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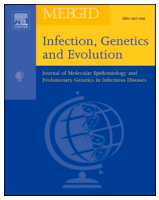

Genetic diversity of tick-borne zoonotic pathogens in ixodid ticks collected from small ruminants in Northern Pakistan

Jehan Zeb [a,b,l,*], Baolin Song [d], Munsif Ali Khan [b,c], Haytham Senbill [e], Muhammad Umair Aziz [d], Sabir Hussain [d], Adrian Alberto Díaz Sánchez [f], Alejandro Cabezas-Cruz [g], Abdulrahman Alzahrani [h], Mohammed Alshehri [j], Rashed Mohammed Alghamdi [i], Olivier Andre Sparagano [k,*]

[a] Center for Immunology and Infection Limited, Hong Kong Science and Technology Park, Hong Kong, SAR, China
[b] School of Public Health, The Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, SAR, China
[c] Vector-Borne Diseases Control Unit, District Health Office, Abbottabad 22010, Pakistan
[d] Jockey Club College of Veterinary Sciences, Department of Infectious Diseases and Public Health, City University of Hong Kong, Hong Kong, SAR, China
[e] Department of Applied Entomology & Zoology, Faculty of Agriculture, Alexandria University, Alexandria 21545, Egypt
[f] Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, Saskatchewan S7N 5E2, Canada
[g] ANSES, INRAE, Ecole Nationale Vétérinaire d'Alfort, UMR BIPAR, Laboratoire de Santé Animale, Maisons-Alfort F-94700, France
[h] Department of Applied Medical Sciences, Applied College, Al-Baha University, Al-Baha City, Kingdom, Saudi Arabia
[i] Department of Laboratory Medicine, Faculty of Applied College, Al-Baha University, Saudi Arabia
[j] Department of Public Health, Faculty of Applied Medical Sciences, Al-Baha University, Al-Baha, Saudi Arabia
[k] Agricultural Sciences and Practice, Royal Agricultural University, Cirencester GL7 6JS, UK
[l] Department of Zoology, GDC Samarbagh, Higher Education Department Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

Mapping tick distribution and pathogens in unexplored areas sheds light on their importance in zoonotic and veterinary contexts. In this study, we performed a comprehensive investigation of the genetic diversity of tick and tick-borne pathogens (TBPs) detection infesting/infecting small ruminants across northern Pakistan. We collected 1587 ixodid ticks from 600 goats and sheep, an overall tick infestation rate of 50.2 %. Notably, gender-based infestation rates were higher in female goats and sheep compared to their male counterparts. Age-wise analysis showed that the tick infestation rate was higher in older animals. This study identified 11 ixodid tick species within three genera: *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*, which were taxonomically classified using *16S rRNA* and cytochrome oxidase I *(cox1)* molecular markers. Sequence analysis indicated that reported ticks are similar to ixodid species found across various Asian and African countries. Tick-borne pathogens were detected by amplifying *16S rRNA* and citrate synthase (*gltA*) for bacterial pathogens and 18S rRNA for apicomplexan parasites. The present study reported a diverse array of TBPs in ticks from the study area, with *Rickettsia massiliae* (24.5 %) and *Theleria ovis* (16.4 %) as the most prevalent bacterial and apicomplexan pathogens. Phylogenetically, detected TBPs shared evolutionary relatedness with identical TBPs from old and new world countries. These findings highlight the presence of zoonotic TBPs in ixodid ticks from Pakistan. In addition, it also provides a foundation for future epidemiological research on ticks and TBPs, emphasizing their relevance in both zoonotic and veterinary contexts.

* Corresponding authors.
E-mail addresses: zebjehan2012@gmail.com, jehan.zeb@c2i.hk (J. Zeb), baolin.Song@my.cityu.edu.hk (B. Song), munsifalikhan@yahoo.com (M.A. Khan), haytham.senbill@alexu.edu.eg (H. Senbill), muhamaziz3-c@my.cityu.edu.hk (M.U. Aziz), sahussain8-c@my.cityu.edu.hk (S. Hussain), adiasanz88@gmail.com (A.A.D. Sánchez), alejandro.cabezas@vet-alfort.fr (A. Cabezas-Cruz), abdulrahmanmuidh@gmail.com (A. Alzahrani), Maalshehri@bu.edu.sa (M. Alshehri), rmalghamdi@bu.edu.sa (R.M. Alghamdi), olivier.sparagano@rau.ac.uk (O.A. Sparagano).

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1. Introduction

Ticks are obligatory hematophagous ectoparasites infesting animals and humans with worldwide geographic distribution (de la Fuente et al., 2017). While tick infestation produces direct damage such as stress, irritation, allergy, anemia, weight loss, and paralysis, the paramount importance of ticks lies in the pathogens they can transmit, including bacteria, fungi, protozoa, rickettsia, spirochetes, and viruses (Anderson and Magnarelli, 2008; Gray, 1994; Jongejan and Uilenberg, 2004). Ticks are second to mosquitoes as vectors of human pathogens but first as vectors of pathogens affecting livestock (Guo et al., 2019). In tropical countries, tick-borne diseases (TBDs) such as anaplasmosis, babesiosis, theileriosis, and cowdriosis cause morbidity and mortality in humans and animals and produce significant economic losses (Jongejan and Uilenberg, 2004).

Ticks and other vectors like mosquitoes have gained attention because of their public and veterinary health concerns (Juliano and Philip Lounibos, 2005; Keirans and Durden, 2001; Lounibos, 2002; White et al., 2021). Even though international authorities regularly monitor ticks and other potential vectors attached to their exotic hosts (Keirans and Durden, 2001), ticks may go unnoticed, leading to the import and possible establishment of such invasive tick species in new territories (White et al., 2021). Unfortunately, the movement of livestock internationally leads not only to the distribution of invasive tick species but to the broadening of the diverse pathogens associated with the animals or their attached ticks (Tan et al., 2021). This scenario leads to the wide infections of TBDs, such as anaplasmosis, babesiosis, ehrlichiosis, rickettsiosis, and theileriosis, from one developing country to another and eventually impairs the livestock economy (Perry and Grace, 2009).

The international movement of livestock significantly impacts the transmission of ticks and TBPs. Ticks may go undetected even with the efforts of international authorities to monitor ticks and other potential vectors attached to exotic hosts (Keirans and Durden, 2001). Due to this mistake, invasive tick species may be brought into and established in new areas (White et al., 2021). Additionally, the transnational movement of livestock not only aids in the spread of invasive tick species but also increases the spread of many diseases linked to the animals or the ticks they carry.

Proper identification of tick species is crucial for effectively controlling and preventing TBDs. Traditionally, tick identification and systematics were based on morphological keys. However, it requires much practice and might be challenging in the case of engorged or physically damaged ticks (Lv et al., 2014). Therefore, molecular approaches have been practical not only in tick identification and phylogenetic inferences but also in the detection of commensals as well as the different types of pathogens they carry (Duron et al., 2015; Ghafar et al., 2020d; Kasi et al., 2020). Given the limited molecular data available on tick-borne pathogens (TBPs) in ticks infesting small ruminants, accurately identifying TBPs necessitates applying molecular techniques such as PCR (Ghafar et al., 2020d; Ghafar et al., 2020a; Karim, Singh, and Ribeiro, 2011; Michelet et al., 2014; Rehman, Conraths, Sauter-Louis, Krücken, and Nijhof, 2019; Salih, El Hussein, and Singla, 2015).

In Pakistan, livestock plays a significant economic role, especially in rural areas (Rehman et al., 2017). Goats (78.2 million) and sheep (30.9 million) are integral components of the livestock sector in the country, and they are raised mainly by rural families and smallholders (Rehman et al., 2017). In countries such as Pakistan, ticks and TBDs' impact on animals is significantly influenced by factors such as poor infrastructure, limited resources, and inadequate access to veterinary services (Nieto, Khan, Uhllah, and Teglas, 2012). Several studies have been conducted on the prevalence of ticks and TBPs in Pakistan, which indicates that the country's climate is conducive to tick development and dissemination of TBPs (Ghafar et al., 2020d, Ghafar et al., 2020a, 2020b). However, compared to other parts of Pakistan, like the semi-arid and arid agro-ecological zones of Punjab and Sindh (Ghafar et al., 2020c; Ghafar et al., 2020a; Hussain et al., 2024; Karim et al., 2017; Rehman, Conraths, Sauter-Louis, Krücken, and Nijhof, 2019), the existing literature lacks comprehensive data on the occurrence of ticks and tick-borne pathogens in the northern and northwestern regions of Pakistan (Zeb et al., 2019a, Zeb et al., 2020). The primary tick species infesting livestock in Pakistan are *Rhipicephalus microplus*, *Rh. turanicus*, *Rh. hemaphysaloides*, *Rh. annulatus*, *Rh. sanguineus* sensu lato (s.l.), *Hy. anatolicum* and *Hy. dromedarii* (Ali et al., 2019). In addition, very few studies in northern Pakistan have been carried out investigating the hemoparasites, such as *Anaplasma ovis*, *Babesia crassa*, *B. ovis*, *B. motasi*, *T. leuwenshuni*, and *T. ovis*, infecting small ruminants (Khan et al., 2020a, b; Niaz et al., 2021). The present study investigated and addressed the knowledge gap regarding tick and TBP molecular epidemiology affecting small ruminants in northern Pakistan.

2. Materials and methods

2.1. Study area, sample size, tick collection, and morphological identification

A cross-sectional epidemiological study was carried out from April 2020 to March 2022 in the Northern (Districts: Astore: 35° 13′ 8.4" N and 74° 52′ 26.76″ E, Ghanche: 35° 9′ 45" N and 76° 20′ 12.99″ E, Gilgit: 35° 48′ 9.36" N and 74° 58′ 59.52″ E and Hunza: 36° 31′ 59.88" N and 75° 9′ 16.2″ E) and North-western (Districts: Chitral: 36° 6′ 40.68" N and 72° 8′ 29.76″ E, Dir (Lower): 34° 54′ 57.96" N and 71° 48′ 34.92″ E, Dir (Upper): 35° 20′ 8.16" N and 72° 2′ 48.48″ E and Swat: 35° 13′ 21.72" N and 72° 25′ 32.88″ E) regions of Pakistan. The sampling sites were chosen based on the following three characteristics: (I) proximity to the international borders with Afghanistan and China, considering livestock transboundary movements; (II) Large population of small ruminants; and (III) location in Pakistan's peripheral region.

The study area is characterized by a typical subtropical scrub forest, mountain dry temperate conifer, subalpine, dry scrub, and dry temperate coniferous forest. Meteorological patterns show that high temperature (35–50 °C; relative humidity: 50–65 %) is prevalent in low-altitude districts of the study area from May–August while the high-altitude districts experience relatively moderate thermal waves (28–35 °C; relative humidity: 65–90 %). The winter season starts in December and lasts till February, while in high-altitude districts, it extends up to April due to heavy snowfall (January–February) with a temperature falling below 0 °C (−7.6 °C–5 °C; relative humidity: 70–82 %) (Fig. 1) (Khan et al., 2020a, b).

