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EDITED BY
Hong Yin,
Chinese Academy of Agricultural Sciences, China

REVIEWED BY
Valentina Virginia Ebani,
University of Pisa, Italy
Marina Eremeeva,
Georgia Southern University, United States

*CORRESPONDENCE
Abid Ali
✉ uop_ali@yahoo.com
Kun-Hsien Tsai
✉ kunhtsai@ntu.edu.tw

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First report of spotted fever group *Rickettsia aeschlimannii* in *Hyalomma turanicum*, *Haemaphysalis bispinosa*, and *Haemaphysalis montgomeryi* infesting domestic animals: updates on the epidemiology of tick-borne *Rickettsia aeschlimannii*

Abdul Majid[1], Mashal M. Almutairi[2], Abdulaziz Alouffi[3], Tetsuya Tanaka[4], Tsai-Ying Yen[5], Kun-Hsien Tsai[5]* and Abid Ali[1]*

[1]Department of Zoology, Abdul Wali Khan University Mardan, Mardan, Pakistan, [2]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, [3]King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia, [4]Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan, [5]Department of Public Health, Institute of Environmental and Occupational Health Sciences, College of Public Health, National Taiwan University, Taipei, Taiwan

Tick-borne *Rickettsia* spp. have long been known as causative agents for zoonotic diseases. We have previously characterized *Rickettsia* spp. in different ticks infesting a broad range of hosts in Pakistan; however, knowledge regarding *Rickettsia aeschlimannii* in *Haemaphysalis* and *Hyalomma* ticks is missing. This study aimed to obtain a better understanding about *R. aeschlimannii* in Pakistan and update the knowledge about its worldwide epidemiology. Among 369 examined domestic animals, 247 (66%) were infested by 872 ticks. Collected ticks were morphologically delineated into three genera, namely, *Rhipicephalus*, *Hyalomma,* and *Haemaphysalis*. Adult females were the most prevalent (number = 376, 43.1%), followed by nymphs (303, 34.74%) and males (193, 22.13%). Overall, genomic DNA samples of 223 tick were isolated and screened for *Rickettsia* spp. by the amplification of rickettsial *gltA*, *ompA*, and *ompB* partial genes using conventional PCR. Rickettsial DNA was detected in 8 of 223 (3.58%) ticks including nymphs (5 of 122, 4.0%) and adult females (3 of 86, 3.48%). The rickettsial *gltA*, *ompA*, and *ompB* sequences were detected in *Hyalomma turanicum* (2 nymphs and 1 adult female), *Haemaphysalis bispinosa* (1 nymph and 1 adult female), and *Haemaphysalis montgomeryi* (2 nymphs and 1 adult female). These rickettsial sequences showed 99.71–100% identity with *R. aeschlimannii* and phylogenetically clustered with the same species. None of the tested *Rhipicephalus microplus*, *Hyalomma isaaci*, *Hyalomma scupense*, *Rhipicephalus turanicus*, *Hyalomma anatolicum*, *Rhipicephalus haemaphysaloides*, *Rhipicephalus sanguineus, Haemaphysalis cornupunctata*, and *Haemaphysalis sulcata* ticks were found positive for rickettsial DNA. Comprehensive surveillance studies should be adopted to update the knowledge regarding tick-borne zoonotic *Rickettsia* species, evaluate their risks

to humans and livestock, and investigate the unexamined cases of illness after tick bite among livestock holders in the country.

KEYWORDS

ticks, *Rickettsia aeschlimannii*, *Hyalomma turanicum*, *Haemaphysalis bispinosa*, *Haemaphysalis montgomeryi*

Introduction

Ticks are important ectoparasites due to their capacity to feed on animals including humans (De la Fuente et al., 2017) and transmit various pathogens including viruses, bacteria, and protozoans to their hosts (Boulanger et al., 2019). Among bacteria, *Rickettsia* species are transmitted by ticks, mites, and fleas, causing rickettsioses (Fournier and Raoult, 2009; Labruna, 2009). The main tick genera that are the potential vectors for *Rickettsia* spp. are *Amblyomma*, *Dermacentor*, *Ixodes*, *Hyalomma*, *Rhipicephalus,* and *Haemaphysalis* (Fournier and Raoult, 2009). Several studies have revealed the extensive diversity of the spotted fever group rickettsiae in different tick species and geographic locations (Socolovschi et al., 2009). Approximately 34 species in the genus *Rickettsia* have been validated, and several others are yet to be determined to the species level (El Karkouri et al., 2022). Spotted fever group *Rickettsia* spp. are distributed in Pakistan and have recently been reported in different ticks including *Rh. microplus, Rh. haemaphysaloides, Rh. turanicus, H. kashmirensis,* and *H. cornupunctata* infesting different animals (Ali et al., 2021, 2022; Khan et al., 2023).

