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Title	Molecular characterization of Rhipicephalus microplus and tick-borne pathogens in cattle in Nepal
Author(s)	Pandey, Gita Sadaula; Pathak, Chet Raj; Sadaula, Amir et al.
Citation	Veterinary Research Communications, 49(article number 346) https://doi.org/10.1007/s11259-025-10913-7
Issue Date	2025-10-04
Doc URL	https://hdl.handle.net/2115/98135
Rights	This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature' s AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/10.1007/s11259-025-10913-7
Type	journal article
File Information	MS_Pandey_HUSCAP_plusFandT.pdf



Molecular characterization of *Rhipicephalus microplus* and tick-borne pathogens in cattle in Nepal

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Abstract

Ticks and tick-borne pathogens (TBPs) have a significant economic impact on livestock. In Nepal, only limited information is available for the molecular identification of ticks and TBPs. This study aims to address this major challenge by providing data on the diversity of TBPs in Nepalese cattle, along with molecular sequences to facilitate accurate identification. A total of 145 cattle blood samples were collected from Pokhara (n = 70) and Madi (n = 75). The cattle were also examined for tick infestations, and the detected ticks were subjected to morphological identification, followed by molecular characterization using the cytochrome *c* oxidase subunit I (COI) gene. The heat shock protein (*groEL*) and citrate synthase (*gltA*) genes were targeted for screening Anaplasmataceae. Piroplasmida infections were initially screened using nested polymerase chain reaction (PCR) targeting the 18S ribosomal RNA gene. *Theileria orientalis* and *Anaplasma marginale* were further characterized by the major surface protein 4 (*MSP4*) gene. *Rhipicephalus microplus* was the only tick species detected in the examined cattle. Overall, 29.7% (43/145) of the cattle examined were infected with at least one TBP, with 3.4% (5/145) being co-infected with more than one TBP. The detection rate of TBPs was 20% (n = 29) for *A. marginale*, 3.4% (n = 5) for *Anaplasma bovis*, 6.2% (n = 9) for *T. orientalis*, and 4.1% (n = 6) for *Theileria annulata*. The phylogenetic analysis showed that two genotypes of *A. marginale* and *T. orientalis* (types 5 and 7) were present in cattle. This study provides preliminary baseline data on the prevalence and genetic diversity of ticks and TBPs in cattle in Nepal.

Keywords: Cattle, Nepal, *Rhipicephalus microplus*, Tick-borne pathogens

Introduction

Cattle have major cultural/economic importance in Nepal and contribute ~50% of the nation's total milk production (Lohani 2010; Paudel et al. 2021; AITC 2024). In Nepal, the dairy sector

contributes 62.6% to the livestock GDP (AITC 2024). However, production losses in dairy cows are attributed to blood feeding of ticks and the diseases caused by tick-borne pathogens (TBPs) (Uilenberg 1992; Singh et al. 2022).

Ticks are hematophagous ectoparasites that can infest a wide range of hosts and transmit a variety of TBPs to humans and animals (Vongphayloth et al. 2016; Jongejan and Uilenberg 2004). *Rhipicephalus microplus* is a one-host tick which affects cattle production directly through weight loss and skin damage, and indirectly through pathogen transmission (De Castro 1997; Burger et al. 2014). The taxonomy of *R. microplus* is complex and not yet fully understood. It was previously classified under the genus *Boophilus*, which is now considered a subgenus within *Rhipicephalus*. *Rhipicephalus microplus* is a member of a species complex that includes several poorly characterized species. Accurate identification often requires input from expert taxonomists in combination with molecular data (Estrada-Peña et al. 2012; Roy et al. 2018).

Tick-borne diseases of cattle are mainly caused by protozoan and Anaplasmataceae pathogens, including babesiosis, theileriosis, anaplasmosis, and ehrlichiosis (Derso and Demessie 2015; Dantas-Torres and Otranto 2017a; Spare et al. 2020; Jacob et al. 2020). Tick-borne diseases impose significant economic losses, exceeding \$300 million annually in Latin America and Africa, while in India, TBPs are associated with an estimated 20% reduction in milk production (Minjauw and McLeod 2003; Singh et al. 2012; Grisi et al. 2014).

Anaplasmosis in bovines is characterized by fever, severe anemia, jaundice, respiratory distress, and abortion, and occurs more frequently in adult animals (Derso and Demessie 2015). *Anaplasma marginale* is the most pathogenic species transmitted by ticks like *Dermacentor* and *Rhipicephalus* (Dantas-Torres and Otranto 2017b; George et al. 2017). *Anaplasma bovis* is transmitted by *Rhipicephalus* and *Haemaphysalis* ticks (Atif 2016; Dantas-Torres and Otranto 2017b). In addition to ticks, the mechanical transmission of *Anaplasma* species via *Tabanus*

76 flies, infected surgical instruments, and needles has been recorded. (Dantas-Torres and Otranto
77 2017b).

78 Major species of *Babesia* infecting bovines include *Babesia bovis*, *Babesia bigemina*, and
79 *Babesia divergens* (Bock et al. 1997; Brown et al. 2006; Filipe et al. 2024). *Rhipicephalus*
80 *microplus* is the primary vector of *B. bovis* and *B. bigemina*, whereas *B. divergens* is transmitted
81 by *Ixodes ricinus* (Filipe et al. 2024). Bovine babesiosis manifests as fever, anemia,
82 hemoglobinuria, and neurological signs (in the case of *B. bovis*), and is associated with moderate
83 to high mortality (Enbiyale et al. 2018).

84 Among the many *Theileria* species, *Theileria parva* is responsible for East Coast fever (ECF)
85 or corridor disease, while *Theileria annulata* is responsible for tropical theileriosis (Singla and
86 Kaur 2022). Theileriosis in bovines is characterized by fever, lymphadenopathy, pulmonary
87 edema, and diarrhea, with a mortality risk in cases of ECF (Norval et al. 1991; Onizawa and
88 Jenkins 2024). Tropical theileriosis presents with similar clinical signs to ECF; however, *T.*
89 *annulata* also invades red blood cells, causing anemia, jaundice, and hemoglobinuria. In
90 addition, *Theileria orientalis* is a member of the relatively benign *Theileria* group (Fujisaki et
91 al. 1994; Yokoyama et al. 2012). In Asia, *Haemaphysalis* ticks serve as vectors of the benign
92 *Theileria* group (Sugimoto and Fujisaki 2002). Based on the sequence analysis of the major
93 piroplasm surface protein (*MPSP*) gene, *T. orientalis* can be divided into 11 genotypes including
94 Chitose (Type 1), Ikeda (Type 2), Buffeli (Type 3), Types 4-8, and N1-N3. Among these, the
95 Chitose and Ikeda genotypes are pathogenic (Sivakumar et al. 2014; Gebrekidan et al. 2016).

96 In Nepal, 9 tick species, including *Haemaphysalis aponomoides*, *Haemaphysalis bispinosa*,
97 *Ixodes acutitarsus*, *Dermacentor everestianus*, *R. microplus*, *Haemaphysalis nepalensis*, *Ixodes*
98 *ovatus*, *Ixodes nuttallianus*, *Hyalomma marginatum isaaci* and one uncharacterized
99 *Amblyomma* sp., have been reported to infest cattle (Hoogstraal 1962, 1971; Clifford et al. 1971,
100 1975; Gupta et al. 2013; Apanaskevich et al. 2014; Bohara and Shrestha 2016; Dhital 2018; Pun

et al. 2018). Microscopic examination of blood smear found *A. marginale* in 5.8% and *B. bovis* in 0.6% of cattle in mid-western Nepal (Bohara and Shrestha 2016). Polymerase Chain reaction (PCR)-based investigations showed the *A. marginale* infection in 7.5% of cattle in Morang, Rupandehi, and Surkhet Districts of Nepal (Pathak et al. 2023) and piroplasma infection in 42.5% of cattle in Kathmandu valley (Dhakal et al. 2023). In *Hyalomma* ticks collected from cattle, 24.1% (114/472) tested positive for *T. annulata* by microscopic examination of stained salivary glands (Gupta et al. 2013).

