

Ticks and Pathogens: Molecular Profiling of *Rhipicephalus microplus* and Their Zoonotic Bacterial Carriage in Pakistani Cattle

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Abstract

Tick-borne bacterial infections caused by *Rickettsia*, *Anaplasma*, and *Ehrlichia* species are of growing concern for livestock production and public health. This study investigated cattle-infesting *Rhipicephalus microplus* ticks from Punjab, Pakistan, and assessed their carriage of selected zoonotic bacterial pathogens. A total of 510 *R. microplus* ticks were collected from cattle between April and October 2024. Tick identity was confirmed through PCR amplification and sequencing of the 16S rRNA gene. Molecular screening showed a significant variation in bacterial detection among the examined ticks ($P = 0.007$). *Rickettsia massiliae* was the most frequently detected pathogen, with a prevalence of 45.5% (232/510), followed by *Anaplasma phagocytophilum* at 34.9% (178/510) and *Ehrlichia* sp. at 32.0% (163/510). Single, double, and triple infections were observed, indicating active co-circulation of these bacterial agents within *R. microplus* populations. Sanger sequencing and BLAST analysis confirmed the identity of the detected pathogens, with sequences showing close similarity to corresponding GenBank records from different geographical regions. Pathogen detection was significantly higher in female ticks than in males for all three bacterial agents. District-wise analysis showed significant variation for *Ehrlichia* sp. ($P = 0.006$), whereas *R. massiliae* and *A. phagocytophilum* showed no significant spatial difference. *In conclusion*, these findings demonstrate that cattle-associated *R. microplus* ticks in Punjab harbor multiple bacterial pathogens with veterinary and zoonotic relevance. The study provides important baseline molecular evidence for targeted surveillance, integrated tick control, and One Health-based monitoring of tick-borne infections in Pakistan.

Introduction

Ticks are obligate blood-feeding ectoparasites play an important role in dispersal of many diseases as they are capable of surviving in diverse geo-climatic conditions (Ali et al., 2020). The close interaction between domestic animals and human permits the ticks and other ecto-parasites to transmit diseases from animals to human (Aggarwal and Ramachandran, 2020). Hard ticks represent the predominant group of ticks and are recognized vectors of bacteria, viruses, protozoa, and helminths. Tick-borne diseases (TBDs) caused by these pathogens pose an increasing threat to both veterinary and public health, particularly in tropical and subtropical regions where environmental conditions favor their survival and spread (de la Fuente et al., 2023).

In Pakistan, *Rhipicephalus microplus* is among the predominant ticks in the semi-arid zone, especially in Punjab province, and it has been found infesting a variety of animals, including cattle, buffalo, goats and even dogs (Abbas et al., 2024, Hussain et al., 2024a). A high prevalence of *Rhipicephalus microplus* has been reported across several districts of Punjab, including Rawalpindi, Kallar Syedan, Gujar Khan, Dera Ghazi Khan and others (Ciarma et al., 2025, Ashraf et al., 2021). This tick species is a well-known vector of *Anaplasma marginale*, *Babesia bigemina*, and *B. bovis*, the causative agents of tick fever in cattle and other ruminants: a potentially fatal disease that reduces livestock productivity and results in substantial economic losses for farmers (Hussain et al., 2024b). In addition, *R. microplus* is also known to transmit other pathogens including species belonging to *Rickettsia* and *Ehrlichia* genera (Some et al., 2023).

Anaplasma phagocytophilum is an obligate intracellular, gram-negative bacterium of the family Anaplasmataceae, which primarily infects host neutrophils in a wide range of animals including dogs, horses, rodents, deer, and both small and large ruminants (El Hamiani Khatat et al., 2021). Besides *R. microplus*, transmission also occurs through *Ixodes ricinus*, *I. scapularis*, and *I. persulcatus* (Ybañez and Inokuma, 2016, Marzok et al., 2024). In both humans and animals, infections caused by *A. phagocytophilum* are effectively treated with doxycycline or oxytetracycline (Abbas et al., 2023).

Members of the genus *Ehrlichia* are gram-negative, obligate intracellular bacteria within the order *Rickettsiales*, characterized by their tropism for host leukocytes (Suartha et al., 2023). Infection with these pathogens causes ehrlichiosis, a disease that can manifest in humans and animals with clinical outcomes ranging from subclinical or mild illness to severe and potentially fatal disease (Yabsley, 2010). Transmission is primarily mediated by ixodid ticks, particularly species within the genera *Amblyomma*, *Ixodes*, and *Rhipicephalus*. Therapeutic management relies mainly on tetracycline-class antibiotics, with doxycycline being the drug of choice (Zeb et al., 2023).

The genus *Rickettsia* comprises small, gram-negative, obligate intracellular bacteria with a primary tropism for endothelial cells, leading to vascular inflammation (Sahni et al., 2019). Based on clinical and epidemiological characteristics, they are classified into three major groups: the spotted fever group, the typhus group, and the scrub typhus group (Thiga et al., 2015). Transmission to humans and animals occurs through arthropod vectors such as ticks, fleas, lice, and mites. Among ticks, species of the genera *Dermacentor*, *Rhipicephalus*, and *Amblyomma* are recognized vectors of diverse *Rickettsia* spp. infecting multiple hosts (Ali et al.,

2021). *Rickettsia massiliae* is a spotted fever group rickettsia transmitted through the bite of infected *Rhipicephalus* ticks, causing a disease characterized by fever, headache, and a maculopapular rash (Monje et al., 2016). Doxycycline remains the treatment of choice for rickettsial infections (Ali et al., 2021).