Hard ticks are important reservoirs for various *Rickettsia* species including *Rickettsia aeschlimannii* (Parola et al., 2013). Previously, *R. aeschlimannii* has been detected in different tick species belonging to different genera such as *Rhipicephalus, Hyalomma, Amblyomma*, *Haemaphysalis*, and *Ixodes* in Morocco, Spain, Senegal, and Bolivia (Beati et al., 1997; Fernández-Soto et al., 2003; Mediannikov et al., 2010; Tomassone et al., 2010). The first human infection by *R. aeschlimannii* was documented serologically in Morocco (Raoult et al., 2002). Till then, several cases of human infection with tick-borne *R. aeschlimannii* have been reported (Raoult et al., 2002; Znazen et al., 2006; Mokrani et al., 2008; Germanakis et al., 2013; Igolkina et al., 2022). Molecular identification and genetic characterization of *Rickettsia* spp. are based on the *gltA* (citrate synthase gene), *ompA* (outer membrane protein A), *ompB* (outer membrane protein B), and *sca*4 (surface cell antigen 4) genes (Roux et al., 1997; Fournier et al., 2003).

Earlier, we characterized pathogenic and undetermined *Rickettsia* spp. associated with different ticks parasitizing a wide range of vertebrate hosts in Pakistan (Karim et al., 2017; Ali et al., 2021, 2022, 2023; Numan et al., 2022; Aneela et al., 2023; Shehla et al., 2023; Ullah et al., 2023). However, there is a paucity of information regarding the presence of *R. aeschlimannii* in different ticks in the country, and its potential risks to the public and animal's health. Updated knowledge regarding the epidemiology, association with ticks infesting different hosts, and phylogenetic position of spotted fever group (SFG) *R. aeschlimannii* is essential for effective management of infection. This study aimed to molecularly characterize tick-borne *Rickettsia* spp. in selected districts of Pakistan and update the information about the global epidemiology of uncharacterized *Rickettsia* spp.

Materials and methods

Study area

Tick specimens were collected from seven districts of Khyber Pakhtunkhwa (KP), a province in northwest Pakistan, namely, Mohmand (34.5356°N, 71.2874°E), Bajaur (34.7865°N, 71.5249°E), Swabi (34.0719°N, 72.4732°E), Charsadda (34.1682°N, 71.7504°E), Mardan (34.1986°N, 72.0404°E), Dir-Upper (35.3356°N, 72.0468°E), and Dir-Lower (34.9161°N, 71.8097°E). Data regarding the host, ticks, climate, and location were noted. Climatic information was taken from the website climate-data.org. Geo-coordinates of location sites were obtained from the global positioning system and were entered into a spreadsheet, and the study map was designed in ArcGIS10.8.1.3 (ESRI, Redlands, CA, USA) (Figure 1).

Ethical statement

The design of the current study has received approval from the members of the Advanced Study and Research Board (Dir/A&R/AWKUM/2023/0014) and the Faculty of Zoology Department, Abdul Wali Khan University Mardan, Pakistan. Permission was obtained from the owner of the animal before collecting ticks from their animals.

Collection and morphological identification of ticks

Tick specimens were collected conveniently from March 2020 to February 2021 from domestic animals in seven districts of Khyber Pakhtunkhwa, Pakistan. The collection was opportunistic occurring whenever tick-infested animals were found within the survey regions. The whole body of the animal was examined for ticks. With the help of curved forceps, ticks were collected carefully so that the morphological features of ticks were not damaged. Every sample was tagged with its location of collection, date, and species of the animal.

The collected tick specimens were morphologically identified through a stereo microscope (CM100, China) by using standard taxonomic keys (Hoogstraal, 1962, 1966, 1973; Walker et al., 2000; Apanaskevich et al., 2008; 2010; Geevarghese and Mishra, 2011; Ali et al., 2022).