Small ruminants were selected as target host populations, and a simple random sampling technique was adopted to collect ticks. The sample size was estimated by considering the expected prevalence of 50 % in the study area with a 95 % confidence interval and 5 % desired precision. A total of 600 small ruminants, including goats = *Capra hircus* ($n = 360$) and sheep = *Ovis aries* ($n = 240$), were carefully checked for tick infestations within 53 small ruminant herds (30 goat and 23 sheep herds). A total of 1587 ixodid ticks were collected using a tweezer from the predilection attachment sites of the hosts' bodies with minimal stress/harm to the host animal. The collected ticks were preserved in 70 % ethanol and 5 % glycerol solution, followed by morphological identification under a stereomicroscope using standard identification keys (Das, Naithani, and Subramanian, 1973; Estrada-Peña, Bouattour, Camicas, and Walker, 2004; Walker, Keirans, and Horak, 2000; Geevarghese and Mishra, 2011). The host-demographic attributes were recorded on pre-designed proforma.

2.2. DNA extraction

A total of 220 ticks (20 ticks for each tick species) were selected for DNA extraction and molecular screening for the presence of TBPs in ixodid tick species prevalent across the study area. The DNA was extracted from each tick using QIAamp DNA Mini Kit (Qiagen, Hilden

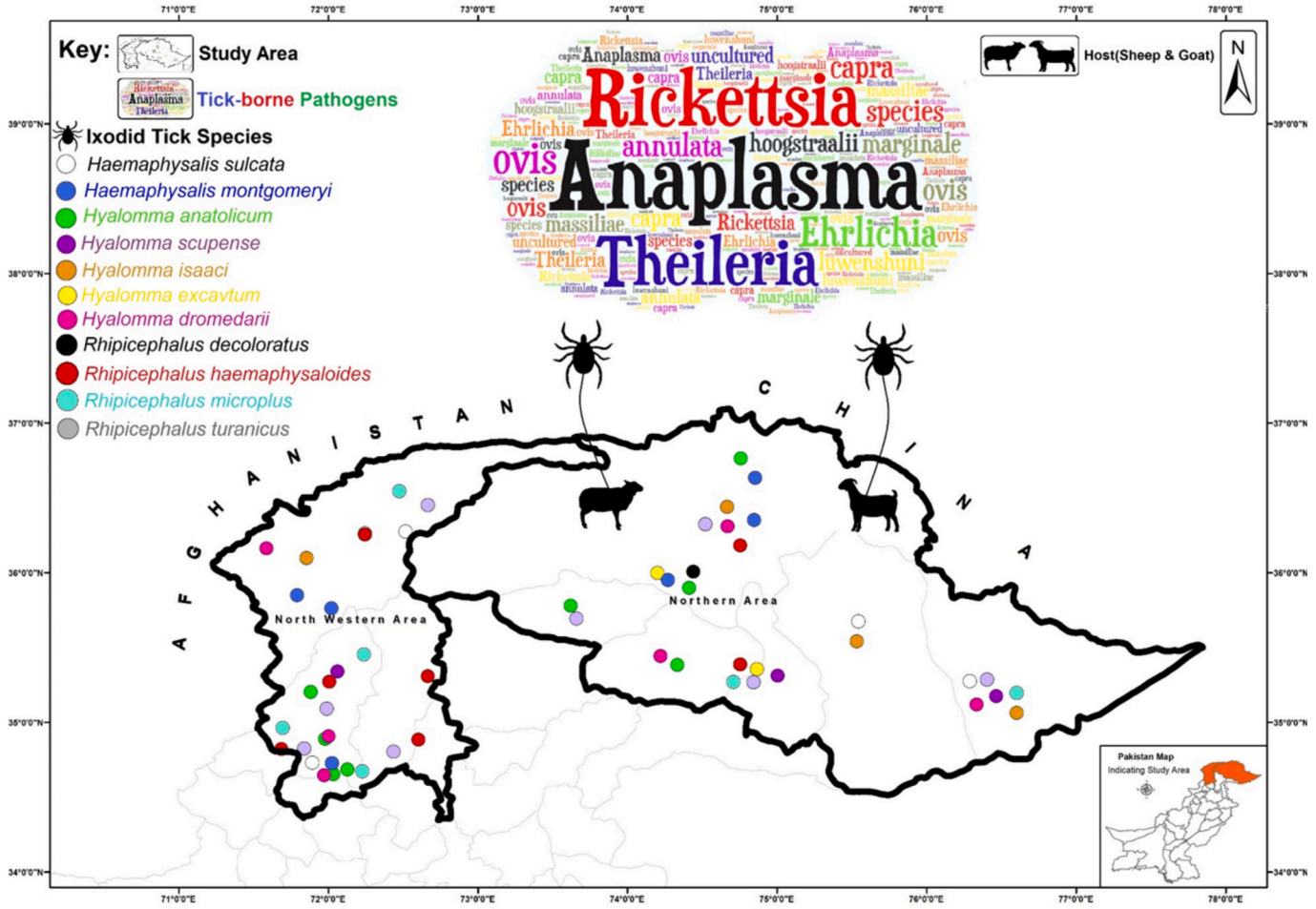

Fig. 1. Study Area map depicts host type, tick species, and their distribution across the Study Area. The word cloud illustrates the diversity of zoonotic TBPs detected in ticks from Northern Pakistan.

Germany) following the manufacturer's instructions. The DNA concentration in each sample was quantified using a spectrophotometer (NanoDrop, Thermo Scientific, USA). All the DNA samples were stored at −20 °C for further analysis.

2.3. Polymerase chain reaction

Extracted DNA samples were subjected to a species-specific polymerase chain reaction (PCR) using specific primers to amplify the ticks' *cox1* and *16S rRNA*. However, for further confirmation of the *Rhipicephalus decoloratus* we used an additional marker of the internal transcribed spacers of the ribosomal *(*ITS2*)* gene (Chitimia et al., 2009). Tick-borne pathogens were detected in collected ticks using TBPs specific primers viz. *16S rRNA/gltA* (*Anaplasma/Ehrlichia/Rickettsia* sp.) and *18S rRNA* (*Theileria* sp.) genes (Table 1). The PCR reactions were carried out according to the previously published protocols for tick and TBP detection (Black and Piesman, 1994; Casati, Sager, Gern, and Piffaretti, 2006; Folmer, Black, Hoeh, Lutz, and Vrijenhoek, 1994; Martin, Brown, Dunstan, and Roberts, 2005; Nijhof et al., 2008; Roux, Rydkina, Eremeeva, and Raoult, 1997). The total PCR reaction volume was 30 µl (2.5

Table 1
List of reference primer sets used to amplify target genes of ticks and tick-borne pathogens.

Organism	Primer name	Primer Sequence (5′–3′)	Target gene	Amplicon Size (bp)	Reference
Ticks	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG			Folmer, Black, Hoeh, Lutz, and Vrijenhoek, 1994
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	*Cox1*	~710 bp	
	16S + 1	CTG CTC AAT GAT TTT TTA AAT TGC TGT GG			Black and Piesman, 1994
	16S-1	CCG GTC TGA ACT CAG ATC AAG T	*16S rRNA*	~460 bp	
	TITS2F	CGAGACTTGGTGTGAATTGCA	*ITS2*	~920–1850 bp	Chitimia et al., 2009
	TITS2R	TCCCATACACCACATTTCCCG			
Ehrlichia spp./ *Anaplasma* spp.	EHR16SF	GGTACCYACAGAAGAAGTCC			Martin, Brown, Dunstan, and Roberts, 2005
	EHR16SR	TAGCACTCATCGTTTACAGC	*16S rRNA*	~345 bp	
Piroplasm	NWF	GTC TTG TAA TTG GAA TGA TGG			Casati, Sager, Gern, and Piffaretti, 2006
	NWR	TAG TTT ATG GTT AGG ACT ACG	*18S rRNA*	~500 bp	
Rickettsia	Rick-F1	GAA CGC TAT CGG TAT GCT TAA CAC A			Nijhof et al., 2008
	Rick-R2	CAT CAC TCA CTC GGT ATT GCT GGA	*16S rRNA*	~364 bp	
	CS-78	GCAAGTATCGGTGAGGATGTAAT			Roux, Rydkina, Eremeeva, and Raoult, 1997)
	CS-238	GCTTCCTAAAATTCAATAAATCAGGAT	*gltA*	~401 bp	

µl of genomic DNA, 1 µl of each primer (10 pmol), 10.5 µl of PCR grade water, and 15 µl PCR master mix). PCR products were run on 2 % agarose gel stained with ethidium bromide and visualized under UV light in a gel documentation machine (Bio-Rad Laboratories, California, USA).

2.4. Purification and sequencing

All amplified PCR products were sent to BGI Tech Solutions (Hong Kong Co. Limited, Hong Kong SAR, China) for purification and sequencing. The obtained sequences were edited, trimmed, and aligned in MEGA X (Kumar, Stecher, Li, Knyaz, and Tamura, 2018) and then blasted (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to the NCBI GenBank database for multiple sequence alignments.

2.5. Phylogenetic analysis

The ticks' partial nucleotide sequences (*16S rRNA* and *COI*) and detected TBPs' sequences (16S rRNA, 18S rRNA, and *gltA*) were phylogenetically analyzed using the maximum likelihood algorithm (ML) and the general time reversible (GTR) model with rapid bootstrapping of 1000 replicates. All the trees were constructed in MEGA X following the Kimura 2-parameter model (Kumar, Stecher, Li, Knyaz, and Tamura, 2018).

2.6. Statistical analysis

All statistical analyses of the present study data set were carried out using R software version 3.5.1 (R Development Core Team). Host demographic parameters were analyzed using summary statistics. The Kruskal-Wallis test (post hoc test: Conover; $P < 0.05$) was used to assess the association between tick species across regions, while the Chi-square test examined host demographic-based prevalence. A confidence interval (*CI*) of 95 % and $p < 0.05$ was considered statistically significant in all the analyses.

3. Results

3.1. Host demographic-based prevalence of tick infestation

Tick infestation rates varied across the study area with respective host demographic parameters, i.e., gender and age. Tick infestation was detected in 50.1 % (301/600) of the small ruminants examined, including 33.6 %% (202/600) goats and 16.5 % (99/600) sheep. Analysis based on host gender revealed significantly higher tick infestation rates among female goats 62.8 % (164/261) compared to male goats 38.3 % (38/99) ($\chi2 = 4.000$, $p = 0.0455$). Similarly, among the male sheep 38.5 % (27/70) while female sheep 42.4 % (72/170) were infested with ticks, showing a statistically significant association between the two variables ($\chi2 = 6.031$, $p = 0.0143$). In addition, the age-based analysis revealed that older goats exhibited significantly higher tick infestation rates 60.5 % (130/215) than younger goats 49.7 % (72/145) ($\chi2 = 6.155$, $p = 0.0147$). Among sheep, young sheep 27.3 % (30/110) showed significantly higher tick infestation rates compared to older sheep 53.1 % (69/130) ($\chi2 = 3.857$, $p = 0.0495$) (Table 2).