Ticks infesting cattle have been extensively described at the morphological level owing to their abundance and diversity; however, molecular data remain comparatively limited. *Rhipicephalus microplus* is one of the most common tick species parasitizing cattle, and a few studies from Pakistan have molecularly characterized this species using markers such as *cox1* and 16S rRNA (Abbasi et al., 2024, Ali et al., 2019, Zeb et al., 2019, Roy et al., 2018). Nevertheless, its role as a vector of pathogens has not been thoroughly explored. To address this gap, the present study reports the molecular prevalence of *Anaplasma*, *Ehrlichia*, and *Rickettsia* species in *R. microplus* collected from cattle across four districts of Punjab, Pakistan.

This study aimed to characterize the ticks that were infesting cattle in Punjab province in Pakistan and also to screen these ticks for the presence of *Anaplasma*, *Rickettsia* and *Ehrlichia* species.

Materials and Methods

2.1 Study Area

Tick specimens infesting apparently healthy cattle were systematically collected from four districts in Punjab Province of Pakistan during April till October 2024. Targeted districts included Faisalabad (31° 25' 7.4" N, 73° 4' 44.8" E), Jhang (31° 17' 8" N, 72° 20' 35" E), Toba Tek Singh (30° 58' 27.6" N, 72° 28' 57.7" E) and Okara (30° 48' 30" N, 73° 27' 33" E) (Fig. 1). We hypothesized that climatic and geographical differences among the sampling sites would lead to variations in tick infestation rates and, consequently, in the prevalence of pathogens carried by these ticks.

2.2 Tick Collection and their Morphological Identification

A total of 351 apparently healthy cattle were inspected for the presence of tick on them during this investigation and 63% of these cattle (221/351) had tick infestation. Each animal underwent a comprehensive clinical examination and their whole bodies were thoroughly and carefully inspected for the presence of ticks. When present, ticks were carefully removed by using curved forceps not to damage their morphological features. A total of 510 ticks were collected from infested cattle. Data was recorded for each infested animal including location of sampling site sampling date and number of ticks collected from each animal. Ticks were first rinsed with distilled water and then transferred to labeled Eppendorf tubes containing a solution of 70% ethanol and 5% glycerol. Morphological identification was subsequently performed under a CM100 stereo microscope (China) by following the morpho-taxonomic characters of ticks using standardized taxonomic keys (Walker, 2003). The morphological analysis focused on structures such as Haller's organs, the capitulum, spiracles, adanal plates, festoons, and other general anatomical features.

2.3 DNA extraction from ticks

The DNA was extracted from individual tick by using the Mini BEST Viral RNA/DNA Extraction Kit Ver. 5.0 (TaKaRa, Dalian, China), following the manufacturers protocol. DNA were stored at – 20°C until further analysis.

2.4 Molecular identification of Ticks

For molecular identification, the 16S rDNA gene was amplified using a PCR reaction mixture comprising DreamTaq PCR Master Mix (Thermo Scientific, Waltham, MA, USA) and the primer pair 5'-CTGCTCAATGATTTTAAATTGCTGTGG-3' and 5'-CCGGTCTGAAGTCAATCAAGT-3'. The thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 60 seconds, annealing at 51°C for 60 seconds, and extension at 72°C for 45 seconds. A final extension step was performed at 72°C for 5 minutes (Black 4th and Piesman, 1994).

2.6 Molecular detection of *Anaplasma Ehrlichia* spp.

Tick DNA samples were analyzed for the presence of *Anaplasma/Ehrlichia* species by amplifying the 16S rRNA gene using previously published primers (Forward: 5'-TAGCACTCATCGTTTACAGC-3', Reverse: 5'-GGTACC(C/T)ACAGAAGAAGTCC-3') as described by Parola et al. (2000). Each 25 µL PCR reaction contained DreamTaq PCR Master Mix (Thermo Scientific, Waltham, MA, USA), 50 ng of template DNA, 0.6 mM of each primer, and nuclease-free water to make up the volume. The thermal cycling conditions included an

initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension step at 72°C for 2 minutes (Parola et al., 2000).

2.7 Molecular detection of *Rickettsia massiliae*.

For the molecular identification of *Rickettsia* species in tick DNA, the citrate synthase (*gltA*) gene was amplified using DreamTaq PCR Master Mix (Thermo Scientific, Waltham, MA, USA). Forward 5'-GGGGACCTGCTCACGGCGG-3' and Reverse 5'-ATTGCAAAAAGTACCGTAAACA-3'. The PCR protocol involved an initial denaturation at 95°C for 5 minutes, followed by 35 cycles comprising denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for 10 minutes (Qi et al., 2022). Each PCR run included both positive and negative controls. The amplified products were visualized through electrophoresis on a 2% agarose gel and documented using a Gel Doc system (BioDoc-It™ Imaging Systems, Upland, CA, USA) stained with ethidium bromide, and visualized.