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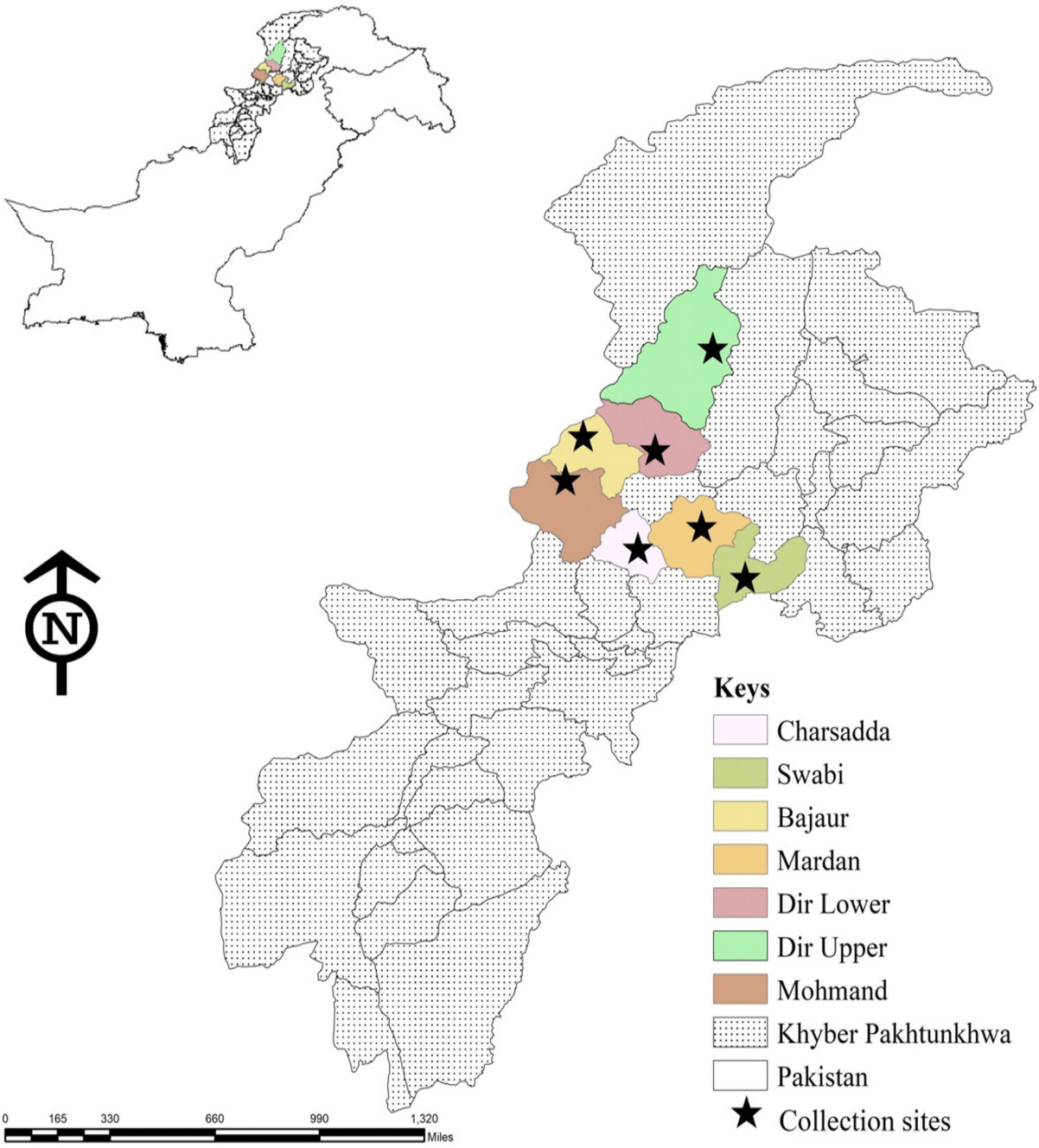

FIGURE 1
Map showing locations in seven districts of Khyber Pakhtunkhwa, where ticks were collected from domestic animals.

Genomic DNA extraction

All ticks were identified morphologically, and genomic DNA was individually extracted from a subset of 223 (122N, 86F, and 15M) ticks. Ticks were cleaned with 70% ethanol, followed by distilled water and phosphate-buffered saline for the elimination of surface contaminants. Each rinsed tick was separately kept in a 1.5 mL tube and subjected to drying within an incubator for 30 min. Using a sterilized scalpel, the samples were cut into pieces inside the Eppendorf tube. The phenol-chloroform method was used for the extraction of genomic DNA from the ticks (Sambrook et al., 1989). The DNA concentration in each extracted sample was quantified with Nanodrop (OPTIZEN, Daejeon, South Korea).