3.2. Distribution and diversity of ixodid tick species

A total of 1587 ticks were collected from small ruminants, including 765 (48.2 %) nymphs, 585 (36.9 %) adults, and 237 (14.9 %) larvae. Ticks were identified to species using morphology and DNA sequencing analysis, revealing representatives belonging to three ixodid tick genera, including *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*. A total of 11 tick species were identified with variable prevalence, where *Hae,aphysalis montgomeryi* (18.5 %) was the most common tick species infesting small ruminants, followed by *Rhipicephalus turanicus* (15.5 %), *Rhipicephalus haemaphysaloides* (13.2 %), *Rhipicephalus*. *microplus* (10.9 %), *Hyalomma dromedarii* (9.2 %), *Hyalomma scupense* (7.7 %), *Haemaphysalis sulcata* (6.6 %), *Hyalomma anatolicum* (5.9 %), *Hyalomma isacci* (5.2 %), *Rhipicephalus decoloratus* (4.4 %), and *Hyalomma excavatum* (2.9 %). The occurrence of single and mixed tick species infestations is summarized in Table 3.

The most frequently reported tick species in goats' populations from the study area was *Rh. turanicus* (19.0 %), followed by *Hae. montgomeryi* (14.3 %), *Rh. haemaphysaloides* (13.2 %), *Rh. microplus* (11.2 %), *Hy. dromedarii* (9.6 %), *Hy. scupense* (8.2 %), *Ha. sulcata* (6.2 %), *Hy. anatolicum* (6.0 %), *Hy. isacci* (5.0 %), *Rh. decoloratus* (4.5 %) and *Hy. excavatum* (2.8 %), respectively. However, no significant association was found statistically among the reported tick species from different regions ($p = 0.164$).

The occurrence pattern of reported tick species in sheep populations across northern Pakistan including *Hae. montgomeryi* (18.0 %), *Rh. turanicus* (17.2 %), *Rh. haemaphysaloides* (13.7 %), *Rh. microplus* (10.3 %), *Hy. dromedarii* (8.4 %), *Hae. sulcata* (7.4 %), *Hy. scupense* (6.8 %), *Hy. isacci* (5.5 %), *Hy. anatolicum* (5.5 %), *Rh. decoloratus* (4.3 %) and *Hy. excavatum* (2.9 %). The association between different tick species across the regions was statistically non-significant ($p = 0.118$).

3.3. Sequence similarity and phylogenetic profile of ixodid tick species

The *16S rRNA* and *cox1* sequences of tick samples from the current study shared genetic identity with similar tick isolates reported from Pakistan and worldwide. Among the characterized ixodid tick species, *Hy. anatolicum*, *Hy. dromedarii*, *Hy. excavatum*, *Hy. isacci*, and *Hy. scupense* demonstrated high sequence similarities (*16S rRNA*: 99.74–100 %; *cox1*: 100 %) with similar isolates from China, Iran, Pakistan, Saudi Arabia, Senegal, South Africa, Sri Lanka, and Turkey. Additionally, *Hae. montgomeryi* and *Hae. sulcata* exhibited sequence homology (*16S rRNA*: 99.47–100 %; *cox1*: 99.54–100 %) with isolates reported from China and Iran. Likewise, *Rh. decoloratus*, *Rh. haemaphysaloides*, *Rh. microplus*, and *Rh. turanicus* shared high sequence resemblance (*16S rRNA*: 99.73–100 %; *cox1*: 100 %) with isolates from China, India, and Pakistan (Tables S1 and S2).

The ML-based *16S rRNA* phylogenetic tree showed that the sequences generated by the present study shared identity with similar isolates published globally, including Africa (South Africa), South America (Senegal), and Asia (China, India, Turkey, and Egypt), with moderate to strong bootstrap values (Fig. 2).

Table 2
Host demography-based prevalence of tick infestation.

Variables	Host type	Demographical attributes	Total no. examined	Tick Infestation n (%)	Chi-square Statistics	*P*-Value
Host Gender	Goats	Male	99	38/99 (38.3)	4.000	0.0455
		Female	261	164/261(62.8)		
	Sheep	Male	70	27/70 (38.5)	6.031	0.0143
		Female	170	72/170 (42.4)		`
Host Age	Goats	Young	145	72/145 (49.7)	6.115	0.0147
		Older	215	130/215 (60.5)		
	Sheep	Young	110	30/110 (27.3)	3.857	0.0495
		Older	130	69/130 (53.1)		

Table 3
Occurrence and distribution of Ixodid ticks collected from goats and sheep across North and North-Western Pakistan.

Study area	Host (n)	Genus *Hyalomma* (*Hy.*)					Genus *Haemaphysalis* (*Ha.*)		Genus *Rhipicephalus* (*R.*)			
		Hy. anatolicum	*Hy. dromedarii*	*Hy. excavatum*	*Hy. isacci*	*Hy. scupense*	*Hae. montgomeryi*	*Hae. sulcata*	*Rh. decoloratus*	*Rh. haemaphysaloides*	*Rh. microplus*	*Rh. turanicus*
Gilgit Baltistan (NA)												
District Astore	Goat (72)	11	18	5	10	15	29	11	8	25	20	35
35° 13′ 8.4" N	Sheep (14)	2	3	1	2	3	10	4	2	5	4	6
74° 52′ 26.76″ E												
District Ghanche	Goat (19)	4	5	2	3	4	8	9	3	7	6	9
35° 9′ 45" N,	Sheep (17)	3	4	1	4	3	9	3	1	6	5	8
76° 20′ 12.99″ E												
District Gilgit	Goat (60)	9	15	4	8	12	24	9	7	21	17	30
35° 48′ 9.36" N	Sheep (32)	5	8	3	5	7	13	6	4	12	10	15
74° 58′ 59.52″ E												
District Hunza	Goat (50)	7	12	3	7	10	20	8	6	17	14	24
36° 31′ 59.88" N	Sheep (37)	6	9	4	6	8	16	7	5	14	11	20
75° 9′ 16.2″ E												
Khyber Pakhtunkhwa (NWA)												
District Chitral	Goat (61)	10	14	5	9	11	8	6	7	22	18	29
36° 6′ 40.68" N	Sheep (35)	5	7	2	5	6	10	7	3	13	12	16
72° 8′ 29.76″ E												
District Dir (Lower)	Goat (57)	8	13	6	8	13	23	10	8	20	16	27
34° 54′ 57.96" N	Sheep (09)	2	3	2	1	2	10	4	1	8	3	8
71° 48′ 34.92″ E												
District Dir (Upper)	Goat (65)	10	16	3	5	14	24	5	4	23	19	31
35° 20′ 8.16" N	Sheep (15)	3	4	1	3	3	11	3	3	7	5	10
72° 2′ 48.48″ E												
District Swat	Goat (45)	7	10	2	4	9	18	8	5	6	10	20
35° 13′ 21.72" N	Sheep (12)	2	5	1	2	3	13	4	3	5	3	5
72° 25′ 32.88″ E												
Total (n)	600	94	146	45	82	123	246	104	70	211	173	293
95 % CI		4.2–7.5	6.5–11.8	1.9–3.7	3.7–6.5	5.3–10.0	12.9–23.7	5.1–7.8	3.1–5.6	9.3–17.0	7.6–14.0	11.8–8.9

NA: Northern Area, NW: North-western Area, *CI*: Confidence interval.

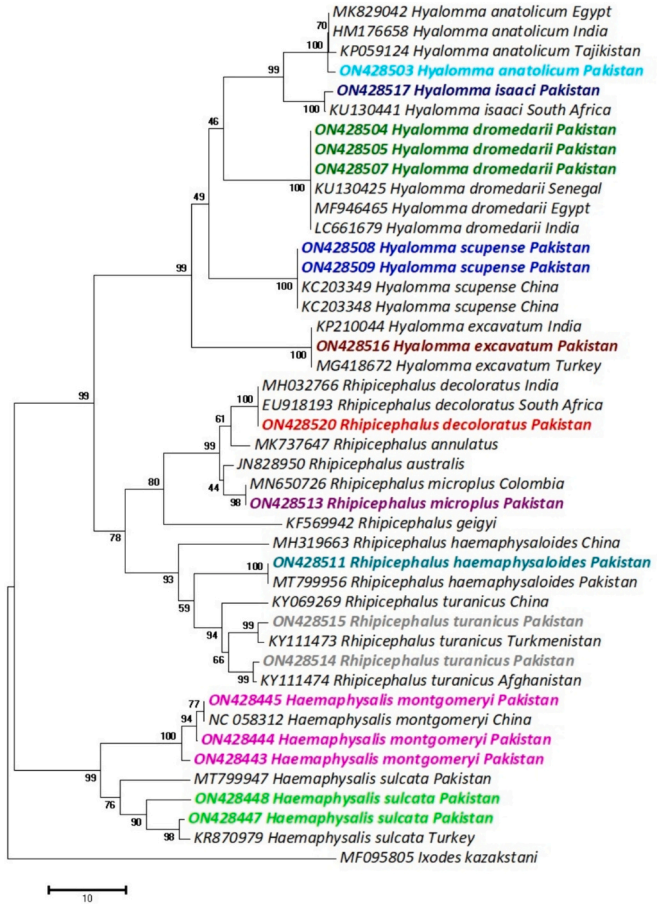

Fig. 2. The phylogenetic tree of 16S rRNA gene partial sequences of the three different Ixodid ticks genera (*Rhipicephalus*, *Haemaphysalis* and *Hyalomma*) in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with the Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Ixodes kazakstani* 16S rRNA gene partial sequences were used as outgroups.

The ML-based *cox1* phylogram provided an excellent resolution of the evolutionary relationships among the reported tick genera with strong bootstrap support. Phylogenetic analysis revealed that tick isolates from this study closely resemble previously reported isolates from various Asian countries such as Afghanistan, China, India, Iran, Saudi Arabia, Sri Lanka, and Turkey. This indicates a shared genetic ancestry or similarity in their genetic makeup among the tick species from different geographic localities (Fig. 3). To further confirm the phylogenetic placement of *Rh. decoloratus*, a maximum likelihood tree was constructed using the ITS2 genetic marker. This analysis showed that the *Rh. decoloratus* samples from the current study clustered together with other isolates from Kenya, confirming the Afrotropical origin of this species (Fig. 4).