2.8 Sequencing and Phylogenetic Analysis

PCR-amplified DNA products were subsequently confirmed by Sanger sequencing (Sangon Biotech Co., Ltd. China). Low-quality sequence reads were trimmed, and consensus sequences were assembled using SeqMan v5.00 (DNASTAR, Inc.). The finalized sequences were submitted to GenBank for accession number assignment. Sequence homology was assessed via BLAST searches on the NCBI platform. Multiple sequence alignments were conducted using BioEdit v7.0.5 with MUSCLE and CLUSTALW algorithms (Thompson et al., 1994). Phylogenetic relationships were inferred using the maximum likelihood approach with 1,000 bootstrap replicates, implemented in MEGA-X software (Kumar et al., 2018).

Results

3.1 Taxonomic identification of the ticks

Morphological identification revealed that all the collected ticks were *Rhipicephalus microplus* including 260 females and 250 males. District wise data analysis indicated that a total of 130 ticks were collected from cattle in Faisalabad (70 female and 60 male), 125 from Toba Tek Singh (65 female and 60 male), 120 from Okara (female and male 60 each) and 135 from Jhang (67 females and 67 males). All the collected ticks were partially fed and adult.

3.2 Molecular confirmation of *Rhipicephalus microplus*

DNA sequencing followed by BLAST analysis of the amplified 16S rRNA gene confirmed the identity of the tick as *Rhipicephalus microplus*, showing 99–100% similarity with previously published 16S rRNA gene sequences of this species. Phylogenetic analysis revealed that both the amplicon used in this study (PV820216 and PV820217) were genetically similar and resembled to the 16S rRNA sequences deposited from Pakistan (accession number MK495912), India (accession numbers PQ215892 and MG811555) and China (PQ681552). *Rhipicephalus microplus* sequences generated in this study were clearly distinct from other 16S rRNA sequences of *Rhipicephalus* species like *R. annulatus*, *R. haemophysaloides*, *R. evertsi evertsi* and *R. sanguineus* infesting canines and ruminants from countries around the globe as well as from 16S rRNA sequence of *Hyalomma anatolicum* that was used as an out group during this phylogenetic analysis (Fig. 2).

3.3 Molecular detection, genetic diversity and epidemiology of *Anaplasma* and *Erlichia* spp. in *Rhipicephalus microplus* ticks

Overall, PCR amplified a 345 base pair fragment from the 16S rRNA gene of *Anaplasma* or *Erlichia* spp. in 341 out of 510 (67%) *Rhipicephalus microplus* ticks collected from Punjab during present investigation. All the positive bacterial products were DNA sequenced and data analysis revealed that 178 out of 510 ticks (34.9%) had *Anaplasma phagocytophilum* while 163 out of 510 (32%) were infected with *Erlichia* sp. (Table 1).

Table 1

Prevalence of *Anaplasma phagocytophilum*, *Ehrlichia* spp. and *Rickettsia massiliae* among *Rhipicephalus microplus* ticks collected from Punjab province during present investigation. % Prevalence of pathogen is given in parenthesis. P –value indicates the results of Chi-square test calculated to compare the prevalence of bacteria among the screened ticks.

<i>Anaplasma phagocytophilum</i> +ve	<i>Ehrlichia</i> sp. +ve	<i>Rickettsia massiliae</i> +ve	χ^2	P value	Co infection			
					A. phagocytophilum and Ehrlichia sp.	A. phagocytophilum and R. massiliae	R. massiliae and Ehrlichia sp.	All three pathogens
178/510 (34.9%)	163/510 (32%)	232/510 (45.5%)	9.816	0.007 **	43/510 (8.4%)	35/510 (6.9%)	39/510 (7.6%)	117/510 (22.9%)
P < 0.01(**) = Significant								

The BLAST analysis confirmed the presence of *A. phagocytophilum* infection in *R. microplus*. The two 16S rRNA sequences included in this analysis displayed genetic diversity as they exhibited nucleotide substitutions upon comparison but they still resided in the same clad and displayed 99–100% similarity to 16S rRNA gene sequence of *A. phagocytophilum* deposited in GenBank from Cyprus (EU090186), Greece (ON415281), Iran (PV175399) and South Korea (MT754357 and MT754358). The isolates generated in this investigation were distinct from 16S rRNA *A. bovis*, *A. ovis*, *Anaplasma* sp. and *A. platys* sequences deposited from across the globe as well as 16S rRNA gene sequence of *Rickettsia hoogstraalii* (PQ223380) used as an outgroup during this phylogenetic analysis (Fig. 3).

Ticks were collected from four districts in Punjab. Chi square test analysis revealed that prevalence of *Anaplasma phagocytophilum* varied non significantly among the ticks that were collected from these four districts (P = 0.9) (Table 2). It was also observed that female ticks were significantly more infected with *Anaplasma phagocytophilum* than males (P < 0.001) (Table 3).

Table 2

Prevalence of *Anaplasma phagocytophilum*, *Ehrlichia* spp. and *Rickettsia massiliae* in *Rhipicephalus microplus* ticks collected from four districts in Punjab. % prevalence of each bacterium is provided in parenthesis. P value indicates the results of Chi-square test calculated to compare the prevalence of each bacterium among the sampling sites.