In Nepal, molecular data on the genotypic profiles of ticks and TBPs in cattle remain limited. This constrains the ability of local researchers to accurately identify tick species and TBPs affecting the cattle. Therefore, this study provides valuable data to support the molecular identification of a key cattle tick species and several important TBPs in Nepal.

Methodology:

Study area and sample collection:

A cross-sectional study was conducted from August to October 2022. The study area was stratified into two regions: the mid hill (Pokhara - 28.2083° N, 83.9888° E; Kaski District) (n = 70) and the Lowland Terai (Madi - 27.4552° N, 84.3184° E; Chitwan District) (n = 75). Pokhara is in the Kaski District of Gandaki Province, whereas Madi Municipality is in the Chitwan District of Bagmati Province. Both are recognized as dairy pocket areas of Nepal. It is surrounded on three sides by the national park and to the south by the Churehill: a buffer zone of the Chitwan National Park (Fig. 1). The objective of selecting two regions was to compare the prevalence of TBPs between them. In both sites, integrated farming practices, either traditional or semi-commercial system, were commonly used.

A two-stage random sampling approach was employed. In the first stage, 109 cattle farms were randomly selected based on the sample size calculation (Daniel 1999): 46 from Pokhara

Metropolitan City and 63 from Madi Municipality (Supplementary File 1). The number of cattle per household in Nepal is generally low; for example, herds of 2–4 animals were most frequently reported in a previous study (Broaddus-Shea et al. 2020). In Pokhara Metropolitan City, the median herd size was 3 animals (mean = 9), while in Madi Municipality the median was 4 animals (mean = 7). In the second stage, proportional sampling was conducted based on herd size. Specifically, one animal was randomly selected from herds comprised fewer than 15 animals; two animals from herds of 16-25 animals; and three animals from herds of 26-34 animals. This sampling strategy was applied consistently throughout the study (Supplementary Table 1). In total, 145 cattle (70 from Pokhara Metropolitan City and 75 from Madi Municipality) were selected. From each individual animal, approximately 5 ml of whole blood was collected from the jugular vein into an EDTA vacutainer tube. The collected samples were stored in the laboratory of the National Trust for Nature Conservation Lab, Sauraha (NTNC – BCC) for further work.

A total of 600 tick samples were collected by hand from 145 cattle. Approximately 2–5 ticks were collected from each animal and preserved in 70% ethanol using separate 2ml tubes.

Morphological identification of ticks

Ticks were morphologically examined and classified into male, female, and nymphs using a stereomicroscope (Olympus SZX16, Tokyo, Japan). The *Rhipicephalus* subgenus *Boophilus* contains five accepted species. Tick specimens in this study were identified to species level using keys and descriptions by Walker et al. (2003) and Feldman-Muhsam and Shechter (1970).

DNA extraction and PCR

DNA was extracted from a 500 µl blood sample with DNAzol® BD (MRC, Cincinnati, OH, USA) following the manufacturer's protocol and stored at – 30 °C for further use. The presence of Anaplasmataceae was first screened by using the EHR PCR assay targeting 16S ribosomal RNA gene (rDNA) (about 345 bp) as described previously (Parola et al. 2000). All samples that tested positive for EHR PCR were further characterized by heat shock protein (*groEL*) gene and citrate synthase (*gltA*) gene PCRs. Additionally, major surface protein 4 (*MSP4*) gene PCR was performed to further characterize *A. marginale* (De la Fuente et al. 2003). *Babesia*, *Theileria*, and *Hepatozoon* parasites were screened by using the BTH nested PCR assay to amplify almost the entire length of the 18S rDNA (1,400 – 1,600 bp) (Masatani et al. 2017). The samples positive for *T. orientalis* were further characterized using the *MPSP* gene as described previously (Ota et al. 2009). The details of the primers and the expected amplicon sizes are provided in Table 1.

After morphological identification, ticks were grouped by developmental stage and sex. From each group, 10 samples were selected for DNA extraction. The ticks preserved in 70% ethanol were washed with distilled water before processing. For molecular identification, DNA was extracted from selected ticks using DNAzol® (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol and stored at -30 °C. The morphological identification of selected ticks was verified by amplification of a 653 bp-fragment of the cytochrome *c* oxidase subunit I (COI) gene (Folmer, et al. 1994).

All PCR reactions were conducted in a 25 µl reaction mixture containing 12.5 µl of 2× TaKaRa Ex Premier™ DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.5 µl of 200 nM of each primer, 1.0 µl of template DNA, and molecular grade water for volume adjustment. The cycling conditions were set with an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at each temperature shown in Table 1 for 15 sec, and extension at 68 °C for 1 min/kb, and final extension at 68 °C for 5 min. In each PCR,

molecular-grade water and DNA from each target organism were included as negative and positive controls, respectively. The PCR products were subjected to 1.5% agarose gel electrophoresis, stained with Gel-Red (Biotium, Hayward, CA, USA), and visualized under UV light.

In-Fusion cloning

Samples exhibiting double peaks in the electropherograms from the direct sequencing of second BTH-PCR products were chosen for In-Fusion cloning to acquire sequences from individual colonies. The PCR products generated using the BTH infusion 2nd F and BTH infusion 2nd R primers were purified and subsequently cloned into the pUC19 vector (TaKaRa Bio Inc.). The In-Fusion reaction was carried out in a 10 µl volume containing 2.0 µl of 5× In-Fusion HD Enzyme Premix vector (TaKaRa Bio Inc.), 1.0 µl of PCR-linearized pUC19 vector, 200 ng of each purified PCR fragment, and molecular-grade water. The infusion reaction condition and circumstances were established as previously outlined (Pandey et al. 2024).

Sanger sequencing

All the amplicons generated by 2nd *gltA* gene PCR and the *MSP4* gene PCR were purified using the NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Nagel, Düren, Germany) and sequenced. Based on the species identified from the obtained *gltA* gene and *MSP4* gene sequences, five samples per *Anaplasma* species were randomly selected from the second *groEL* gene PCR for sequencing. Sequencing was performed using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3130 x genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic analysis

Sequence editing, including the trimming of primer annealing sites, was conducted using ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis was performed with Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al. 2016). Sequence alignment was performed with ClustalW, comparing the obtained sequences with those of closely related organisms from GenBank. The best substitution model was determined prior to constructing the phylogenetic tree using the find best DNA/protein model in MEGA version 7. The maximum likelihood phylogenetic trees were constructed in MEGA using the General Time Reversible model for *groEL* gene and Kimura 2 parameter model for *gltA* gene of *A. marginale* and *A. bovis*, Tamura-Nei 93 model for 18S rDNA of *T. orientalis* and *T. annulata*, Tamura 3 parameter model for *MPSP* gene of *T. orientalis* and COI gene of *R. microplus*. Bootstrap values were obtained with 1,000 replicates.

Statistical analysis

To assess the influence of various host related factors (age, sex, breed, and location) on the prevalence of TBPs in the cattle studied, Fisher's exact test was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). In addition, the odds ratios and the corresponding 95% confidence intervals were evaluated. $P < 0.05$ was considered statistically significant.

Results

Tick identification

In total, 600 ticks at different stages of feeding were collected from cattle in two districts. All collected ticks were identified as *R. microplus* (160 male, 415 Female, 25 nymph) (Fig. 2). A total of 30 samples were randomly selected for sequencing comprising 10 samples each from males, females and nymphs. The COI sequencing confirmed the identity of *R. microplus*. Six

different haplotypes were obtained among the 30 sequenced samples. In the phylogenetic analysis, all *R. microplus* from this study clustered within clade C (Fig. 3).