3.4. Genetic diversity of detected TBPs

Among the molecularly screened tick species, 104 (47.3 %) ticks were found positive for at least one TBP DNA. The present study detected 11 different TBPs belonging to four genera: *Rickettsia* (50.0 %), *Theileria* (36.7 %), *Anaplasma* (34.2 %), and *Ehrlichia* (8.3 %). Among the detected TBPs, the most prevalent was *R. massiliae* (14.5 %), followed by *T. ovis* (10.5 %), *Ehrlichia* sp. (uncultured) (9.0 %), *A. capra* (8.0 %), *A. ovis* (8.0 %), *T. annulata* (7.5 %), *T. luwenshuni* (7.5 %), *A. marginale* (7.0 %), *R. hoogstraalii* (3.0 %), *Anaplasma* sp. (2.5 %) and *Rickettsia* sp. (2.5 %) (Table 4).

The occurrence of TBPs in hard ticks also varied, with the highest percentage occurring in *Hae. montgomeryi* (95.0 %) *Rh. turanicus* (60.0 %), and *Rh. haemaphysaloides* (55.0 %), followed by *Rh. microplus* (50.0 %), *Hy. dromedarii* (50.0 %), *Hy. anatolicum* (50.0), *Hae. sulcata* (45.0 %), *Hy. scupense* (45.0 %), *Hy. excavatum* (35.0 %), *Rh. decoloratus* (25.0 %) and *Hy. isacci* (10.0 %) (Fig. 5).

3.5. The co-infection pattern of TBPs in ixodid ticks

Among the 104 (47.3 %) TBPs positive ticks, single, double, and multiple (triple and quadruple) TBPs co-infection was observed among different tick species. DNA of at least a single pathogen was found in all reported tick species. Tick-borne pathogens double co-infection was observed in one *Rh. decoloratus* tick for *A. marginale* and *R. massiliae*; three *Hy. excavatum* for *Ehrlichia* sp. and *R. massiliae*, and one *Hy. isacci* tick for *Ehrlichia* sp. and *T. annulata*. In the case of triple co-infection TBPs, five *Hy. scupense* ticks tested positive for *A. capra*, *R. massiliae*, and *T. ovis*. On the other hand, four TBPs were simultaneously detected in many tick species, for instance, in two *Rh. microplus* ticks for *A. ovis*, *Anaplasma* sp., *R. massiliae*, and *A. marginale*; three *Hy. dromedarii* for *A. capra*, *Anaplasma* sp., *R. massiliae*, and *T. ovis*; five *Hae. montgomeryi* for *A. capra*, *R. hoogstraalii*, *R. massiliae*, and *Rickettsia* spp.; three *Rh. haemaphysaloides* for *A. ovis*, *R. massiliae*, *T. ovis*, and *T. luwenshuni*; five *Rh. haemaphysaloides* for *Anaplasma* sp., *R. massiliae, Rickettsia* sp., and *T. ovis* and six ticks (three each of *Rh. turanicus* and *Hae. sulcata*) *Ehrlichia* sp., *R. massiliae*, *T. annulata*, and *T. luwenshuni* TBPs infections were reported (Table 4).

3.6. Sequencing and phylogenetic profile of TBPs

Tick-borne pathogens of the genus *Anaplasma*, including *A. capra*, *A. marginale, A. ovis*, and other *Anaplasma* sp., exhibited genetic similarities ranging from 99.84 % to 100 %, with corresponding isolates from China, Iraq, and Kenya. Among these TBPs, uncultured *Ehrlichia* sp., a member of the genus *Ehrlichia*, demonstrated a 99.66 % similarity with isolates previously identified in Pakistan. Additionally, TBPs of the genus *Rickettsia*, specifically *R. hoogstraalii* and *R. massiliae*, were identical to isolates from Greece, India, South Africa, and the United States. Moreover, piroplasms from the genus *Theileria*, namely *T. annulata*, *T. luwenshuni*, and *T. ovis,* showed 100 % genetic identity with respective species previously reported from Italy, Pakistan, and the United Arab Emirates (Table S3).

In the present study, the *16S rRNA* phylogenetic tree clustered the present study isolates of the genus *Anaplasma* with identical isolates previously reported from Asia (Iraq and Pakistan), Africa (Mozambique), and South America (Brazil and Portugal) (Fig. 6). Similarly, isolates of uncultured *Ehrlichia* sp. were grouped with the identical isolates published from Asia (China, Pakistan, and Malaysia), Europe (France), Africa (South Africa), and South America (Portugal), with robust bootstrap support (Fig. 6). Likewise, species of the genus *Rickettsia* (*R. hoogstraalii*, *R. massiliae*, and *Rickettsia* sp.) were classified within the same clade as their respective species, with moderate to strong bootstrap support, as reported from Asia (China, India, Lebanon, Thailand, and Turkey), Europe (Greece, Spain, and Slovakia), America (Mexico, USA), and South Africa (Figs. 7 & 8). The *18S rRNA* phylogram showed that the present study *Theileria* isolates clustered with similar sequences deposited in the NCBI database from Asia (China, India, Myanmar, Pakistan, Thailand) and the Middle East (Egypt, Saudi Arabia, United Arab Emirates), with strong nodal support in the NJ phylogenetic tree (Fig. 9).

4. Discussion

Climate change/global warming has favored tick habitat expansion and TBP emergence into new geographic areas, thus placing public and

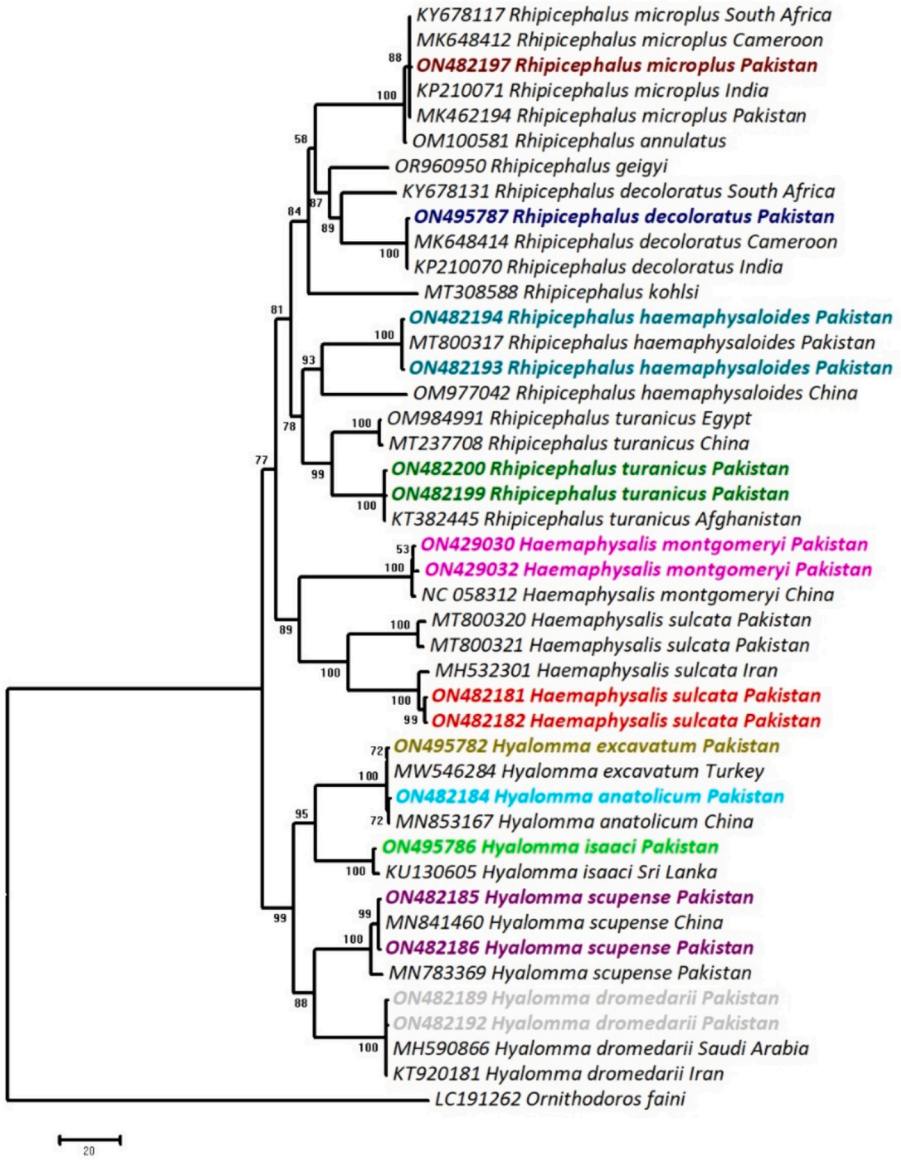

Fig. 3. The phylogenetic tree of *COI* gene partial sequences of the three different Ixodid ticks' genera (*Rhipicephalus*, *Haemaphysalis,* and *Hyalomma*) in this study (Highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with the Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Argas reflexus COI* gene partial sequences were used as outgroups.

veterinary health at risk of infectivity (Ali et al., 2019; Zeb et al., 2023). Small ruminant farming is a vital component of the livestock industry and contributes significantly to Pakistan's agricultural economy. Goats and sheep are constantly exposed to tick infestation/TBP infection due to grazing practices. These small ruminants often graze in pastures, rangelands, and mountainous areas where ixodid ticks thrive. During grazing, the animals come into close contact with the vegetation and environments harboring questing ticks, increasing the chances of tick attachment and infestation.

In Pakistan, molecular-based surveys investigating the molecular epidemiology of ticks and TBPs are still at an early stage, with only a limited number of studies conducted. In the current study, we documented and characterized molecularly 11 ixodid tick species infesting small ruminants. Our findings align with previous studies that reported the presence of hard tick infestations in small ruminants in different geographic locations within Pakistan. However, these prior studies were limited to morphological identification or focused on only a few tick species (Ali et al., 2019; Ghafar et al., 2020b; Karim et al., 2017; Rehman et al., 2017). We have observed that the genus *Rhipicephalus* tick species occur most frequently, followed by *Hyalomma* and *Haemaphysalis*. This tick infestation pattern aligns with previous studies conducted in other agroecological zones of Pakistan (Ali et al., 2019; Ghafar et al., 2020b; Zeb et al., 2019b). However, contrasting patterns of tick occurrences have been observed in different localities of Pakistan, such as Kashmir, Balochistan, and Punjab, where *Hyalomma* ticks dominate (Ali et al., 2019; Nizhat Sultana et al., 2015; Rafiq et al., 2017; Rehman et al., 2017). Our results are consistent with previous research, suggesting that different ecological zones, particularly arid and semi-arid regions, provide favorable environmental conditions for specific tick species (Ali et al., 2019; Estrada-Peña et al., 2006; Jyotika Kapur-Ghai, Singh, and Singh, 2008; Rehman, Conraths, Sauter-Louis, Krücken, and Nijhof, 2019). This is evident because *Rhipicephalus* species are more common in semi-arid agroecological zones in Pakistan and elsewhere (Ali et al., 2019; Rehman et al., 2017; Singh and Rath, 2013; Zeb et al., 2019).