Sampling districts	Number of screened ticks	Pathogens		
		<i>Anaplasma phagocytophilum</i> +ve	<i>Ehrlichia</i> spp.+ve	<i>Rickettsia massiliae</i> +ve
Faisalabad	130	46/130 (35.4%)	24/130 (18.4%)	60/130 (46.2%)
Toba Tek Singh	125	40/125 (32%)	35/125 (28%)	50/125 (40%)
Okara	120	42/120 (35%)	44/120 (36.6%)	53/120 (44.2%)
Jhang	135	50/135 (37%)	60/135 (44.4%)	69/135 (51.1%)
Total	510	178/510 (34.9%)	163/510 (32%)	232/510 (45.5%)
χ^2		0.354	11.902	1.253
P value		0.9	0.006 **	0.7
P > 0.05 = Non significant; P < 0. 01(**) = Significant				

Table 3

Association of the tick sex with the prevalence of *Anaplasma phagocytophilum*, *Rickettsia massiliae* and *Ehrlichia* sp. among the *Rhipicephalus microplus* ticks screened during present study. N represents the total number of collected ticks. % prevalence of pathogen is given in parenthesis. P-value represents the results of Chi square test calculated for each pathogen.

Tick species	Sex	N	<i>Anaplasma phagocytophilum</i> +ve	χ ² /P-value	<i>Ehrlichia</i> sp. +ve	χ ² /P-value	<i>Rickettsia massiliae</i> +ve	χ ² /P-value
Rhipicephalus microplus	Female	260	157/260 (60.4%)	76.57/P < 0.001***	154/260 (59.2%)	98.72/P < 0.001***	216/260 (83%)	123.03/P < 0.001***
	Male	250	21/250 (8.4%)		9/250 (3.6%)		16/250 (6.4%)	
P < 0.001 = Highly significant (***)								

The BLAST analysis confirmed the presence of *Ehrlichia* sp. in *R. microplus*. The two 16S rRNA sequences included in this analysis displayed genetic diversity but they resided in the same clad and displayed 100% similarity to 16S rRNA gene sequence of *Ehrlichia* sp. deposited in GenBank from Pakistan (PV717250) and Eswatini (MW628648, MZ351090). These isolates were distinct from 16S rRNA of *A. bovis*, *E. canis*, *E. chaffeensis*, *E. ruminantium* as well as *Candidatus neohrlichia*, *A. bovis*, *A. ovis*, *A. camelii* and the out group: *Rickettsia amblyommatis* (PV037360) (Fig. 4).

For *Ehrlichia* sp., Chi square test results indicated that prevalence of this bacterium varied significantly among the ticks that were collected from four districts in Punjab (P = 0.006). The highest prevalence of *Ehrlichia* sp. was observed in *Rhipicephalus microplus* that were collected from Jhang district (44.4%) followed by Okara (36.6%), Toba Tek Singh (28%) and Faisalabad (18.4%) (Table 2). It was also observed that female ticks were significantly more infected with *Anaplasma phagocytophilum* than males (P < 0.001) (Table 3).

3.4 Molecular detection, genetic diversity and epidemiology of *Rickettsia* spp. in *Rhipicephalus microplus* ticks

PCR amplified a 381 base pair fragment from the *gltA* gene of *Rickettsia* spp. in 232 out of 510 (45.5%) *Rhipicephalus microplus* ticks collected from Punjab. (Table 1).

DNA sequencing followed by the BLAST analysis confirmed the presence of *Rickettsia massiliae* infection in *R. microplus*. The two *gltA* sequences included in this analysis were genetically similar as they resided in the same clad and displayed 99–100% similarity to *gltA* gene sequence of *R. massiliae* deposited in GenBank from Iran (PV684243 and PV685004), Guinea (PV261013) and Pakistan (OQ599533, OQ599534, OQ599536). The isolated generated in this investigation were distinct from *gltA* gene sequence of *R. hoogstraalii*, *R. asembonensis*, *R. helvetica*, *R. monacensis*, *R. aeschlimannii*, *R. africae* and *R. rickettsii* sequences deposited from various countries as well as *gltA* gene sequence of *Anaplasma capra* (PQ473723) used as an outgroup during this phylogenetic analysis (Fig. 5).

Chi square test analysis revealed that prevalence of *Rickettsia massiliae* varied non significantly among the ticks that were collected from these four districts (P = 0.7) (Table 2). It was also observed that female ticks were significantly more infected with *Rickettsia massiliae* than males (P < 0.001) (Table 3).

3.5 Bacterial co-infection among *Rhipicephalus microplus* ticks

Prevalence of the three screened pathogens varied significantly among the screened *Rhipicephalus microplus* ticks (P = 0.007). *Rickettsia massiliae* (45.5%) had the highest prevalence followed by *Anaplasma phagocytophilum* (34.9%) and *Ehrlichia* spp. (32%). Co infection with two bacteria was also observed: 43 ticks (8.4%) were co-infected with *Anaplasma phagocytophilum* and *Ehrlichia* spp., 39 (7.6%) were co-infected with *Ehrlichia* spp. and *Rickettsia massiliae* while 35 (6.9%) were co-infected with *Anaplasma phagocytophilum* and *Rickettsia massiliae*. 117 (22.9%) *Rhipicephalus microplus* ticks were simultaneously infected with all three pathogens that were screened during present investigation (Table 1).