Amplification of targeted rickettsial DNA

The extracted DNA was used in conventional PCR (Thermo Fisher Scientific, and Walham, MA, USA), to amplify fragments of three genes of *Rickettsia,* namely, *gltA* (citrate synthase gene), *ompA* (outer membrane protein A), and *ompB* (outer membrane protein B). PCR was conducted in a 25 μL reaction mixture, containing 1 μL of

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each primer (forward and reverse primers), 8.5 μL of PCR water, 2 μL of template DNA, and 12.5 μL of DreamTaq PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA). The primers used in the current study are presented in Table 1, and thermocycling conditions were set as previously used (Regnery et al., 1991; Roux and Raoult 2000; Labruna et al., 2004). Positive and negative control samples were *Rickettsia massiliae* DNA and "nuclease-free" water, respectively (Shehla et al., 2023). The amplified products of each PCR were observed by electrophoresis on 2% agarose gel and stained with ethidium-bromide, and the results were visualized on the Gel Doc system (BioDoc-It™ Imaging Systems, Upland, CA, USA).

Sequencing and phylogenetic analysis

The amplified DNA fragments were purified using the GENECLEAN II Kit (Qbiogene, Illkirch, France) and sequenced using the Sanger sequencing (Macrogen, Inc., Seoul, South Korea) in both forward and reverse directions. Poor-quality sequences were removed by trimming all the obtained sequences using SeqMan v 5.00 (DNASTAR, Inc.), and a consensus sequence was generated. Sequences with the highest identity were selected from GenBank using the Basic Local Alignment Search Tool (BLAST) on the user interface of National Center for Biotechnology Information, and these sequences were aligned with the obtained sequences by BioEdit v. 7.0.5 using CLUSTALW multiple alignments (Thompson et al., 1994), followed by MUSCLE alignment (Edgar, 2004). The Neighbor-Joining method was applied for obtaining phylogenies with 1,000 bootstrap replicates in Molecular Evolutionary Genetic Analysis (MEGA-X) software (Kumar et al., 2018). The sequences that were obtained made up the final positions in the dataset.

Data analysis

The recorded data of tick-infested hosts and tick distribution were described with frequency and percentage using descriptive statistics. Fisher's exact test was used to determine the association between host, tick species, rickettsial species, and locations in GraphPad Prism software (V 5.0). p-value <0.05 was considered as significant standard.

Literature-based search

The literature-based search was carried out by using different databases including ScienceDirect, PubMed, Web of Sciences, and Google Scholar to collect published data regarding *Rickettsia aeschlimannii* in different tick species, wild and domestic animals, humans, environment, and vegetation. The search was conducted by using some keywords, such as ticks, tick-borne pathogens, domestic animals, small ruminants, zoonosis, livestock, and *Rickettsia aeschlimannii*. Complete research articles, short communication, review papers, and conference articles were downloaded by using a combination of the above mentioned keywords. Lists of references from downloaded studies were examined to relevant articles (Table 2).

Results

Identified ticks

A total of 369 domestic animals were examined in 7 districts including Mohmand (23 cattle, 14 goats, and 12 sheep), Dir Upper (20 cattle, 15 goats, 12 sheep, and 7 dogs), Dir lower (26 cattle, 17 goats, 13 sheep, and 5 dogs), Bajaur (19 cattle, 16 goats, 13 sheep, and 11 dogs), Charsadda (17 cattle, 15 goats, 13 sheep, and 5 dogs), Mardan (16 cattle, 13 goats, 20 sheep, and 5 dogs), and Swabi (17 cattle, 13 goats, 10 sheep, and 4 dogs) ($p = 0.248$). Of the examined hosts, 66% (247 of 369) of hosts were parasitized by 872 ticks of various life stages (Table 3), having a mean intensity of 3.5 ticks/infested host while the mean abundance was 2.3 ticks/examined host. The highest tick infestation was found on goats (74 of 103, 71.8%) compared with cattle (99 of 138, 71.7%), sheep (62 of 91, 60%), and dogs (12 of 37, 32.4%) ($p < 0.0001$). All collected ticks belonged to three different genera of ixodid ticks, namely, *Rhipicephalus, Hyalomma,* and *Haemaphysalis.* Adult female including engorged ticks were the most prevalent (376, 43.1%), followed by nymphs (303, 34.74%) and males (193, 22.13%) (Table 3) ($p < 0.0001$). The highest tick burden was observed on domestic animals in the Bajaur district (19.3%), followed by Mardan (15%), Swabi (14.9%), Charsadda (13.9%), Mohmand (13.3%), Dir Upper (12.7%), and Dir Lower (10.6%) ($p < 0.0001$). The most dominant species was *Rhipicephalus microplus* (19.0%), followed by *Hyalomma anatolicum* (15.7%), *Rhipicephalus turanicus* (11.5%), *Haemaphysalis sulcata* (10.6%), *Haemaphysalis bispinosa* (9.8%), *Haemaphysalis montgomeryi* (9.1%), *Rhipicephalus sanguineus* (8.8%), *Hyalomma scupense* (4.9%), *Rhipicephalus haemaphysaloides* (3.8%), *Haemaphysalis cornupunctata* (2.7%) *Hyalomma isaaci* (1.9%), and *Hyalomma turanicum* (1.60%) ($p < 0.0001$).