Detection of bacterial pathogens

Anaplasmatataceae was detected in 23.4% (34 /145) of the tested cattle by EHR-PCR. Nested PCRs targeting the *groEL*, *gltA*, and *MSP4* genes detected *A. marginale* with an infection rate of 25.7% (n = 18) in Pokhara and 14.7% (n = 11) in Madi. The infection rate of *A. bovis* was 1.5 % (n = 1) in Pokhara and 5.3% (n = 4) in Madi, as determined by the nested PCR targeting the *groEL* and *gltA* genes.

The infection rate of *Anaplasma* (n = 8; 32.0%) was higher in calves than in adult cattle (n = 26; 21.7%), which was not statistically significant. There was also no significant difference in infection rates between sex and breed of cattle ($p > 0.05$). The infection rate of *Anaplasma* was higher in Pokhara (n = 19; 27.1%) than in Madi (15; 20.0%), but no significant association was found between location and infection rates.

The *groEL* gene sequences of *A. marginale* (LC872887 – LC872891) showed 99.41 – 100.00 % (1,108/1,109 bp – 1,109/1,109 bp) identity to *A. marginale* (CP006847) reported from cattle in Australia. The *groEL* gene sequences of *A. bovis* from this study (LC872882 – LC872886) were 96.17% – 99.25% (1,005/1,045 bp – 1,002/1,045 bp) identity to *A. bovis* (KX987399) found in a tick in China. The *gltA* gene sequences of *A. marginale* (LC871906 – LC871934) showed 99.29% – 100.00% (419/422 bp – 422/422 bp) identity with *A. marginale* (JQ839004) reported from cattle in the Philippines. The *gltA* gene sequences of *A. bovis* (LC871935 – LC871939) showed 91.45 – 92.40% (385/421 bp – 389/421 bp) identity to *A. bovis* (MH255913) reported from goat in China. The *MSP4* sequences of *A. marginale* (LC871940 – LC871968) showed 99.74% – 100.00% (769/771 bp – 771/771 bp) identity with

248 *A. marginale* (KX989514) from cattle in India and *A. marginale* (JN564648) from cattle in
249 Mexico.

250 Phylogenetic analysis based on *groEL* and *gltA* genes showed that the sequences of *A.*
251 *marginale* (LC872887, LC872891, LC871906, and LC871923) and *A. bovis* (LC872882,
252 LC872886, LC871939, and LC871935) obtained in this study were identical (or almost so) to
253 *A. marginale* (JQ839013, FJ226455, LC664078, and AF414864) and *A. bovis* (KX987399,
254 OQ185211, OM648128, MH255906, and OM648127) reported from other countries (Figs 4,
255 5). The phylogenetic analysis revealed that the *MSP4* gene sequences of *A. marginale* were
256 divided into two clusters (Fig. 6). One sequence (LC871940) clustered with *A. marginale*
257 sequences previously reported from Taiwan (EU677383), India (OM039457), and China
258 (MW772454, KX904527), while the other sequence (LC87960) clustered with sequences from
259 Thailand (MH939156) and Mexico (JN564646).

260 **Detection of protozoan pathogens**

261 BTH PCR targeting *Babesia*, *Theileria*, and *Hepatozoon* parasites was positive in 8.9%
262 (13/145) of tested cattle; 10.0% (7/70) in Pokhara and 6.0% (8/75) in Madi (Table 4). In
263 Pokhara, the infection rates of *T. orientalis* and *T. annulata* were 4.3% (3/70) and 7.1% (5/70),
264 respectively. In Madi, the infection rates of *T. orientalis* and *T. annulata* were 8.0% (6/75) and
265 1.3% (1/75) respectively. However, none of the samples tested positive for *Babesia* and
266 *Hepatozoon*. BLAST analysis revealed that the 18S rDNA sequences of *T. annulata* obtained
267 in this study (LC872902 – LC872906) had 99.93% (1,492/1,493 bp) identity with *T. annulata*
268 (MN944852) reported from cattle in Italy. The 18S rDNA sequences of *T. orientalis* (LC872892
269 – LC872900) showed 99.93% (1,507/1,508 bp) identity to *Theileria* sp. reported from cattle in
270 India (OR067890), 99.47% – 100.00% (1,496/1,504 bp – 1,424/1,424 bp) identity with *T.*
271 *orientalis* reported from cattle in India (MF576178), and Japan (LC576817). The *MPSP* gene
272 sequences of *T. orientalis* (LC872594 – LC872602) showed 99% – 100.00% (676/677 bp –

659/659 bp) identity with *T. orientalis* reported from Thailand (KU886281), Myanmar (AB871365), India (PV565058) and Bangladesh (PP297118).

The infection rate of *Theileria* spp. was higher in calves (12.0%, 3/25) than in adult cattle (8.3%, 10/120) with no statistical significance. Similarly, no significant association was found between *Theileria* spp. infection rates and sex, breed of cattle, or sampling location.

The phylogenetic tree based on 18S rDNA sequences revealed that the *Theileria* sequences obtained in this study clustered with *T. orientalis* and *T. annulata* reported from other countries.

The 18S rDNA phylogenetic tree showed that the *T. orientalis* sequences obtained in this study are divided into two clusters. One cluster consists of isolates from Korea, Australia, Myanmar, China, India, and Pakistan, while the other included isolates from Myanmar, China, and Japan.

The *T. annulata* sequences obtained in this study clustered with the *T. annulata* sequences reported from China (KF559356 and EU083799) (Fig. 7).

Phylogenetic analysis of *MPSP* genes revealed two genotypes of *T. orientalis* belonging to types 5 and 7. Six samples from Madi belonged to *T. orientalis* type 7 and were closely related to *T. orientalis* reported from Thailand, Japan, and Indonesia, while three samples from Pokhara belonged to *T. orientalis* type 5 (AB218430, AB081329, and AF102500) (Fig. 8).

Detection of mixed infections

The overall infection rate with at least one TBP was 29.7% (43/145). A single infection was detected in 26.2% (38/145) of the cattle, while 3.4% (5/145) were co-infected with multiple TBPs (Table 3). Among the TBPs in cattle, *A. marginale* was the most common with a detection rate of 20% (29/145). This was followed by *T. orientalis* with 6.2% (9/145), *T. annulata* with 4.1% (6/145), and *A. bovis* with 3.4% (5/145) (Table 2).

Discussion

298 Ticks are obligatory hematophagous ectoparasites infesting animals as well as humans and acts
 299 as vectors of numerous pathogens that cause serious diseases. The impacts of ticks and TBPs
 300 results in considerable economic losses worldwide (Jongejan and Uilenberg 2004). There have
 301 been several molecular studies of ticks and TBPs in the countries neighboring Nepal, including
 302 China and India. However, molecular investigations of the ticks and TBP in cattle in Nepal have
 303 been extremely limited until now. This is the first molecular study of ticks and Anaplasmatataceae
 304 pathogens and the second study on piroplasms in cattle in Nepal.

305 Our study detected only *R. microplus* on cattle sampled from Pokhara and Madi. This is in
 306 contrast with previous studies conducted in Nepal, where several other species in the genera
 307 *Haemaphysalis*, *Ixodes*, *Amblyomma*, and *Hyalomma* were apparently detected (Gupta et al.
 308 2013; Bohara and Shrestha 2016; Dhital 2018). For example, Bohara and Shrestha (2016)
 309 reported *R. microplus* as the dominant species, with a 96.8% (150/155) prevalence, followed
 310 by *Haemaphysalis* spp. at 1.9% (3/155), *Ixodes* sp. at 0.6% (1/155) and *Amblyomma* sp. at
 311 0.6% (1/155) in cattle in mid-western Nepal. Similarly, Dhital (2018) documented a prevalence
 312 of *R. microplus* of 95.2% (278/292) in cattle from Chitwan, *Haemaphysalis* spp. at 2.3%
 313 (7/292), *Ixodes* spp. at 1.4% (4/292), and *Amblyomma* spp. at 1.1% (3/292). The difference
 314 may be attributed to variations in sampling location, season, and tick management programs in
 315 each area (Monakale et al. 2024).