Discussion

The predominantly subtropical climate of Pakistan provides favorable conditions for tick survival and reproduction, contributing to a higher incidence of TBDs in the region (Younas et al., 2023). Furthermore, increased animal movement has facilitated the expansion

of tick habitats, promoting the spread of novel pathogens into new geographic areas and raising significant concerns for both veterinary and public health (Ali et al., 2019). Infestation of cattle with *Rhipicephalus microplus* is prevalent worldwide and is considered a significant socio-economic issue in many developing countries (Al-Hoshani et al., 2023). While *Rhipicephalus microplus* has been identified as a vector for several pathogens, the role of this tick in harboring and potentially transmitting pathogens remains underexplored, particularly in Pakistan.

A number of studies from Pakistan have reported the morphological identification of *Rhipicephalus microplus* from Pakistan but limited data is available regarding their molecular confirmation. Molecular confirmation is essential because species within the *Rhipicephalus* complex exhibit close morphological resemblance. Such similarity often leads to misidentification, especially in cases where ticks are engorged, damaged, or at immature developmental stages (Zeb et al., 2023). Therefore, the 16S rRNA gene of the collected ticks was amplified for molecular identification. Owing to its high copy number, conserved regions, and discriminatory power among closely related species, the 16S rRNA gene serves as a reliable marker for both phylogenetic analysis and species-level identification within the *Rhipicephalus* genus (Low et al., 2015). The 16S rRNA gene sequences of *R. microplus* obtained in the present study clustered within the same branch of the phylogenetic tree, reflecting a close genetic relationship. Our results are in line with the previous studies as they had also documented genetic homogeneity among the screened *R. microplus* from Pakistan (Abbasi et al., 2024, Ali et al., 2019, Zeb et al., 2023). This genetic uniformity is likely due to the long-standing endemic presence of *R. microplus* in South Asia, where the species originally evolved, and the limited influence of long-range gene flow on its population structure (Guglielmone et al., 2014). Moreover, *R. microplus* is a one-host tick species, completing its entire life cycle on a single animal. Studies have shown that once its larvae attach to cattle, they may remain parasitic for up to 149 days, allowing the tick to travel with its host over considerable distances. Such host-mediated dispersal promotes gene flow within regions of livestock movement, contributing to the observed low genetic variation (Estrada-Peña et al., 2018). Adding to this, the relative absence of genetic differentiation may also be influenced by increased opportunities for inbreeding in *R. microplus* populations (Koffi et al., 2006). The Pakistani sequences showed close resemblance to 16S rRNA sequences of *R. microplus* previously reported from Pakistan (Zeb et al., 2023). As well as from India and China (unpublished data) (Fig. 2). Given the scarcity of available molecular data, there is a strong need to collect *R. microplus* from different regions of Pakistan and neighboring countries to assess their genetic diversity. Such information will be essential for formulating sustainable tick management approaches, including the development of region-specific vaccines and the optimization of chemical control strategies to mitigate the economic burden posed by this pest.

Although traditionally linked with substandard hygiene and low socio-economic conditions, ticks are now increasingly recognized as not only ecto-parasitic pests but also significant reservoirs and vectors of diverse infectious pathogens (Wikel, 2018). A high prevalence of *A. phagocytophilum* (34.4%) was recorded in the DNA of *R. microplus* during this investigation (Table 1). *Anaplasma phagocytophilum* is classically associated with *Ixodes* ticks in temperate climates (Bakken and Dumler, 2015). However, its detection at relatively high prevalence in *R. microplus* from subtropical areas raises concerns about its expanding vector range and indicates a notable risk of its zoonotic transmission in the studied regions. The presence of *A. phagocytophilum* DNA in *R. microplus* has previously been documented in Pakistan (Ghaffar et al., 2020), China (Lu et al., 2022), and Madagascar (Matysiak et al., 2016). The 16S rRNA gene sequences of *A. phagocytophilum* generated in this study resided in the same clad of the phylogenetic tree indicating their genetic similarity. These sequences resembled the 16S rRNA gene sequences of *A. phagocytophilum* detected in sheep and goats from Cyprus (Chochlakis et al., 2009), sheep in Greece (unpublished data), goats from Iran (unpublished data) and in domestic ruminants from South Korea (unpublished data) (Fig. 3).

A notably high prevalence of *Ehrlichia* spp. (32%) was detected in the screened *R. microplus* ticks (Table 1). Several studies from different geographic regions, including China, Brazil, Tibet, Thailand, Madagascar, and Pakistan, have similarly reported diverse *Ehrlichia* species in *R. microplus*, such as *E. minasensis*, *E. canis*, *E. chaffeensis*, *E. muris*, and other unclassified *Ehrlichia* spp. (Wen et al., 2002, Cruz et al., 2012, Cabezas-Cruz et al., 2016, Carvalho et al., 2016). The 16S rRNA gene sequences of *Ehrlichia* spp. generated in this study displayed nucleotide substitutions indicating their genetic variations and indicating that probably more than one species of *Ehrlichia* genera are infecting the *R. microplus* ticks in Punjab. The sequences generated in this investigation resembled the 16S rRNA gene sequences of *Ehrlichia* spp. detected in *R. microplus* ticks from Pakistan (unpublished data) and in ticks from Eswatini (Ledger et al., 2021). (Fig. 4).