Among the identified tick species, *Hae. montgomeryi*, an Asian Himalayan tick, commonly found infesting domestic animals, including

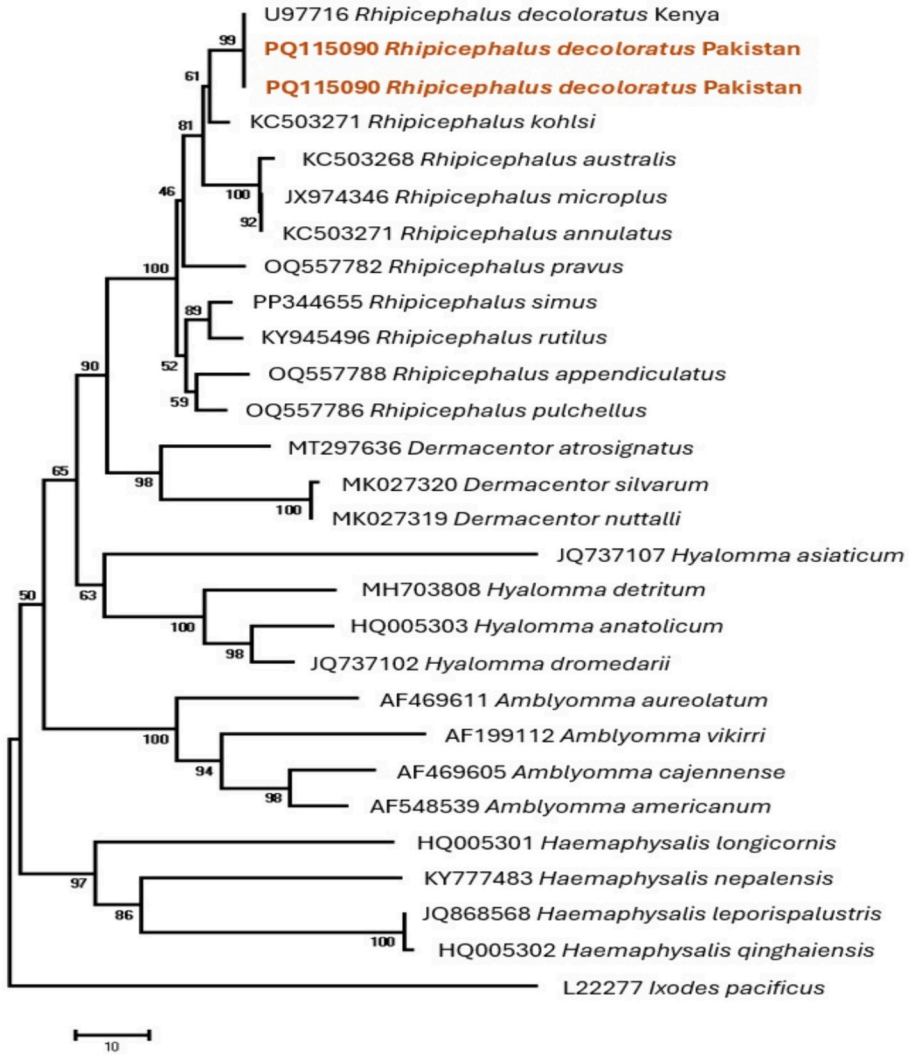

Fig. 4. The phylogenetic tree of *ITS-2* gene partial sequences of the *Rhipicephalus decoloratus* in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with the Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and are shown in the tree. *Ixodes pacificus ITS-2* gene partial sequences were used as outgroups.

cattle, goats, sheep, buffalo, dogs, and occasionally humans (Karim et al., 2017), has a distribution spanning the subtropical and lower temperate regions of the Himalayas in west Pakistan, Nepal, India, and China (Hoogstraal, Trapido, and Kohls, 1966; Karim et al., 2017). The presence of this tick species in Pakistan could be attributed to transboundary animal movements, trade, or the migration of birds from neighboring countries, considering that Pakistan imported live animals primarily from China, India, Nepal, and Afghanistan. In addition, *Rh. decoloratus*, originally native to the Afrotropical region, has become widespread in India and could have entered Pakistan from Africa by infesting African imported bovine hosts brought to India, resembling the introduction of *Rh. microplus* (originating from Asia) to Africa when Indian cattle were imported through Madagascar (Cohen, Auckland, Marra, and Hamer, 2015; Hasle, 2013; Hoogstraal, Trapido, and Kohls, 1966).

The present study findings on TBPS align with earlier research that described comparable TBPs from various agroecological zones in this country (Ghafar et al., 2020d; Niaz et al., 2021; Rehman, Conraths, Sauter-Louis, Krücken, and Nijhof, 2019). A total of 11 TBP species were detected in collected tick samples. Among them, *R. massiliae* was the most prevalent TBP. A previous study detected a high prevalence of *R. massiliae* in several tick species taken from sheep and goats (Ghafar et al., 2020b). Furthermore, our findings align with similar studies conducted in different geographic regions, both locally and internationally, which have reported comparable TBPs in diverse tick species (Ghafar et al., 2020b; Hakimi, Sarani, Takeda, Kaneko, and Asada, 2019; Niaz et al., 2021; Omondi et al., 2017; Rehman, Conraths, Sauter-Louis, Krücken, and Nijhof, 2019; Zeb et al., 2019). Moreover, the observed variations in prevalence patterns could be attributed to differences in sample size or the inclusion of different geographic locations with diverse climates.

Herein, our results molecularly documented the detection of *R. hoogstraalii* from *Hae. montgomeryi* infesting small ruminants is consistent with a recent study in the Punjab province of Pakistan (Hussain et al., 2024). However, no previous record is available from the study area. Phylogenetically, *R. hoogstraalii* is closely related to *R. felis* species, an emerging zoonotic pathogen known to be transmitted by arthropods, including fleas and possibly mosquitoes (Dieme et al., 2015; Parola et al., 2003). Among several *Rickettsia* species of the SFG, *R. hoogstraalii* had been previously reported in *Haemaphysalis* (*Hae. punctata* and *Hae. sulcata*) infesting goats and sheep from other parts of the world (Chisu et al., 2017; Chochlakis et al., 2012; Duh et al., 2010; Márquez, 2008). Although the exact vector remains uncertain, our results, along with previous studies, provide support to the hypothesis that several *Haemaphysalis* species, including *Hae. montgomeryi*, could potentially play a significant role in the transmission of various

Table 4
Positive infections and co-infections detected in the tick species of the present study (Skardu: 35° 19′ 28.9596" N; 75° 33′ 3.4452″ E, Astore: 45° 24′ 1.8324" N; 10° 31′ 24.924″ E, Diamir: 17° 35′ 8.2356" N; 33° 58′ 6.8556″ E, Tangir: 35° 46′ 13.404" N; 5° 48′ 12.996" W, Swat: 35° 13′ 21.756" N; 72° 25′ 32.9232″ E, Chitral: 32° 51′ 3.2" N; 71° 47′ 24.7″ E, Lower Dir: 35° 5′ 36.0" N; 72° 1′ 4.3″ E and Dir Upper: 35° 8′ 45.7" N; 72° 0′ 45.4″ E).*

Study area (District)	Tick species	No. of positive specimens (out of 20 screens)	Single/co-infections																	
			Single								Double			Triple	Quadruple					
			Ac	Am	Ao	Rh	Rm	Ta	Tl	To	Am+Rm	Esp. + Rm	Esp. + Ta	Ac + Rm+ To	Am+Ao+ Asp. + Rm	Ac + Asp.+ Rm + To	Ac + Rh + Rm+ Rsp.	Ao + Rm + To+Tl	Asp. + Rm + Rsp. + To	Esp. + Rm + Ta + Tl
Astore, Chitral, Dir (L), Gilgit, Hunza, Swat	*Hy. anatolicum*	10					2	1	2	2										3
Chitral, Dir (L), Dir (U), Gilgit, Ghanche, Hunza	*Hy. dromedarii*	10	1		1				2	3						3				
Astore, Chitral, Gilgit	*Hy. excavatum*	7					4					3								
Chitral, Ghanche, Hunza	*Hy. isacci*	2						1					1							
Astore, Chitral, Dir (U), Ghanche, Hunza,	*Hy. scupense*	9	2				1			1				5						
Astore, Chitral, Dir (L), Gilgit, Hunza	*Hae. montgomeryi*	19	1		4	4	5										5			
Chitral, Dir (L), Ghanche	*Hae. sulcata*	9	1			2				3										3
Chitral, Gilgit, Hunza	*Rh. decoloratus*	5		2			2				1									
Astore, Chitral, Dir (L), Dir (U), Ghanche, Hunza, Swat	*Rh. haemaphysaloides*	11	1		1		4		1	1								3		
Astore, Dir (L), Dir (U), Gilgit, Swat	*Rh.microplus*	10		5			3								2					
Astore, Chitral, Dir (L), Dir (U), Gilgit, Ghanche, Hunza, Swat	*Rh. turanicus*	12	1		2					4									5	
Total		104	7	7	8	6	21	2	5	14	1	3	1	5	2	3	5	3	5	6

* Ac: *Anaplasma capra*, Am: *Anaplasma marginale*, Ao: *Anaplasma ovis*, Rh: *Rickettsia hoogstraali*, Rm: *Rickettsia massiliae*, Ta, *Theileria annulata*, Tl: *Theileria luwenshuni*, To: *Theileria ovis*, Esp.: *Ehrlichia* sp., Asp.: *Anaplasma* sp., Rsp.: *Rickettsia* sp.