316 Previous studies have indicated that the *R. microplus* complex consists of at least 5 taxa
 317 including *R. annulatus*, *R. australis*, and three clades of *R. microplus* (clades A, B, and C)
 318 (Burger et al. 2014; Low et al. 2015). The phylogenetic analysis in our study revealed that the
 319 obtained *R. microplus* sequences belonged to clade C of the *R. microplus* complex which is
 320 widespread on the Indian subcontinent, in Southern China, and Malaysia (Low et al. 2015). The
 321 transport of live cattle from India to other region may have contributed to the distribution of
 322 clade C (Low et al. 2015). *Rhipicephalus microplus* is a tick species of significant veterinary

323 importance due to its invasive capabilities and its role as a vector of several pathogens such as
324 *A. marginale*, *B. bovis*, and *B. bigemina* (Jongejan and Uilenberg, 2004; Kanduma et al., 2020).
325 The control of *R. microplus* is particularly challenging due to the widespread occurrence of
326 acaricide-resistant populations worldwide (Abbas et al. 2014; Roy et al. 2018).
327 The overall infection rate of *Anaplasma* spp. was 23.4% (34/145), which is higher than those
328 reported in the previous microscopic examinations conducted in Nepal as PCR-based assays
329 have higher sensitivity than microscopic examination. Previous microscopic examinations of
330 *Anaplasma* spp. in cattle detected prevalences of 3.5% – 9.7% in the eastern part of the country
331 (Shretha and Singh 1999; Acharya and Pradha, 1996; Shrestha et al. 2017) and 8.8% in Gorkha
332 District (Khankhawash, 2018). Similarly, Pathak et al. (2023) reported that 7.5% (9/120) of
333 cattle in the Morang, Rupandehi, and Surkhet Districts tested positive for *Anaplasma* spp.
334 *Anaplasma marginale* was the most common pathogen, with an overall prevalence of 20.0%
335 (29/145) with 25.7% (18/70) in Pokhara and 14.7 % (11/75) in Madi. This prevalence is notably
336 higher than that reported by Bohara and Shrestha (2016) in the mid-western region of Nepal
337 with infection rate of 5.8% (9/155). *Rhipicephalus microplus* is considered to be the primary
338 vector of *A. marginale* (Futse et al. 2003), which is the dominant tick species infesting cattle
339 in Nepal (Bohara and Shrestha, 2016). *Anaplasma marginale* has also been reported from other
340 Asian countries, with varying detection rates 9 – 18.3% in Pakistan (Ashraf et al. 2021; Zafar
341 et al. 2022), 10.5% in Bangladesh (Mohanta et al. 2023), 43.3 % in Sri Lanka (Zhyldyz et al.
342 2019), 16.4 – 23.7% in India (George et al. 2017), and 31.6% in China (Yang et al. 2017). This
343 variation may be due to factors such as sample size, geographical location, climatic conditions,
344 abundance of vector, time of sampling, difference in detection methodology, and other
345 husbandry practices (Fatima et al. 2024). The phylogenetic analysis based on *gltA* and *MSP*
346 genes showed that two genotypes of *A. marginale* are present in Nepal.

347 In the present study, we reported an overall prevalence of *A. bovis* of 3.4% (5/145), 1.4% (1/70)
 348 in Pokhara and 5.3% (4/75) in Madi. Our results are consistent with previous study of South
 349 India reporting infection rate of 3.3% (5/150) (Nair et al. 2013). *Anaplasma bovis* has been
 350 reported from various countries across the world with 63.7% in Bangladesh (Mohanta et al.
 351 2023), 11.4 % in China (Zhou et al. 2023), 7.8% in Pakistan (Iqbal et al. 2019), and 2.0% in
 352 Malawi (Chatanga et al. 2022). *Anaplasma bovis* is considered non-pathogenic but can be fatal
 353 in case of co-infection with other TBPs (Jurković et al. 2020). In China, *A. bovis* has also been
 354 detected in humans with severe health implications (Lu et al. 2019). Two distinct *A. bovis*
 355 sequences were obtained in this study, and in the phylogenetic tree based on *gltA* and *groEL*
 356 genes, these sequences clustered with *A. bovis* reported from other countries (Fig. 4 and Fig. 5).
 357 Overall infection rate of *T. annulata* was 4.1% (6/145), with 7.1% (5/70) in Pokhara and 1.3%
 358 (1/75) in Madi. The infection rate is comparable to the one reported from China, with an
 359 infection rate of 2.9% (Junlong et al. 2015) but notably lower than those reported in *H.*
 360 *marginatum isaaci* in Eastern Nepal (24.2%; Gupta et al. 2013), in cattle from China (22.5%;
 361 Qi et al. 2018), and in cattle from India (32.4%; George et al. 2015). The infection rate of *T.*
 362 *orientalis* was 4.3% in Pokhara (3/70) and 8.0% (6/75) in Madi, with the overall prevalence of
 363 6.2% (9/145). The presence of *T. orientalis* has been reported in other Asian countries, with
 364 varying prevalence rates, including 4.8% in India (George et al. 2015), 13.8% in Vietnam
 365 (Khukhuu et al. 2011), 53.5% in Sri Lanka (Sivakumar et al. 2013), 36.2% in Myanmar (Bawm
 366 et al. 2014), 0.6% in China (Qi et al. 2018), and 10.0-64.8% in Japan (Ota et al. 2009).
 367 In the phylogenetic tree based on 18S rDNA, the obtained *T. annulata* sequence was identical
 368 and belonged to the same clade as the one reported from China (Fig. 7). However, the *T.*
 369 *orientalis* sequences obtained in this study belong to two different clades. Similarly,
 370 phylogenetic analysis of *T. orientalis* based on the *MPSP* gene revealed the presence of two
 371 genotypes (types 5 and 7), with type 5 being the most abundant. These genotypes have been

372 reported in India (George et al. 2015), Thailand (Altangerel et al. 2011b), Vietnam (Khukhuu et
 373 al. 2011), Sri Lanka (Sivakumar et al. 2013), Mongolia (Altangerel et al. 2011a), China (Qi et
 374 al. 2018), Myanmar (Bawm et al. 2014), and Japan (Ota et al. 2009; Yokoyama et al. 2011).
 375 In this study, 29.7% (43/145) of cattle were positive for at least one TBP, with an overall
 376 infection rate of 20.0% for *A. marginale*, 3.4% for *A. bovis*, 6.2% for *T. orientalis*, and 4.1% for
 377 *T. annulata*. This is higher than previous studies conducted in different regions of Nepal.
 378 Previous microscopic-based surveys showed an overall prevalence of TBP in cattle of 6.4%
 379 (10/155) in the mid-western region of Nepal (Bohara and Shrestha 2016), 16.3% (13/80) in
 380 Gorkha (Khankhawash 2018), and 9.1% (26/285) in Kathmandu valley (Poudel et al. 2023).
 381 The co-infection of different TBPs is complex and includes ecological, epidemiological, and
 382 clinical factors (Baneth 2014; Diuk-Wasser et al. 2016). In this study, co-infection of *Anaplasma*
 383 spp. and *Theileria* spp. was 3.4% (5/145), which is comparable to the 4.9% reported by Zeb et
 384 al. (2020), in cattle from Pakistan. The co-infection of two pathogens is a significant
 385 consideration in farm management, since it hinders accurate diagnosis and management of
 386 infection in animals (Fatima et al. 2024). In some cases, co-infection with multiple pathogens
 387 can increase disease severity (Diuk-Wasser et al. 2016; Liu et al. 2023).
 388 Age-wise analysis showed that young cattle had higher infection rates than adults for
 389 *Anaplasma* spp. (32.0% vs 21.7%) and *Theileria* spp. (12.0% vs 8.3%) although this pattern
 390 was not statistically significant. These results are consistent with Fatima et al. (2024) who
 391 reported no significant age-related pattern of *Theileria* infections in the cattle in India, but
 392 contrast with Ullah et al. (2021), who reported a significant association between age and *T.*
 393 *annulata* infections. The higher infection in young cattle may be due to immunological naivety
 394 (Ullah et al. 2021). Our study showed that female cattle had a higher prevalence of both
 395 *Anaplasma* spp. (23.6%) and *Theileria* spp. (9.4%) compared to male, which had prevalence
 396 rates of 22.2% and 5.6%, respectively. However, these differences were not statistically