TABLE 1 Primers used for the amplification of rickettsial DNA in the current study.

Gene	Primer	Sequence(5′-3′)	Amplicon size	Annealing Temperatures	Reference
gltA	CS-78	GCAAGTATCGGTGTGAGGATGTAAT	401 bp	56°C	Labruna et al. (2004)
	CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT			
ompA	Rr190.70p	ATGGCGAATATTTCTCCAAAA	532 bp	55°C	Regnery et al. (1991)
	Rr190.602n	AGTGCAGCATTCGCTCCCCCT			
ompB	120-M59	CCGCAGGGTTGGTAACTGC	862 bp	50°C	Roux and Raoult (2000)
	120–807	CCTTTTAGATTACCGCCTAA			

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TABLE 2 Global epidemiology of *Rickettsia aeschlimannii* detected in different ticks and vertebrate hosts including human.

Continents	Country	Tick specie/Source	Host	Reference
Africa	Algeria	*Hy. scupense*	Sheep	Bitam et al. (2006)
		Hy. marginatum marginatum	Cattle, sheep	Bitam et al. (2006)
		Hy. aegyptium	Tortoises	Bitam et al. (2009)
		Hy. marginatum rufipes	Camels	Djerbouh et al. (2012)
		Hy. scupense, *Hy. anatolicum*	Cattle	Leulmi et al. (2016)
		Hy. marginatum, *Hy. scupense*, *Hy. impeltatum*	Cattle, goats, sheep, and equids	Sadeddine et al. (2020)
	Angola	*Hy. turncatum*	Cattle	Palomar et al. (2022)
	Benin	*A. variegatum*, *Rh. microplus*, *Hy. rufipes*	Cattle	Yessinou et al. (2023)
	Cameroon	*Hy. rufipes*, *Hy. truncatum*	Cattle	Vanegas et al. (2018)
		Hy. rufipes, *Hy. turncatum*	Nile Monitor, Hedgehog	Paguem et al. (2023)
	Chad	*Hy. marginatum rufipes*	Camels	Mura et al. (2008b)
	Egypt	*Hy. dromedarii*	Camels	Loftis et al. (2006)
		Hy. marginatum rufipes	Cows	
		Hy. impeltatum	Cows	
		Hy. anatolicum excavatum, *Hy. impeltatum*, *Hy. dromedarii*, *Hy. marginatum marginatum*	Ruminants, camels, sheep, and cattle	Allam et al. (2018)
	Ethiopia	*Hy. marginatum rufipes*	Cattle	Mura et al. (2008a)
	Ghana	*A. variegatum*, *Hy. rufipes*, *Rh. evertsi*, *Rhipicephalus* sp.	Cattle, goats, and sheep	Addo et al. (2023)
	Kenya	*Hy. truncatum*, *Hy. marginatum*, *Rh. pulchellus*, *A. variegatum*, *Hy. truncatum*, *Hyalomma* spp.	Camel, cattle, and goat	Koka et al. (2017)
		Hy. truncatum, *Hy. marginatum rufipes*, *Rh. pulchellus*	Sheep and goats	Omondi et al. (2017)
		Hy. truncatum	Domestic animals	Mutai et al. (2013)
		Rh. sanguineus, *Hy. truncatum*, *Hy. marginatum rufipes*	Domestic animals	Diarra et al. (2017)
		Rh. pulchellus, *Hy. dromedarii*, *Hy. rufipes*, *Hy. truncatum*, *Hy. impeltatum*	Camels and sheep	Getange et al. (2021)
		A. gemma	Cattle	Godani et al. (2022)
	Mali	*Hy. marginatum rufipes*	Cattle	Parola et al. (2001)
	Morocco	Unknown	Human	Raoult et al. (2002)
		Hy. marginatum	Cattle	Beati et al. (1997)
	Niger	*Hy. marginatum rufipes*	Cattle	Parola et al. (2001)
	Nigeria	*Hy. impeltatum*, *Hy. rufipes*	Camels	Kamani et al. (2015)
		Hy. dromedarii, *Hy. truncatum*, *Hy. rufipes*, *Hy. impeltatum*	Camels	Onyiche et al. (2020)
	Senegal	*Hy. marginatum rufipes*, *Hy. truncatum*, *Rh. evertsi evertsi*	Cows, donkeys, sheep, goats, and horses	Mediannikov et al. (2010)
		Hy. m. rufipes, *Rh. evertsi*, *Hy. impeltatum*	Domestic animals	Sambou et al. (2014)
	South Africa	*Rh. appendiculatus*	Human	Pretorius and Birtles (2002)
		Hy. marginatum	Domestic animals	Sarih et al. (2008)
		Rh. appendiculatus	Cattle	Pretorius and Birtles (2002)
		Hy. rufipes, *Rh. appendiculatus*, *Rh. evertsi*	Donkeys	Halajian et al. (2018)
	Sudan	*Hy. dromedarii*, *Hy. truncatum*	Camels	Morita et al. (2004)
		Rh. evertsi, *Hy. rufipes*	Domestic animals	Springer et al. (2020)
		Hy. rufipes, *Hy. dromedarii*	Camels	Shuaib et al. (2020)
	Tunisia	*Hy. dromedarii*	Camel	Demoncheaux et al. (2012)
		Unknown	Human	Znazen et al. (2006)
		Rh. sanguineus	Dogs	Khrouf et al. (2014)
		Hy. impeltatum, *Hy. dromedarii*	Camels	Selmi et al. (2020)
		Hy. marginatum, *Rh. sanguineus*	Cattle	Kratou et al. (2023)