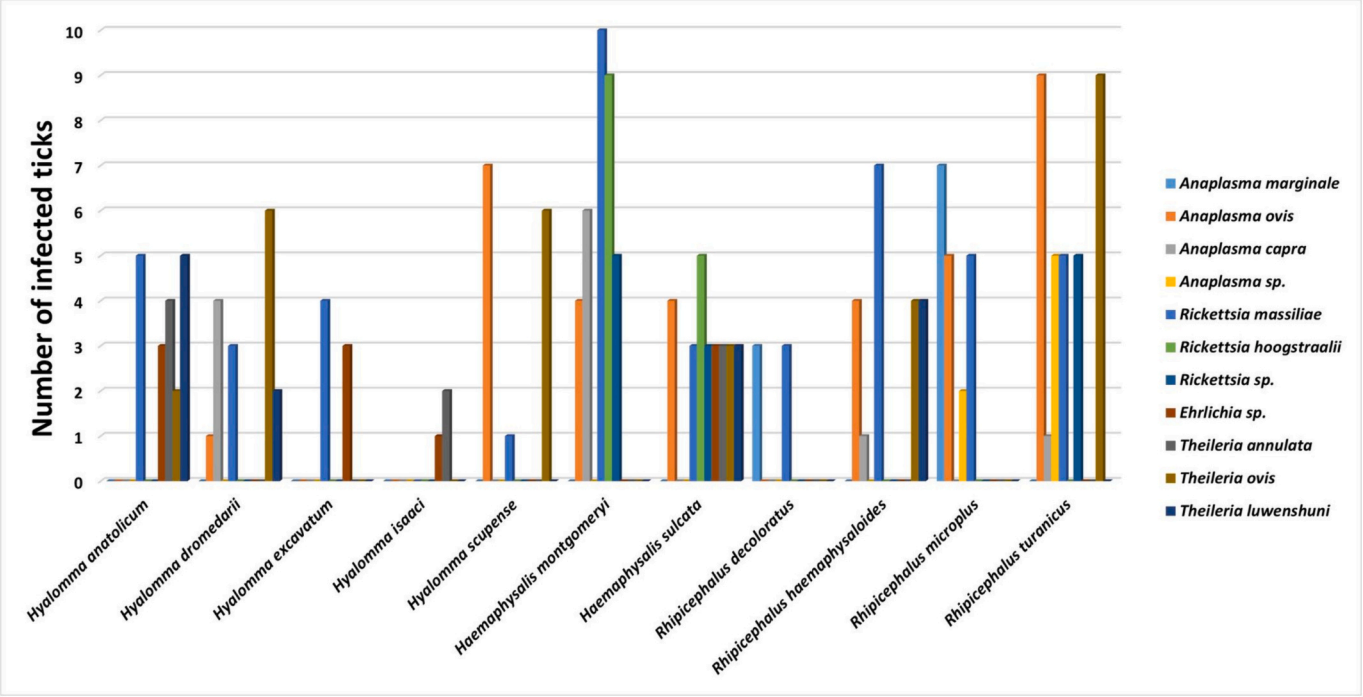

Fig. 5. Prevalence of tick-borne pathogens in ixodid ticks collected from small ruminants across northern Pakistan.

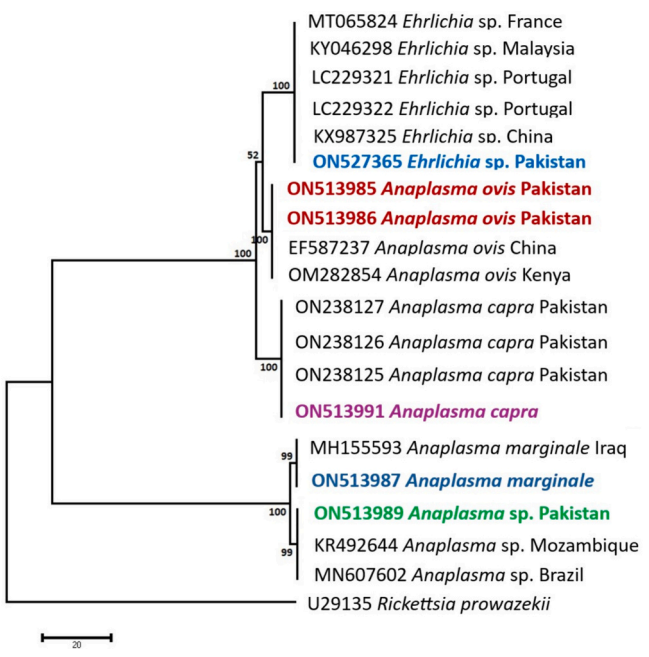

Fig. 6. The phylogenetic tree of 16S rRNA gene partial sequences of the genera (*Ehrlichia* and *Anplasma*) in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with the Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Rickettsia prowazekii* 16S rRNA gene partial sequences were used as outgroups.

Rickettsia species, including *R. hoogstraalii*. However, further investigations are warranted to examine the vector competence specifically for this pathogen. In addition, *A. capra*, a human pathogen, has also been detected in several tick species, including *Hy. dromedarii* and *Hy. scupence*, *Hae. montgomeryi*, *Hae. sulcata*, *Rh. haemaphysaloides*, and *Rh. turanicus* corroborates the previous findings reported from China (Li et al., 2015; Sun, Zhao, Wen, Luo, and Yu, 2015; Yu et al., 2015). However, the vector competence of these ticks for transmission of *A. capra* has not yet been confirmed. Previous findings, together with the results of this study, suggest that additional tick species and animals, respectively, may act as vectors and reservoirs for *A. capra,* which warrants further investigation.

The current study also observed co-infection of different TBPs in other tick species. Among them, *Hy. excavatum*, *Hy. isacci* and *Rh. decoloratus* were found to be infected with two pathogens, *Hy. scupense* with three pathogens, while *Hy. anatolicum*, *Hy. dromedarii*, *Hae. montgomeryi*, *Hae. sulcata*, *Rh. haemaphysaloides*, *Rh. microplus* and *Rh. turanicus* was detected positive with four TBPs. Our findings also corroborate the previous findings across the country and elsewhere, who reported the co-occurrence of up to seven TBPs in bovine and ovine/caprine tick species (Ghafar et al., 2020b; Hussain et al., 2024; Omondi et al., 2017). The ability of the pathogen to multiply in the salivary glands and midgut of the tick vector, as well as the host's vulnerability during tick attachment, are some factors contributing to the co-occurrence of pathogens. Furthermore, several host-related and environmental factors linked to pathogen development impact this interaction. For example, the coexistence of *Borrelia burgdorferi* and *Babesia microti* within *Ixodes* ticks increases the transmission of *B. microti* in locations endemic to Lyme disease despite their low ecological fitness (Diuk-Wasser, Vanacker, and Fernandez, 2021). This work opens a new avenue of investigation into the pattern of interactions between different TBDs in tick vectors and their mammalian hosts. It is necessary to conduct more experimental research to clarify the fundamental mechanisms influencing the co-occurring ecological community within the tick vector.

Phylogenetic inferences showed that hard ticks from the present study were successfully clustered with similar tick species reported from Asia, Europe, South America, and Africa. These findings corroborate published literature that used similar taxonomic markers to establish the phylogenetic profile of ixodid ticks from Pakistan and elsewhere (Ghafar et al., 2020a).

The phylogenetic profile of the reported TBPs was also inferred by

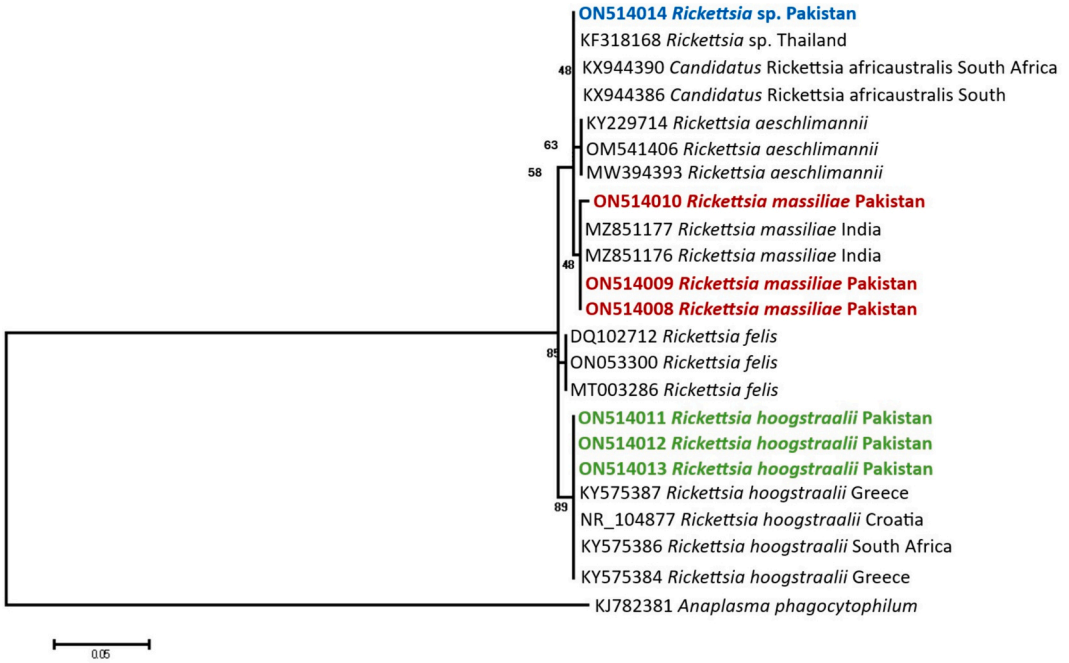

Fig. 7. The phylogenetic tree of 16S rRNA gene partial sequences of the genus (*Rickettsia*) in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Anaplasma phagocytophilum* 16S rRNA gene partial sequences were used as outgroups.

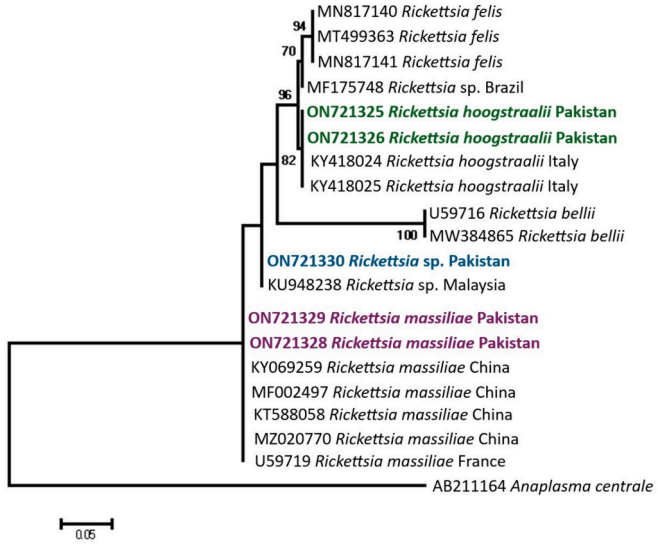

Fig. 8. The phylogenetic tree of citrate synthase (*gltA*) synth gene partial sequences of the genus (*Rickettsia*) in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with the Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Anaplasma centrale gltA* gene partial sequences were used as outgroups.

multi-locus analysis of 16S rRNA, *gltA*, and 18S rRNA phylogenetic markers. The molecular phylogenies of TBPs from the study area were successfully established. Our findings are consistent and support the results of the previous research reports from Pakistan and other countries including Iran, and Afghanistan (Ghafar et al., 2020a; Hakimi, Sarani, Takeda, Kaneko, and Asada, 2019; Duh et al., 2010). These studies have reported and characterized molecularly the same reported TBPs and inferred their phylogenetic profile using the same taxonomic markers.