significant, which is consistent with a previous report conducted in Nepal (Pathak et al., 2023). The infection rates of TBPs were higher in Pokhara than in Madi, but there was no significant difference between infection rate and sampling site. The different prevalence rates of TBPs in the study area may be influenced by different climatic conditions in different ecological zones as well as the breed of cattle, farming system, and the biology of the tick vector (Ali et al. 2019; Zeb et al. 2020; Ullah et al. 2021). The presence of other wildlife reservoirs may also impact the prevalence of these TBPs in cattle (Gebrekidan et al. 2016). Previous studies have reported *Babesia* spp. in other regions of Nepal (Bohara and Shrestha 2016; Poudel et al. 2023; Dhakal et al. 2023). However, no *Babesia* infection was found in this study, which may be related to several factors including vector abundance and sampling season. Further comprehensive investigations are needed to assess *Babesia* infection in cattle in Nepal. The limitations of this study include sampling restricted to two areas, a relatively short sampling period, a small sample size, and the selective collection of ticks from animals. It is also essential to evaluate the clinical significance of the detected TBPs in cattle in future studies.

Conclusion

The present study revealed the occurrence and genetic diversity of *A. marginale*, *A. bovis*, *T. annulata*, and *T. orientalis* in cattle in Nepal. These findings contribute to the existing data and support future research aimed at understanding the prevalence of ticks and TBPs in the study area. The absence of *Babesia* infection in the tested samples highlights the need for further investigation in Nepal. Further studies should incorporate larger sample sizes from diverse climatic regions to better understand the nationwide distribution of ticks and TBPs in cattle.

Acknowledgements

The authors express gratitude to the animal owners for their cooperation during the sampling. The authors would like to thank the staff of Veterinary Hospital and Livestock Service Expert Center, Kaski, for their assistance in sampling, to all the staff of the Center for Biotechnology, Agriculture and Forestry University, Chitwan, for their cooperation in the laboratory work. and to Lalit Pathak for his support to this work.

Author contribution:

Conceptualization: G.S.P. and R.N.; Data curation: G.S.P. and N.H.; Funding acquisition: R.N.; Investigation: G.S.P., C.R.P., A.S., and P.M., R.B., and N.H.; Methodology: G.S.P., R.N., and N.H.; Project administration: N.N. and R.N.; Resources: G.S.P., A.S., R.B., N.N., and R.N.; Supervision: N.N. and R.N.; Visualization: G.S.P. and N.H.; Writing– original draft: G.S.P.; Writing– review & editing: G.S.P., A.S., N.N., N.H., C.R.P., M.L.K., and R.N. All authors read and approved the final manuscript.

Funding:

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI (grant numbers JP24KK0133, and JP25K02166); the Japan Agency for Medical Research and Development (AMED) (grant number JP23wm0225034); Heiwa Nakajima Foundation; the World-leading Innovative and Smart Education (WISE) Program (1801) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability

The sequence obtained were submitted to the DNA Data Bank of Japan (DDBJ) under accession number LC874703 – LC874732 for COI gene of *R. microplus*, LC871906 –

LC871934 for *gltA* gene of *A. marginale*, LC871935 – LC871939 for *gltA* gene of *A. bovis*,
LC872887 – LC872891 for *groEL* gene of *A. marginale*, LC872882 – LC872886 for *groEL*
gene of *A. bovis*, LC871940 – LC871968 *MSP4* gene of *A. marginale*, LC872902 – LC872906
for 18S rDNA gene of *T. annulata*, LC872892 – LC872900 for 18S rDNA gene of *T. orientalis*,
LC872594 – LC872602 *MPSP* gene of *T. orientalis*.

Declaration

Competing interest

The authors declare that there is no conflict of interest.

Ethical approval

Both ticks and blood samples were collected with the consent of the animal owners and in
accordance with the ethical guidelines of the Nepal Veterinary Council. Ethical approval for
this research was obtained from the Ethical Clearance Committee of the Nepal Veterinary
Council (Ref No: 56/2079.80).

Submission declaration and verification

This manuscript has not been published and is not under consideration for publication
elsewhere.

Informed consent

Informed consent was obtained from all owners of cattle involved in this study.

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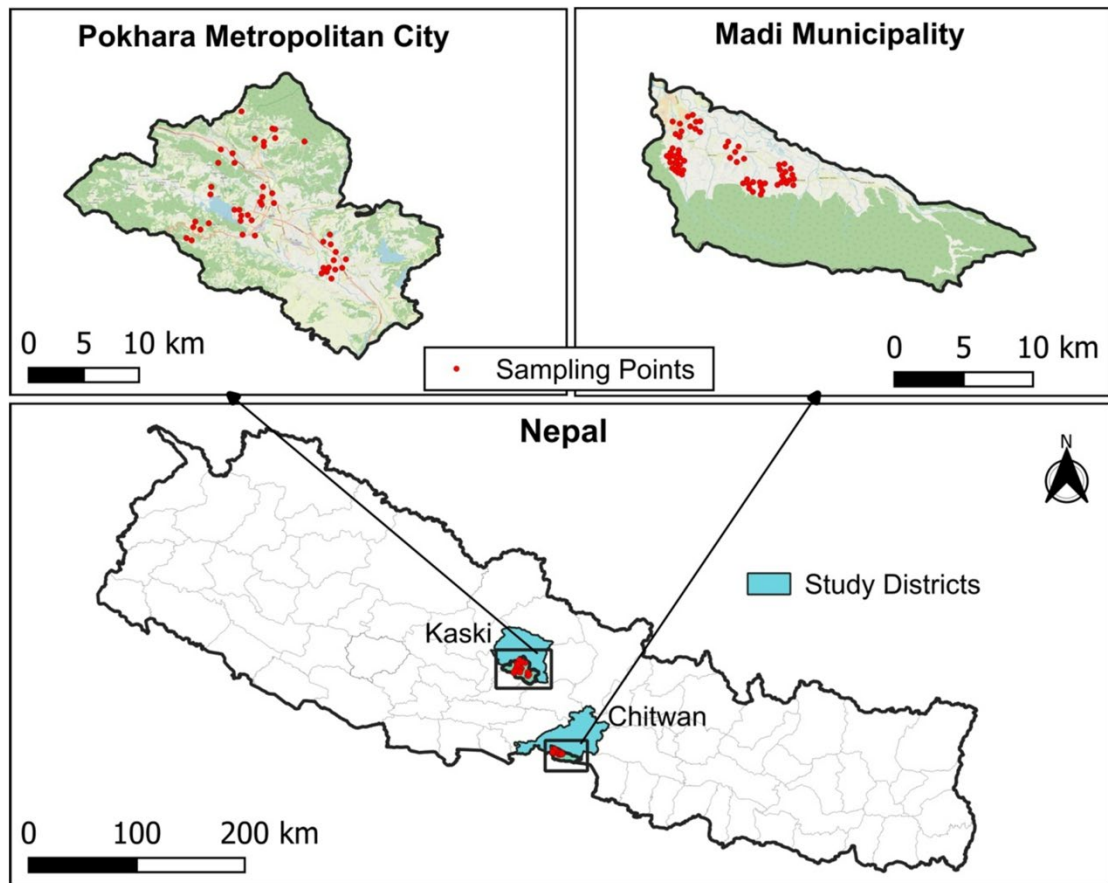


Fig. 1 Map showing the sample collection site in Chitwan and Kaski Districts in Nepal