In comparison of the other two bacteria screened during this investigation, the highest infection rate was recorded for *Rickettsia massiliae* (45.5%) among the examined *R. microplus* ticks (Table 1). Our findings corroborate a couple of recent investigation from Pakistan, where (Shehla et al., 2023), and (Ali et al., 2021), reported the presence of *R. massiliae* in local tick species, including *R. turanicus*, *R. haemaphysaloides* and *R. microplus*. It is noteworthy that *R. massiliae* has been identified in several *Rhipicephalus*

species, including *R. sanguineus s.l.*, *R. turanicus* and *R. lunulatus*, across diverse regions such as Argentina, France, the USA, Africa, and China (Cicuttin et al., 2014, Jiao et al., 2021, Jumpertz et al., 2023). However, to date, the detection of *R. massiliae* in *R. microplus* has only been documented in Pakistan. It is plausible that the *R. massiliae* strains circulating in Pakistan have specifically adapted to *R. microplus* as a competent vector, whereas strains from other regions appear to maintain stronger associations with different *Rhipicephalus* species. Moreover, genetic variability in both the tick populations and *R. massiliae* itself may underlie these vector–pathogen compatibilities, warranting further investigation. The citrate synthase gene (*gltA*) is commonly employed in bacterial phylogenetic studies, as it is well conserved among diverse bacterial taxa while retaining sufficient sequence variation to distinguish closely related species (Inokuma et al., 2001). Our *gltA* gene sequences of *R. massiliae* resembled the *gltA* sequences of *R. massiliae* detected in hedgehogs from Iran (unpublished data), ticks in Guinea (unpublished data) and livestock in Pakistan (Hussain et al., 2024a). (Fig. 5). For all the three bacteria, the reported prevalence rates differ considerably across the studies cited above and the observed differences were most likely due to geographic variation in ecological conditions, host density and tick biology, all of which shape pathogen acquisition and transmission dynamics, resulting in region-specific infection levels (Parola and Raoult, 2001).

Risk factor assessment revealed marked variation in the prevalence of *Ehrlichia* spp. among *Rhipicephalus microplus* ticks collected across the four surveyed districts of Punjab (Table 2). Such differences may stem from variations in cattle density, breed composition, and husbandry practices, as well as district-specific environmental factors such as temperature, humidity, and vegetation cover, all of which can influence tick population dynamics and pathogen persistence (Ahmad et al., 2025). For all three bacterial pathogens that were screened during this investigation, the infection rates were significantly higher in female *R. microplus* than in male (Table 3). Our findings are consistent with previous reports showing that female ticks, due to their prolonged feeding duration and higher blood intake compared to males, have greater chances of acquiring blood-borne pathogens. In addition, females are often characterized by a richer and more diverse microbiota, which can create favorable conditions for pathogen survival and co-infections (Bonnet et al., 2017, De la Fuente et al., 2017).

Conclusion

This study provides molecular evidence that cattle infesting *Rhipicephalus microplus* ticks in Punjab harbor bacterial agents of veterinary and zoonotic importance, including *Anaplasma phagocytophilum*, *Ehrlichia* sp., and *Rickettsia massiliae*. Overall pathogen detection was high (34.4%), and mixed infections were observed in nearly a quarter of the examined ticks, indicating active circulation of multiple bacterial agents within local tick populations. The presence of these pathogens in ticks collected from rural livestock farms highlights a potential exposure risk for farmers, animal handlers, veterinarians, and other livestock associated communities. These findings establish an important molecular baseline for tick-borne disease surveillance in Punjab and support the need for broader, district-level epidemiological investigations integrated with One Health-based tick control and public health strategies in Pakistan.

Declarations

Ethical Approval

Ethical Research Committee of the Bahauddin Zakariya University Multan (Pakistan) approved all the experimental procedures and protocols applied in this study via letter number BZU./ Ethics/24-21.

ARRIVE guidelines

All the methods were performed in accordance with ARRIVE guidelines laws and regulations.

Statement of Informed Consent

Informed verbal consent was obtained from livestock owners before including their animal in this study.

Conflict of interest/Competing interests

The authors declare no competing interests with anyone.

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Availability of data and material

The datasets generated and/or analyzed during the current study are available in the GenBank repository, with Accession numbers PV820216 and PV820217 (*Rhipicephalus microplus*), PV717247 and PV717248 (*Anaplasma phagocytophilum*), PV752233 and PV752234 (*Rickettsia massiliae*) and PV717249 and PV717250 (*Ehrlichia* sp.).

<https://www.ncbi.nlm.nih.gov/nuccore/PV820216>

<https://www.ncbi.nlm.nih.gov/nuccore/PV820217>

<https://www.ncbi.nlm.nih.gov/nuccore/PV717247>

<https://www.ncbi.nlm.nih.gov/nuccore/PV717248>

<https://www.ncbi.nlm.nih.gov/nuccore/PV752233>

<https://www.ncbi.nlm.nih.gov/nuccore/PV752234>

<https://www.ncbi.nlm.nih.gov/nuccore/PV717249>

<https://www.ncbi.nlm.nih.gov/nuccore/PV717250>

Authors' contributions

M.A designed and supervised this study. MA, KI and SU collected the ticks' samples and epidemiological data. MA and AE performed the wet lab experiments. SU, KI and AE performed the phylogenetic analysis. All authors contributed to the writing of manuscript and approved the final version.