5. Conclusion

The current study focused on detecting ticks and TBPs in goats and sheep northern Pakistan. The results revealed the presence of 11 ixodid tick species from three genera (*Hyalomma*, *Haemaphysalis*, and *Rhipicephalis*) using *16S rRNA* and *COI* genetic markers. Additionally, three genera of TBPs (*Anaplasma*, *Rickettsia*, and *Theileria*) with 11 TBP species were identified using *16S rRNA*, *gltA,* and *18S rRNA* markers, including the detection of zoonotic pathogens *A. capra*, *R. massiliae,* and *R. hoogstraalii*. This research documented preliminary data on ticks and TBPs in small ruminants in the study area. It will facilitate researchers in devising large-scale epidemiological investigations to map tick and TBP distribution and their associated risks of public and veterinary health concerns.

Authors contributions

JZ, OS conceived the study. JZ drafted the initial manuscript draft. JZ, BS, MAK, MUA, SH, and HS edited and revised the manuscript. JZ, MAK, and HS performed a genetic analysis. JZ and MAK developed the study area map. JZ, BS, and MAK performed statistical analysis. OS, AADS, AA, MA, RMA, and ACC critically reviewed and provided intellectual inputs. All authors read and approved the final manuscript.

Ethical statement

The current research was performed following the rules approved by the ethical committee on animal care (No. FCLS/AWKUM/227) at the College of Veterinary Science and Animal Husbandry, Abdul Wali Khan University Mardan, Pakistan.

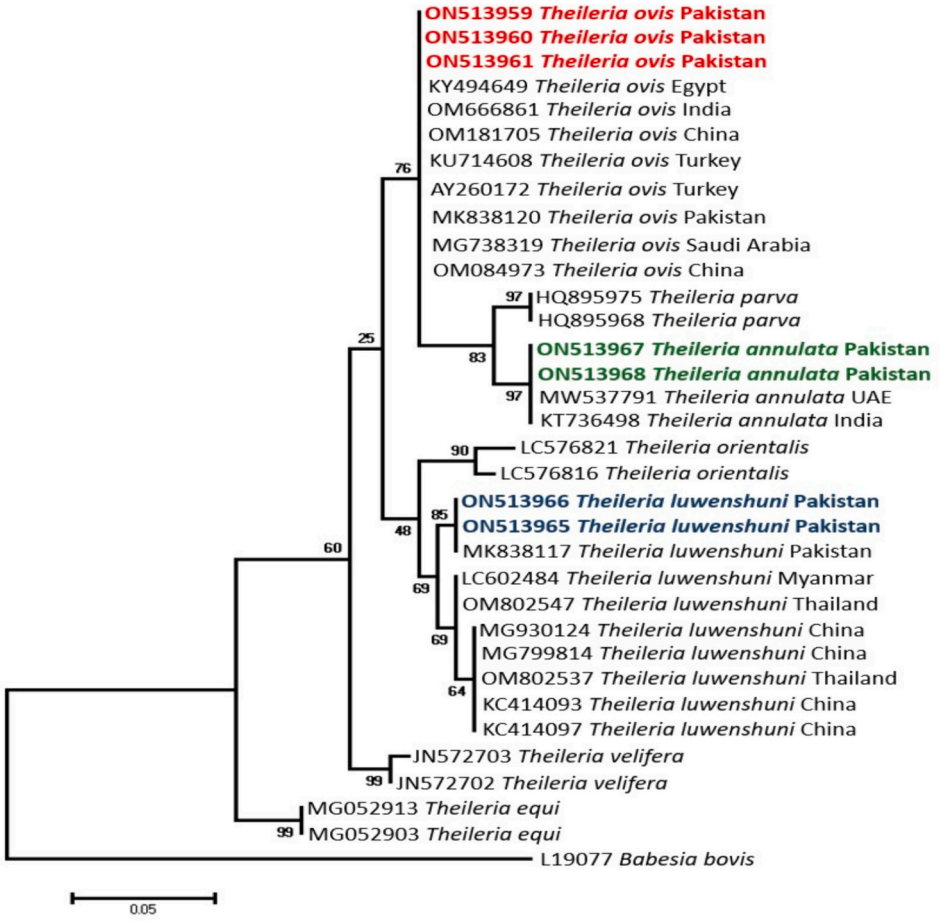

Fig. 9. The phylogenetic tree of 18S rRNA gene partial sequences of the genus (*Theileria*) in this study (highlighted with different colors) and representative sequences from the NCBI GenBank. The tree was constructed using a maximum likelihood algorithm with Kimura model method with 1000 bootstrap replications. Bootstrap values are indicated at each node and the numbers over 50 are shown in the tree. *Babesia bovis* 18S rRNA gene partial sequences were used as outgroups.

CRediT authorship contribution statement

Jehan Zeb: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Baolin Song:** Visualization, Software, Formal analysis, Data curation. **Munsif Ali Khan:** Writing – review & editing, Visualization, Validation, Software, Formal analysis. **Haytham Senbill:** Writing – review & editing, Visualization, Validation, Software, Formal analysis. **Muhammad Umair Aziz:** Visualization, Data curation. **Sabir Hussain:** Visualization, Validation. **Adrian Alberto Díaz Sánchez:** Writing – review & editing, Visualization. **Alejandro Cabezas-Cruz:** Visualization. **Abdulrahman Alzahrani:** Visualization, Funding acquisition, Formal analysis. **Mohammed Alshehri:** Visualization, Funding acquisition. **Rashed Mohammed Alghamdi:** Visualization, Funding acquisition. **Olivier Andre Sparagano:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

There is no competing interest among the authors.

Data availability statement

All the relevant data supporting the findings of this study have been comprehensively included in the manuscript, both in the main text and the supplementary materials. The dataset is fully transparent and accessible to readers for further analysis and reference. Any additional information will be provided upon request.

References

Ali, A., Khan, M.A., Zahid, H., Yaseen, P.M., Qayash Khan, M., Nawab, J., Ur Rehman, Z., Ateeq, M., Khan, S., Ibrahim, M., 2019. Seasonal dynamics, record of ticks infesting humans, wild and domestic animals, and molecular phylogeny of *Rhipicephalus microplus* in Khyber Pakhtunkhwa Pakistan. Front. Physiol. 10, 793.

Anderson, J.F., Magnarelli, L.A., 2008. Biology of ticks. Infect. Dis. Clin. N. Am. 22, 195–215.

Black, W.C., Piesman, J., 1994. Phylogeny of hard-and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. USA 91, 10034.

Casati, S., Sager, H., Gern, L., Piffaretti, J.-C., 2006. Presence of potentially pathogenic Babesia sp. for humans in Ixodes ricinus in Switzerland. Ann. Agric. Environ. Med. 13.

Chisu, V., Leulmi, H., Masala, G., Piredda, M., Foxi, C., Parola, P., 2017. Detection of *Rickettsia hoogstraalii*, *Rickettsia helvetica*, *Rickettsia massiliae*, *Rickettsia slovaca* and *Rickettsia aeschlimannii* in ticks from Sardinia, Italy. Ticks Tick Borne Dis 8, 347–352.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Braila, P., Song, H.Q., Zhu, X.Q., 2009. Molecular characterization of hard and soft ticks from Romania by sequences of the internal transcribed spacers of ribosomal DNA. Parasitol. Res. 105, 907–911.

Chochlakis, D., Ioannou, I., Sandalakis, V., Dimitriou, T., Kassinis, N., Papadopoulos, B., Tselentis, Y., Psaroulaki, A., 2012. Spotted fever group Rickettsiae in ticks in Cyprus. Microb. Ecol. 63, 314–323.

Cohen, E.B., Auckland, L.D., Marra, P.P., Hamer, S.A., 2015. Avian migrants facilitate invasions of neotropical ticks and tick-borne pathogens into the United States. Appl. Environ. Microbiol. 81, 8366–8378.

Das, H.L., Naithani, R.C., Subramanian, G., 1973. On the larva and nymph of *Hyalomma* (*Hyalomma*) *marginatum isaaci* (Sharif, 1928) (Acari: Ixodidae). Acarologia, XV I, 33–36.

De la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Peña, A., Johnson, N., Kocan, K.M., Mansfield, K.L., Nijhof, A.M., 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front. Cell. Infect. Microbiol. 7, 114.