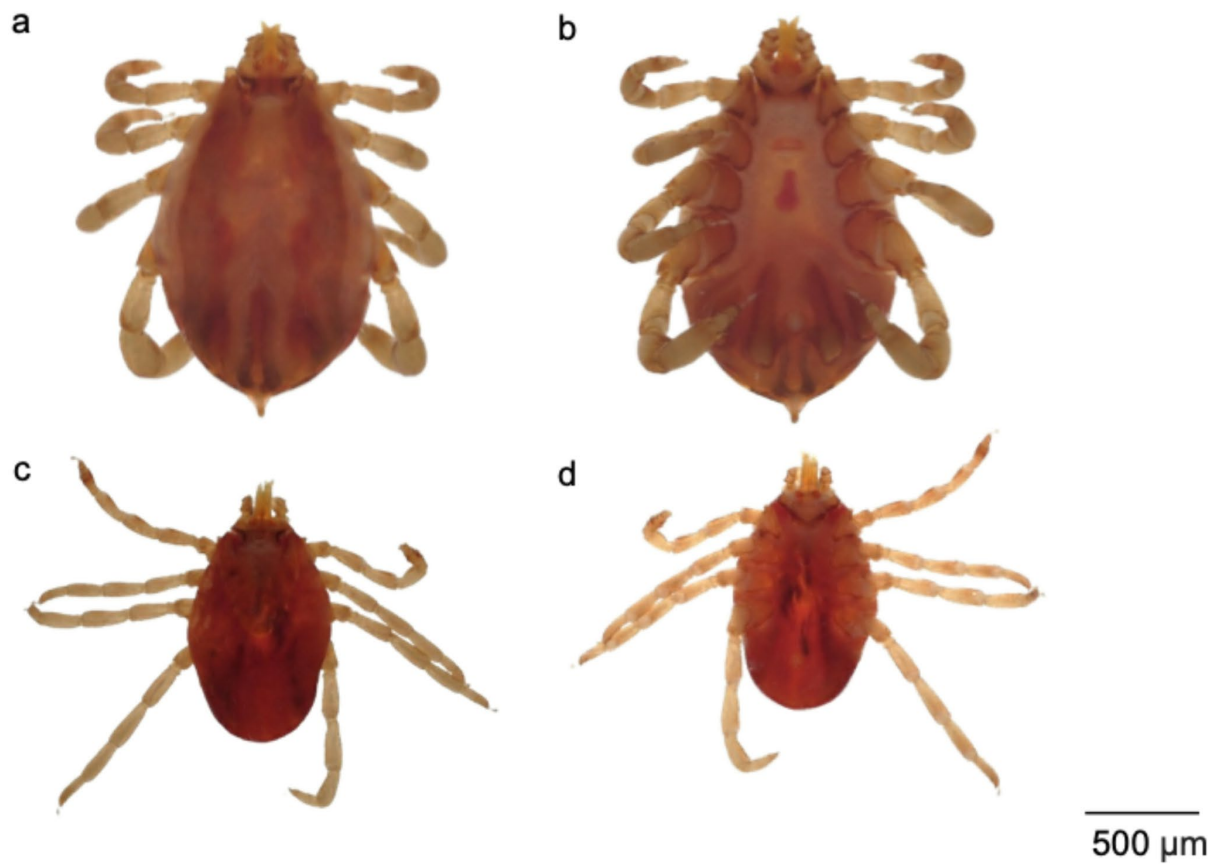


Fig. 2 Representative images of *Rhipicephalus microplus* collected from cattle in Nepal. Male: a) dorsal, b) ventral; female c) dorsal, d) ventral

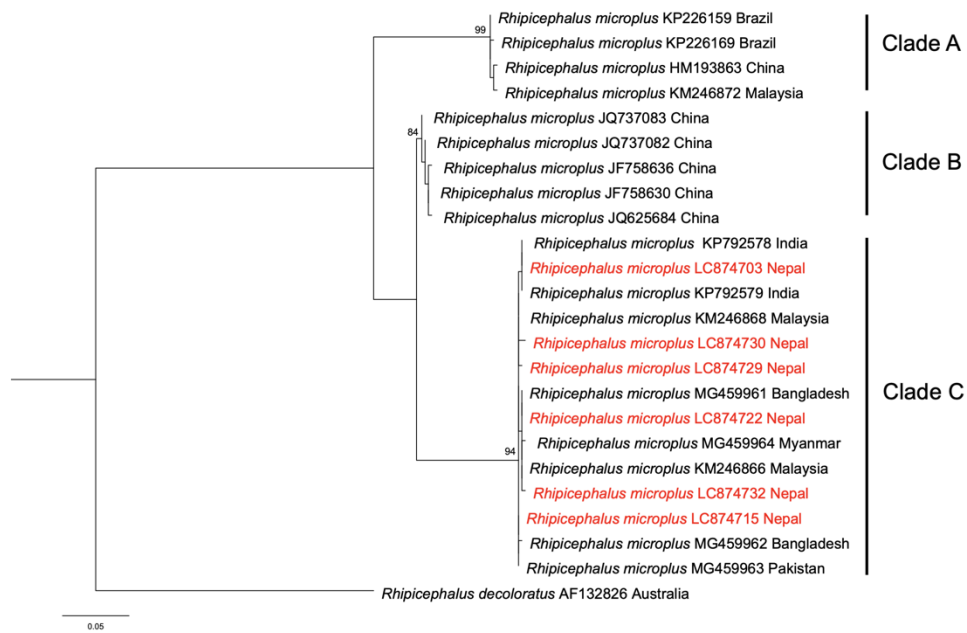


Fig. 3 Maximum likelihood phylogenetic tree based on the COI gene of *Rhipicephalus microplus* collected from cattle using the Tamura-3 parameter model. The bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red

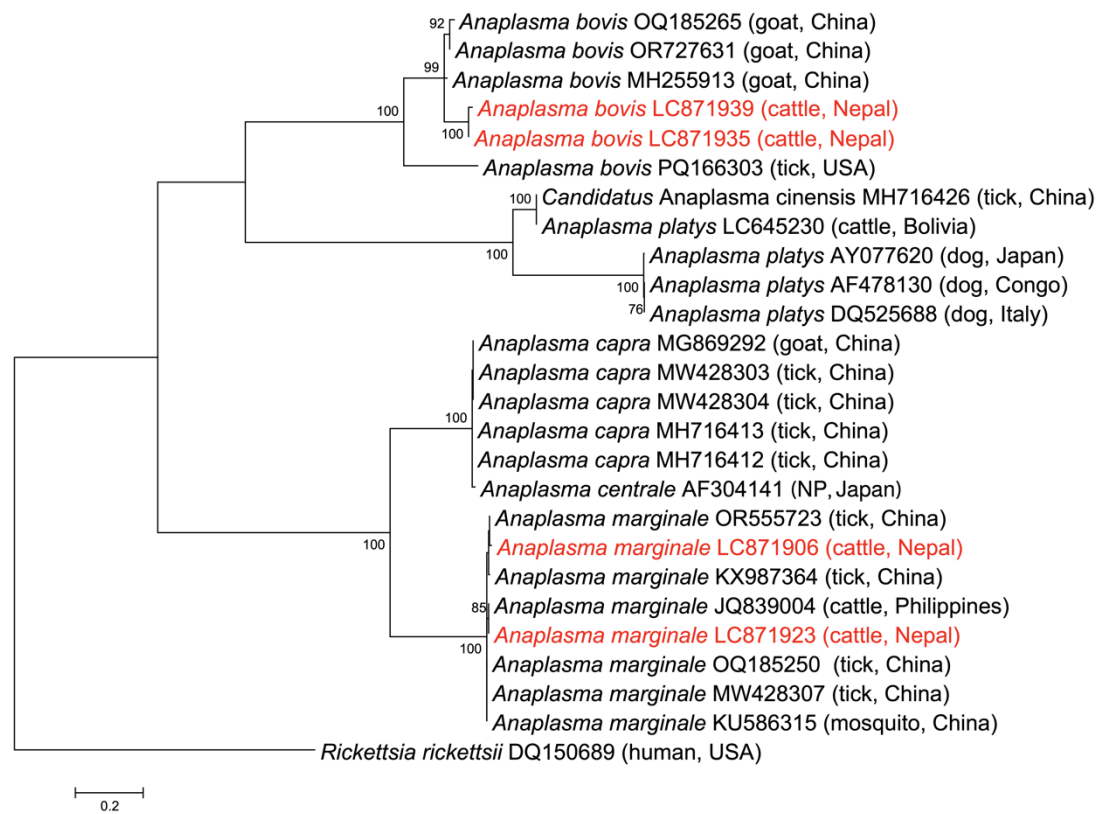


Fig. 4 Maximum likelihood phylogenetic tree based on the *gltA* gene of *Anaplasma marginale* and *Anaplasma bovis* detected in cattle. The bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red. The host and country of origin are indicated in parenthesis, NP means host information not provided