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Figures

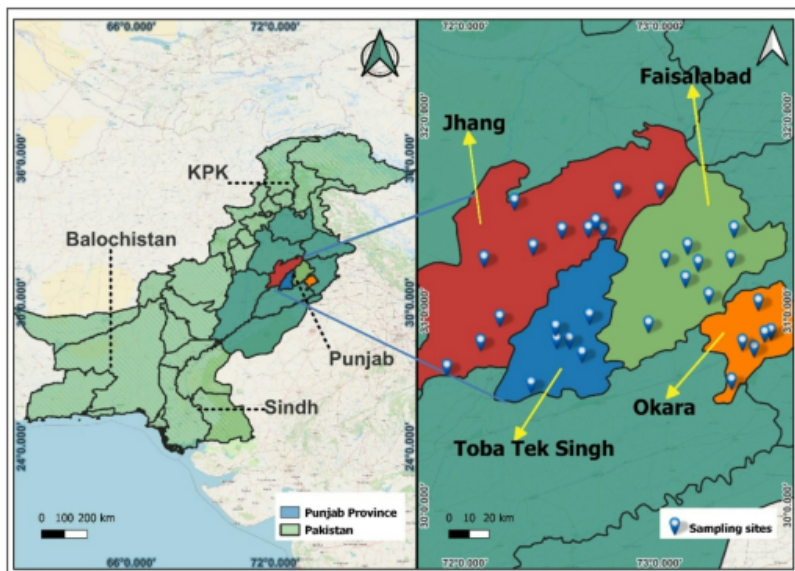


Figure 1

Map of Pakistan with sampling districts in Punjab province are represented with various colors. This study area map was generated using ArcGIS 10.8.1.3 (ESRI, Redlands, CA, USA).

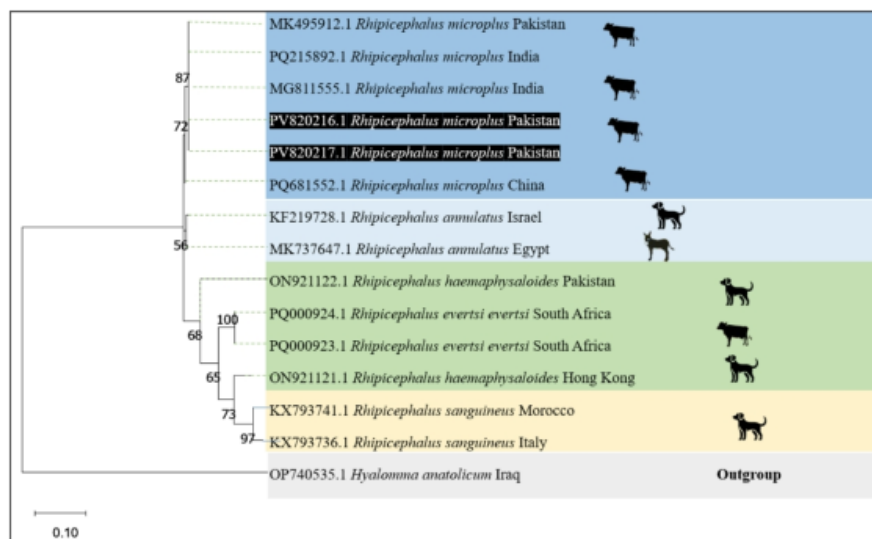


Figure 2

Phylogenetic tree of *Rhipicephalus microplus* based on the partial 16S rRNA gene sequences available in GenBank. The two haplotypes (PV820216 and PV820217) generated in this study are highlighted in black. The tree was constructed using maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications.

