GTGACACTCAACGTTGTCTG	*Babesia gibsoni*	*BgTRAP*	1st PCR	1300	[38]
BgTRAPtR1	TGTTGATCCTCGTACAGTCC					
BgTRAPtF2	GGTTAACAGTGGTTCTGTGAG	*B. gibsoni*	*BgTRAP*	2nd PCR	1100	[38]
BgTRAPtR2	CTGGCGTCCATATCATAGTC					
CS-F	TCGCAAATGTTCACGGTACTTT	*Rickettsia*	*gltA*	Real-time PCR	74	[31]
CS-R	TCGTGCATTTCTTTCCATTGTG					
CS-P	TGCAATAGCAAGAACCGTAGGCTGGATG					
InFusin_BTH 2ndF	AAAACGACGGCCAGTGGCTCATTACAACAGTTATAGTTT	*Babesia, Theileria* & *Hepatozoon*	18S rDNA	cloning	1400–1600	[39]
InFusin_BTH_2ndR	GACCATGATTACGCCCGGTCCGAATAATTCACCGG					

Table 2: Tick-borne pathogens detected in the community dogs in Nepal.

Location	**Sex**	**No. of dogs**	***A. platys* (%)**	***E. canis* (%)**	***B. gibsoni* (%)**	***B. vogeli* (%)**	***Rickettsia* spp.**	***Theileria* spp.**	***Hepatozoon* spp.**
Lumbini	Male	51	11 (21.5)	3 (5.9)	5 (9.8)	3 (5.9)	0	0	0
	Female	59	6 (10.2)	4 (6.8)	3 (5.1)	5 (8.5)	0	0	0
Subtotal		110	17 (15.5)	7 (6.4)	8 (7.3)	8 (7.3)	0	0	0
Kathmandu	Male	63	0	16 (25.4)	1 (1.6)	5 (7.9)	0	0	0
Valley	Female	57	4 (7.0)	9 (15.8)	6 (10.5)	7 (12.3)	0	0	0
Subtotal		120	4 (3.3)	25 (20.8)	7 (5.8)	12 (10.0)	0	0	0
Total		230	21 (9.1)	32 (13.9)	15 (6.5)	20 (8.6)	0	0	0

Table 3: The occurrence of single and mixed infections of tick-borne pathogens in the community dogs in Nepal.

Tick-borne pathogen(s) detected		**No. of dogs (%)**
Single infection	*B. gibsoni*	5 (2.2 %)
	B. vogeli	10 (4.3 %)
	A. platys	21 (9.1 %)
	E. canis	28 (12.2 %)
Mixed infection	*B. gibsoni* + *B. vogeli*	7 (3.0 %)
	B. gibsoni + *E. canis*	1 (0.4 %)
	B. vogeli + *E. canis*	1 (0.4 %)
	B. gibsoni + *B. vogeli* + *E. canis*	2 (0.9 %)

Table 4. The association between different variables (age, sex, and location) and the infection with tick-borne pathogens.

Variable	Category	No. of dogs tested	*A. platys*			*E. canis*			*Babesia* spp.		
			No. of positives (%)	OR (95 % CI)	p value	No. of positives (%)	OR (95 % CI)	p value	No. of positives (%)	OR (95 % CI)	p value
Age	Young (≤ 1 yr)	69	8 (11.6)	1.49 (0.59–3.78)	0.455	10 (14.5)	1.07 (0.48–2.40)	0.838	7 (10. 1)	0.84 (0.34–2.11)	0.823
	Adult (≥ 1 yr)	161	13 (8.1)	0.67 (0.26–1.67)		22 (13.7)	0.93 (0.42–2.09)		19 (11.8)	1.19 (0.47–2.96)	
Sex	Male	114	11 (9.7)	1.13 (0.46–2.78)	0.823	19 (16.7)	1.59 (0.74–3.38)	0.257	13 (11.4)	1.02 (0.45–2.31)	1.000
	Female	116	10 (8.6)	0.88 (0.36–2.17)		13 (11.2)	0.63 (0.3–1.35)		13 (11.2)	0.98 (0.43–2.22)	
Location	Kathmandu Valley	120	4 (3.3)	0.19 (0.06–0.58)	0.002	25 (20.8)	3.87 (1.60–9.37)	0.002	14 (11.7)	1.08 (0.48–2.45)	1.000
	Lumbini	110	17 (15.5)	5.30 (1.72–16.30)		7 (6.4)	0.26 (0.11–0.62)		12 (10.9)	0.93 (0.41–2.10)	

OR, odds ratio; CI, confidence interval.