Fig. 5 Maximum likelihood phylogenetic tree based on the *groEL* gene of *Anaplasma marginale* and *Anaplasma bovis* detected in cattle. The bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red

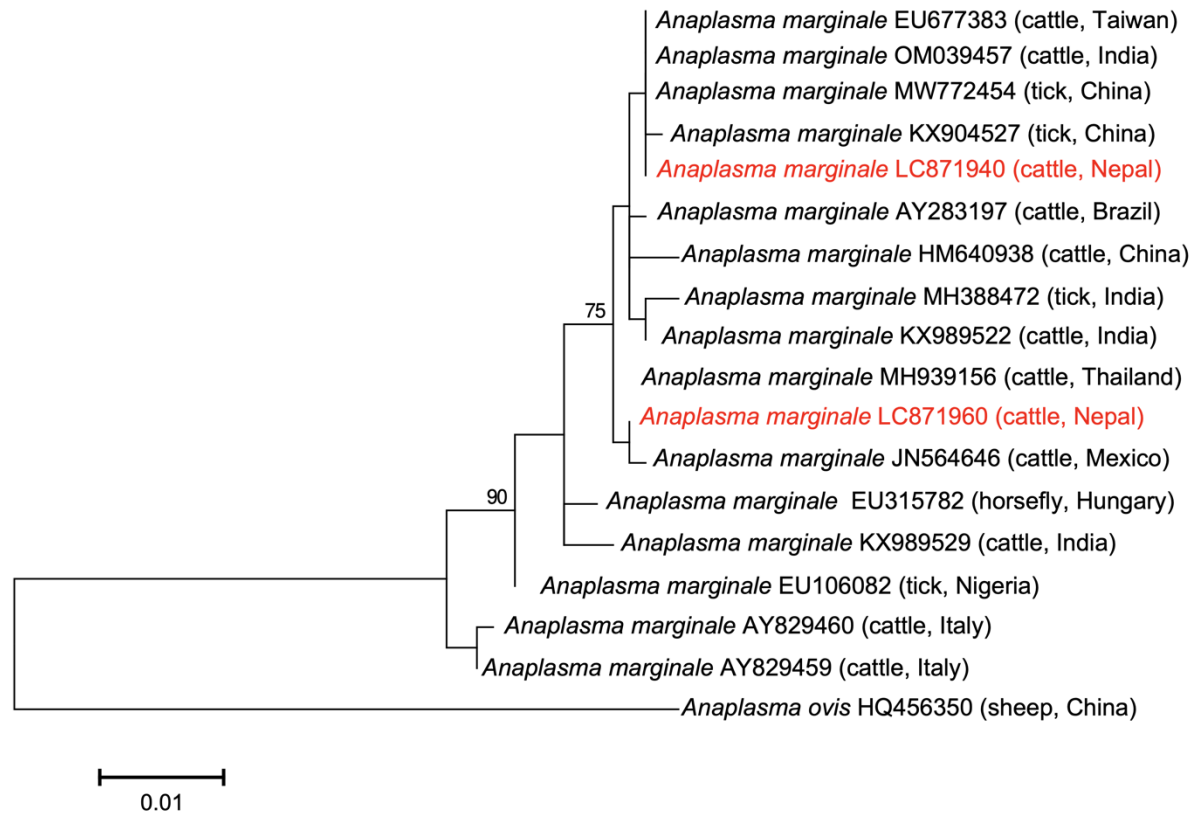


Fig. 6 Maximum likelihood phylogenetic tree based on the *MSP4* gene of *Anaplasma marginale* detected in cattle. Bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red

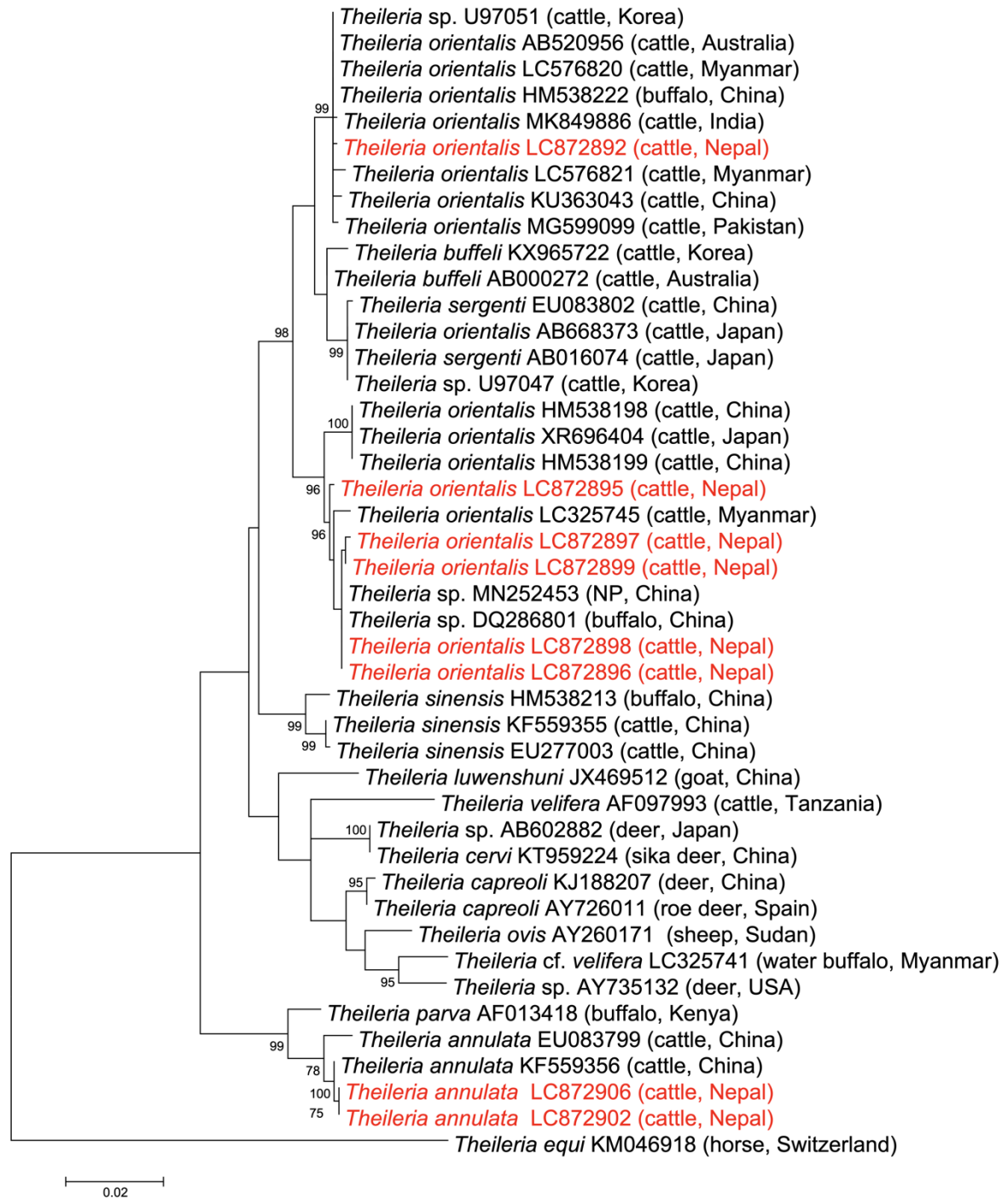


Fig. 7 Maximum likelihood phylogenetic tree based on the 18S rDNA gene of *Theileria annulata* and *Theileria orientalis* detected in cattle. The tree was rooted with *Theileria equi* and bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red. NP means host information not provided