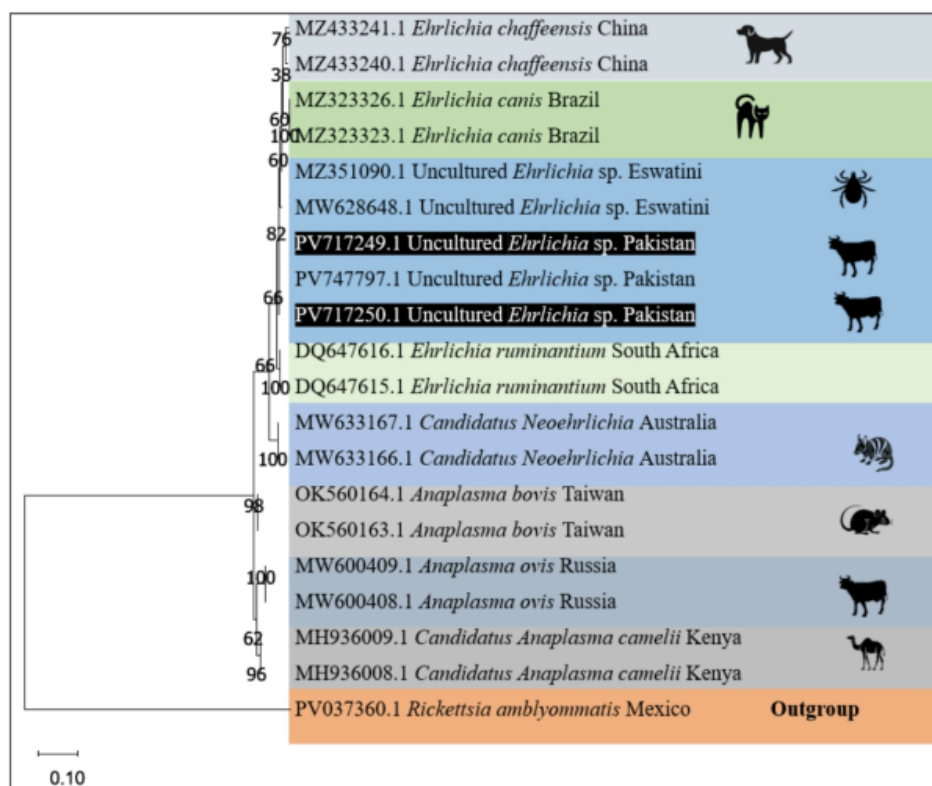


Figure 3

Phylogenetic tree of *Anaplasma phagocytophilum* based on the partial 16S rRNA gene sequences available in GenBank. The two haplotypes (PV717247 and PV717248) generated in this study are highlighted in black. The tree was constructed using maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications.

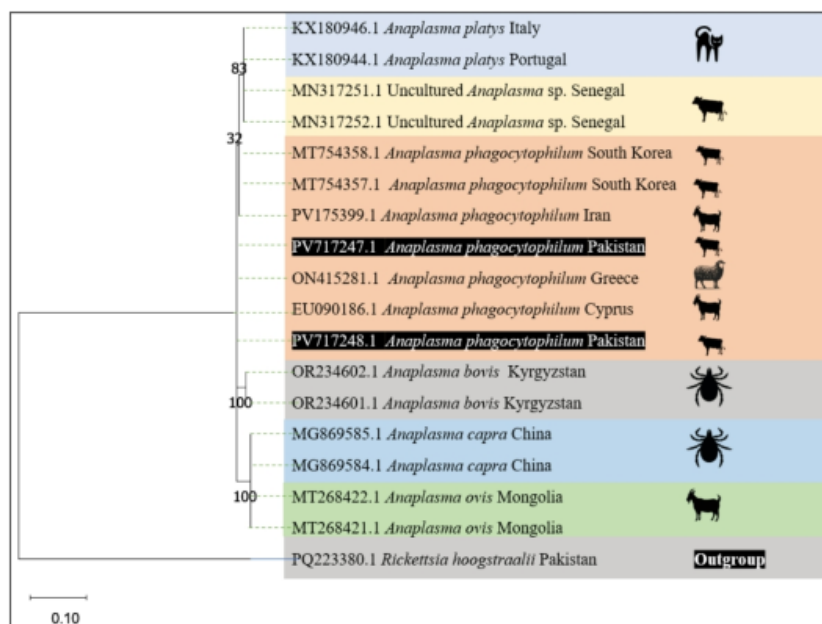


Figure 4

Phylogenetic tree of *Ehrlichia* sp. based on the partial *gltA* gene sequences available in GenBank. The two haplotypes (PV717249 and PV717250) generated in this study are highlighted in black. The tree was constructed using maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications.

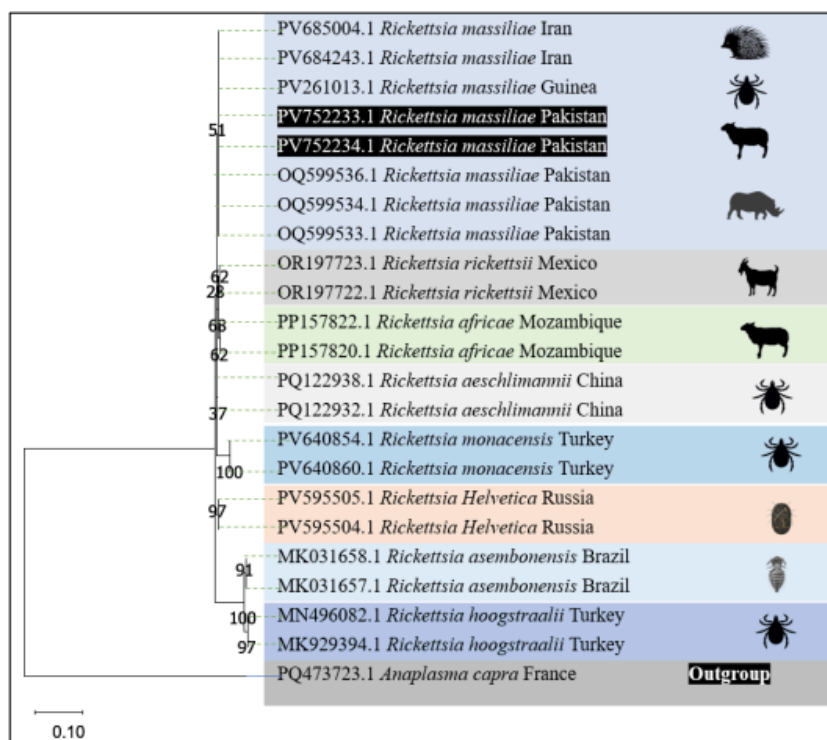


Figure 5

Phylogenetic tree of *Rickettsia massiliae* based on the partial *gltA* gene sequences available in GenBank. The two haplotypes (PV752233 and PV752234) generated in this study are highlighted in black. The tree was constructed using maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications.

Supplementary Files

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