Dieme, C., Bechah, Y., Socolovschi, C., Audoly, G., Berenger, J.-M., Faye, O., Raoult, D., Parola, P., 2015. Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes. Proc. Natl. Acad. Sci. 112, 8088–8093.
Diuk-Wasser, M.A., Vanacker, M.C., Fernandez, M.P., 2021. Impact of land use changes and habitat fragmentation on the eco-epidemiology of tick-borne diseases. J Med Entomol 58.
Duh, D., Punda-Polic, V., Avsic-Zupanc, T., Bouyer, D., Walker, D.H., Popov, V.L., Jelovsek, M., Gracner, M., Trilar, T., Bradaric, N., 2010. *Rickettsia hoogstraalii* sp. nov., isolated from hard-and soft-bodied ticks. Int. J. Syst. Evol. Microbiol. 60, 977–984.
Duron, O., Noël, V., McCoy, K.D., Bonazzi, M., Sidi-Boumedine, K., Morel, O., Vavre, F., Zenner, L., Jourdain, E., Durand, P., 2015. The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. PLoS Pathog. 11, e1004892.
Estrada-Peña, A., Bouattour, A., Camicas, J.L., Walker, A.R., 2004. Ticks of Domestic Animals in the Mediterranean Region. University of Zaragoza, Spain, p. 131.
Estrada-Peña, A., Bouattour, A., Camicas, J.-L., Guglielmone, A., Horak, I., Jongejan, F., Latif, A., Pegram, R., Walker, A.R., 2006. The known distribution and ecological preferences of the tick subgenus *Boophilus* (Acari: Ixodidae) in Africa and Latin America. Exp. Appl. Acarol. 38, 219–235.
Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3 (5), 294–299.
Geevarghese, G., Mishra, A.C., 2011. Haemaphysalis Ticks of India. https://doi.org/10.1016/C2010-0-68435-5.
Ghafar, A., Cabezas-Cruz, A., Galon, C., Obregon, D., Gasser, R.B., Moutailler, S., Jabbar, A., 2020a. Bovine ticks harbor a diverse array of microorganisms in Pakistan. Parasit. Vectors 13, 1–15.
Ghafar, A., Khan, A., Cabezas-Cruz, A., Gauci, C.G., Niaz, S., Ayaz, S., Mateos-Hernández, L., Galon, C., Nasreen, N., Moutailler, S., Gasser, R.B., Jabbar, A., 2020c. An assessment of the molecular diversity of ticks and tick-borne microorganisms of small ruminants in Pakistan. Microorganisms 8 (9), 1428.
Ghafar, A., Koehler, A.V., Hall, R.S., Gauci, C.G., Gasser, R.B., Jabbar, A., 2020d. Targeted next-generation sequencing and informatics as an effective tool to establish the composition of bovine piroplasm populations in endemic regions. Microorganisms 9, 21.
Gray, J.S., 1994. Biology of ticks. Volume 2. By Daniel E. Sonenshine.(New York: Oxford university press, 1993). Xvii+ 465 pp. hard cover£ 75.00. ISBN 0 19 508431 4. Bull. Entomol. Res. 84, 590–591.
Guo, H., Moumouni, P.F.A., Thekisoe, O., Gao, Y., Liu, M., Li, J., Galon, E.M., Efstratiou, A., Wang, G., Jirapattharasate, C., 2019. Genetic characterization of tick-borne pathogens in ticks infesting cattle and sheep from three south African provinces. Ticks Tick Borne Dis 10, 875–882.
Hakimi, H., Sarani, A., Takeda, M., Kaneko, O., Asada, M., 2019. Epidemiology, risk factors, and co-infection of vector-borne pathogens in goats from Sistan and Baluchestan province. Iran. PLoS One 14, e0218609.
Hasle, G., 2013. Transport of ixodid ticks and tick-borne pathogens by migratory birds. Front. Cell. Infect. Microbiol. 3, 48.
Hoogstraal, H., Trapido, H., Kohls, G.M., 1966. Studies on southeast Asian Haemaphysalis ticks (Ixodoidea, Ixodidae). Speciation in the H.(*Kaiseriana*) obesa group: *H. Semermis Neumann, H. Obesa Larrousse, H. Roubaudi Toumanoff, H. Montgomeryi Nuttall*, and *H. Hirsuta* sp. n. J. Parasitol. 169–191.
Hussain, S., Hussain, A., Aziz, M.U., Song, B., Zeb, J., Moutailler, S., Foucault-Simonin, A., Smith, R.L., Cabezas-Cruz, A., George, D., 2024. Exploring the coinfection and genetic diversity of multiple tick-borne pathogens in livestock population of Punjab, Pakistan. Transbound. Emerg. Dis. 2024 (1), 9958535.
Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129, S3–S14.
Juliano, S.A., Philip Lounibos, L., 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol. Lett. 8, 558–574.
Jyotika Kapur-Ghai, J.K.-G., Singh, M., Singh, A., 2008. Population dynamics of ixodid ticks infesting cattle in Bathinda and Hoshiarpur districts in the Punjab state. J. Vet. Parasitol. 22 (2), 79–84.
Karim, S., Singh, P., Ribeiro, J.M.C., 2011. A deep insight into the sialotranscriptome of the gulf coast tick. *Amblyomma maculatum*. PLoS One 6, e28525.
Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S., Dowd, S.E., Apanskevich, D., Arijo, A., 2017. A study of ticks and tick-borne livestock pathogens in Pakistan. PLoS Negl. Trop. Dis. 11, e0005681.
Kasi, K.K., Sas, M.A., Sauter-Louis, C., von Arnim, F., Gethmann, J.M., Schulz, A., Wernike, K., Groschup, M.H., Conraths, F.J., 2020. Epidemiological investigations of Crimean-Congo haemorrhagic fever virus infection in sheep and goats in Balochistan, Pakistan. Ticks Tick Borne Dis 11, 101324.
Keirans, J.E., Durden, L.A., 2001. Invasion: exotic ticks (Acari: Argasidae, Ixodidae) imported into the United States. A review and new records. J. Med. Entomol. 38, 850–861.
Khan, S., Javed, Z.H., Wahid, A., Ranjha, A.N., Hassan, M.U., 2020a. Climate of the Gilgit-Baltistan province, Pakistan. Int. J. Econ. Environ. Geol. 11, 16–21.
Khan, A., Niaz, S., Khan, Aisha, Ahmed, H., Khattak, I., Zeb, J., Naeem, H., Hassan, M.A., Ulucesme, M.C., Ozubek, S., 2020b. Molecular detection of small ruminant piroplasmosis and first report of *Theileria luwenshuni* (Apicomplexa: Theileridae) in small ruminants of Pakistan. Exp. Parasitol. 212, 107872.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547.
Li, H., Zheng, Y.-C., Ma, L., Jia, N., Jiang, B.-G., Jiang, R.-R., Huo, Q.-B., Wang, Y.-W., Liu, H.-B., Chu, Y.-L., 2015. Human infection with a novel tick-borne *Anaplasma* species in China: a surveillance study. Lancet Infect. Dis. 15, 663–670.
Lounibos, L.P., 2002. Invasions by insect vectors of human disease. Annu. Rev. Entomol. 47, 233–266.
Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., Wang, Q., 2014. Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasit. Vectors 7, 1–11.
Márquez, F.J., 2008. Spotted fever group *Rickettsia* in ticks from southeastern Spain natural parks. Exp. Appl. Acarol. 45, 185–194.
Martin, A.R., Brown, G.K., Dunstan, R.H., Roberts, T.K., 2005. *Anaplasma platys*: an improved PCR for its detection in dogs. Exp. Parasitol. 109, 176–180.
Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., Chirico, J., van der Wal, F.J., Sprong, H., Boye Pihl, T.P., 2014. High-throughput screening of tick-borne pathogens in Europe. Front. Cell. Infect. Microbiol. 4, 103.
Niaz, S., Rahman, Z.U., Ali, I., Cossío-Bayúgar, R., Amaro-Estrada, I., Alanazi, A.D., Khattak, I., Zeb, J., Nasreen, N., Khan, A., 2021. Molecular prevalence, characterization and associated risk factors of *Anaplasma* spp. and *Theileria* spp. in small ruminants in northern Pakistan. Parasite 28.
Nieto, N.C., Khan, K., Uhllah, G., Teglas, M.B., 2012. The emergence and maintenance of vector-borne diseases in the Khyber Pakhtunkhwa province, and the federally administered tribal areas of Pakistan. Front. Physiol. 3, 250.
Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., Jebbink, F., Jongejan, F., 2008. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector-borne and zoonotic diseases 7 (4), 585–596.
Nizhat Sultana, N.S., Asim Shamim, A.S., Awan, M.S., Usman Ali, U.A., Murtaz-ul-Hassan, M.-H., Siddique, R.M., 2015. First pilot study on the prevalence of tick infestation in livestock of tehsil Hajira, Rawalakot, Azad Kashmir. Adv. Anim. Vet. Sci. 3 (8), 430–434.
Omondi, D., Masiga, D.K., Fielding, B.C., Kariuki, E., Ajamma, Y.U., Mwamuye, M.M., Ouso, D.O., Villinger, J., 2017. Molecular detection of tick-borne pathogen diversities in ticks from livestock and reptiles along the shores and adjacent islands of Lake Victoria and Lake Baringo, Kenya. Front Vet Sci 4, 73.
Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Van Thien, H., Gonzalez, J.P., Raoult, D., Telford, S.R., Wongsrichanalai, C., 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. J. Clin. Microbiol. 41, 1600–1608.
Perry, B., Grace, D., 2009. The impacts of livestock diseases and their control on growth and development processes that are pro-poor. Philos. Trans. R. Soc. B 364, 2643–2655.
Rafiq, N., Kakar, A., Ghani, A., Iqbal, A., Achakzai, W.M., Sadozai, S., Shafiq, M., Mengal, M.A., 2017. Ixodid ticks (Arachnida: Acari) prevalence associated with risk factors in the bovine host in district Quetta, Balochistan. Pak. J. Zool. 49, 2113–2121.
Rehman, A., Nijhof, A.M., Sauter-Louis, C., Schauer, B., Staubach, C., Conraths, F.J., 2017. Distribution of ticks infesting ruminants and risk factors associated with high tick prevalence in livestock farms in the semi-arid and arid agro-ecological zones of Pakistan. Parasit. Vectors 10, 1–15.
Rehman, A., Conraths, F.J., Sauter-Louis, C., Krücken, J., Nijhof, A.M., 2019. Epidemiology of tick-borne pathogens in the semi-arid and the arid agro-ecological zones of Punjab province, Pakistan. Transbound. Emerg. Dis. 66, 526–536.
Roux, V., Rydkina, E., Eremeeva, M., Raoult, D., 1997. Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. Int. J. Syst. Bacteriol. 47, 252–261.
Salih, D.A., El Hussein, A.M., Singla, L.D., 2015. Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. J. Vet. Med. Animal Health 7, 45–56.
Singh, N.K., Rath, S.S., 2013. Epidemiology of ixodid ticks in cattle population of various agro-climatic zones of Punjab, India. Asian Pac J Trop Med 6, 947–951.
Sun, X.-F., Zhao, L., Wen, H.-L., Luo, L.-M., Yu, X.-J., 2015. *Anaplasma* species in China. Lancet Infect. Dis. 15, 1263–1264.
Tan, L.P., Hamdan, R.H., Hassan, B.N.H., Reduan, M.F.H., Okene, I.A.-A., Loong, S.K., Khoo, J.J., Samsuddin, A.S., Lee, S.H., 2021. *Rhipicephalus* tick: A contextual review for Southeast Asia. Pathogens 10, 821.
Walker, J.B., Keirans, J.E., Horak, I.G., 2000. The Genus *Rhipicephalus* (Acari, Ixodidae): A Guide to the Brown Ticks of the World. Cambridge University Press.
White, S.A., Bevins, S.N., Ruder, M.G., Shaw, D., Vigil, S.L., Randall, A., Deliberto, T.J., Dominguez, K., Thompson, A.T., Mertins, J.W., 2021. Surveys for ticks on wildlife hosts and in the environment at Asian longhorned tick (*Haemaphysalis longicornis*)-positive sites in Virginia and New Jersey, 2018. Transbound. Emerg. Dis. 68, 605–614.
Yu, Z., Wang, H., Wang, T., Sun, W., Yang, X., Liu, J., 2015. Tick-borne pathogens and the vector potential of ticks in China. Parasit. Vectors 8, 1–8.
Zeb, J., Szekeres, S., Takács, N., Kontschán, J., Shams, S., Ayaz, S., Hornok, S., 2019. Genetic diversity, piroplasms and trypanosomes in *Rhipicephalus microplus* and *Hyalomma anatolicum* collected from cattle in northern Pakistan. Exp. Appl. Acarol. 79, 233–243.
Zeb, J., Shams, S., Ayaz, S., Din, I.U., Khan, A., Adil, N., Ullah, H., Raza, A., 2020. Epidemiology of ticks and molecular characterization of *Rhipicephalus microplus* in cattle population in North-Western Pakistan. Int. J. Acarol. 46, 335–343.
Zeb, J., Song, B., Khan, M.A., Senbill, H., Aziz, M.U., Hussain, S., Waris, A., E-Tabor, A., Sparagano, O.A., 2023. Genetic diversity of vector-borne pathogens in ixodid ticks infesting dogs from Pakistan with notes on Ehrlichia canis, *Rickettsia raoultii* and *Dirofilaria immitis* detection. Parasit. Vectors 16 (1), 214.