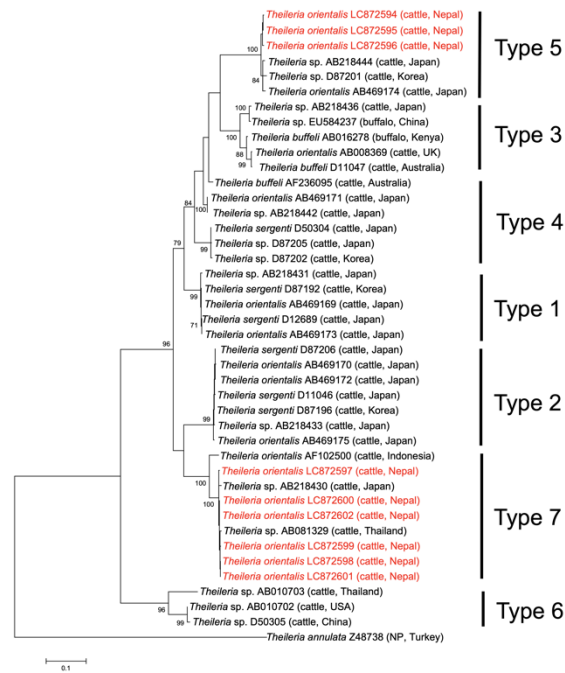


Fig. 8 Maximum likelihood phylogenetic tree based on the *MPSP* gene of *Theileria orientalis* detected in cattle. The tree was rooted with *Theileria annulata* and bootstrap values >70 from 1,000 replicates are shown above the branch. The sequences obtained in this study are shown in red. NP means host information not provided

Table 1 The list of primers used in this study

Primer	Sequence (5'-3')	Target organism(s)	Target gene	Purpose	Product size (bp)	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	ticks	COI	PCR	653	(Folmer et al. 1994)
HC02198	TAAACTTCAGGGTGACCAAAAAATCA					
EHR16SD	GGTACCYACAGAAGAAGTCC	Anaplasmataceae	16S rRNA	PCR	345	(Parola et al. 2000)
EHR16SR	TAGCACTCATCGTTTACAGC	Anaplasmataceae	<i>groEL</i>	1 st PCR	1,300	(Rar et al. 2011)
HS1-F	CGYCAGTGGGCTGGTAATGAA					
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT					
HSV-R	TCAACAGCAGCTCTAGTWG	<i>Anaplasma</i>	<i>groEL</i>	2 nd PCR	1,256	(Liz et al. 2000)
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG					
groEL_rev2	GCCGACTTTTAGTACAGCAA	<i>Ehrlichia</i>	<i>groEL</i>	2 nd PCR	1,100	(Gofton et al. 2016)
F4B	CCGGGTTTTATGTCTACTGC					
HG1085R	ACTATACCKGAGTAAAAGTC	<i>Anaplasma</i> and <i>Ehrlichia</i>	<i>gltA</i>	1 st PCR	950	(Inokuma et al. 2001)
F1b	GATCATGARCARAATGCTTC					
HG1085R	ACTATACCKGAGTAAAAGTC					
MSP43	CCGGATCCTTAGCTGAACAGGAATCTT					
MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	<i>Anaplasma marginale</i>	<i>msp4</i>	PCR	851	(De la Fuente et al. 2003)
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC					
BTH 1st R	AAGTGATAAGGTTCACAAAACCTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTG					
BTH 2nd R	CGGTCCGAATAATTCACCGGAT	<i>Babesia</i> , <i>Theileria</i> , and <i>Hepatozoon</i>	18S rRNA	1 st PCR	1,400-1,600	(Masatani et al. 2017)
MPSP-F	CTTTGCCTAGGATACTTCCT					
MPSP-R	ACGGCAAGTGGTGAGAACT					
InFusin_BTH 2ndF	AAAACGACGGCCAGTGGCTCATTACAACAGTTATAGTTT					
InFusin_BTH 2ndR	GACCATGATTACGCCCGGTCCGAATAATTCACCGG	<i>Theileria orientalis</i>	<i>MPSP</i>	PCR	776	(Ota et al. 2009)
		<i>Babesia</i> , <i>Theileria</i> , and <i>Hepatozoon</i>	18S rRNA	cloning	1,400-1,600	(Pandey et al. 2024)

Table 2 Tick-borne pathogens detected in cattle in Nepal

Location	Sex	No. cattle	<i>Anaplasma marginale</i> (%)	<i>Anaplasma bovis</i> (%)	<i>Theileria orientalis</i> (%)	<i>Theileria annulata</i> (%)
Pokhara	Male	2	0	0	1 (50.0)	0
	Female	68	18 (26.4)	1 (1.5)	2 (2.9)	5 (7.4)
Subtotal		70	18 (25.7)	1 (1.4)	3 (4.3)	5 (7.1)
Madi	Male	16	4 (25.0)	0	0	0
	Female	59	7 (11.9)	4 (6.8)	6 (10.2)	1 (1.7)
Subtotal		75	11 (14.7)	4 (5.3)	6 (8.0)	1 (1.3)
Total		145	29 (20.0)	5 (3.4)	9 (6.2)	6 (4.1)

Table 3 The occurrence of single and mixed infections of tick-borne pathogens in cattle in Nepal

	Tick-borne pathogen(s) detected	No. cattle (%)
Single infection	<i>Anaplasma marginale</i>	27 (18.6)
	<i>Anaplasma bovis</i>	3 (2.1)
	<i>Theileria orientalis</i>	4 (2.8)
	<i>Theileria annulata</i>	4 (2.8)
Mixed infection	<i>A. marginale</i> + <i>T. orientalis</i>	2 (1.4)
	<i>A. bovis</i> + <i>T. orientalis</i>	1 (0.7)
	<i>T. orientalis</i> + <i>T. annulata</i>	1 (0.7)
	<i>A. bovis</i> + <i>T. orientalis</i> + <i>T. annulata</i>	1 (0.7)

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

Variable	Category	No. cattle	<i>Anaplasma (A. marginale + A. bovis)</i>			<i>Theileria (T. orientalis + T. annulata)</i>		
			No. positives (%)	OR (95% CI)	p value	No. positives (%)	OR (95% CI)	p value
Age	Young (≤ 1 yr)	25	8 (32.0)	1.70 (0.66-4.38)	0.30	3 (12.0)	1.50 (0.38-5.90)	0.69
	Adult (≥ 1 yr)	120	26 (21.7)	0.59 (0.23-1.51)		10 (8.3)	0.67 (0.17-2.62)	
Sex	Male	18	4 (22.2)	0.92 (0.28-3.02)	1.00	1 (5.6)	0.56 (0.07-4.62)	1.00
	Female	127	30 (23.6)	1.08 (0.33-3.54)		12 (9.4)	1.77 (0.22-14.52)	
Breed	Holstein Friesian cross	69	16 (23.2)	0.97 (0.45-2.10)	1.00	4 (5.8)	0.46 (0.13-1.56)	0.25
	Jersey cross	76	18 (23.7)	1.03 (0.48-2.22)		9 (11.8)	2.18 (0.64-7.44)	
Location	Pokhara	70	19 (27.1)	1.49 (0.69-3.23)	0.33	7 (10.0)	1.28 (0.41-4.01)	0.77
	Madi	75	15 (20.0)	0.67 (0.31-1.45)		6 (8.0)	0.78 (0.25-2.45)	

Abbreviations: OR, odd ratio; CI, confidence interval