(Continued)

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TABLE 2 (Continued)

Continents	Country	Tick specie/Source	Host	Reference
	Zambia	*Hy. truncatum*	Dogs and cattle	Qiu et al. (2022)
		Hyalomma spp.	Cattle	Chitanga et al. (2021)
Asia				
		H. punctata	Cattle, sheep	Song et al. (2018)
		Rh. turanicus	Sheep	Wei et al. (2015)
		Hy. marginatum	Yaks	Jiao et al. (2022)
	Iran	*Rh. sanguineus, Hy. rufipes,*	Unknown	Saman and Chegeni (2022)
		Hy. marginatum, Rh. sanguineus	Dogs, sheep	Ghasemi et al. (2022)
	Israel	*Hy. turanicum, Hy. dromedarii, Hy. excavatum*	Camel	Kleinerman et al. (2013)
		Rh. sanguineus	Dogs	Mumcuoglu et al. (2022)
		Hy. marginatum	Horse	Azagi et al. (2017)
	Kazakhstan	*H. punctata*	Vegetation	Shpynov et al. (2004)
	Lebanon	*Rh. annulatus, Hy. anatolicum, Rh. sanguineus*	Farms	De Mera et al. (2018)
Europe	Austria	*Hy. marginatum*	Migratory birds	Duscher et al. (2018)
	Bulgaria	*Hy. anatolicum, Hy. marginatum, Hy. excavatum, Rhipicephalus* spp.	Dogs and cattle	Nader et al. (2018)
	Croatia	*Hy. marginatum*	Cattle	Punda-Polic et al. (2003)
	England	*Hy. marginatum*	Human	McGinley et al. (2021)
	France	*Hy. marginatum*	Sheep, humans, cattle, vegetation, wild boar, cattle, sheep, humans (on clothes), and vegetation	Matsumoto et al. (2004)
		Hyalomma sp.	Migratory birds	Socolovschi et al. (2011)
		Hy. marginatum rufipes	Migratory birds	Matsumoto et al. (2004)
		Hy. marginatum, Rh. sanguineus, Hy. scupense, Rh. bursa	Domestic and wild animals	Grech-Angelini et al. (2020)
	Germany	*Hy. marginatum*	Migratory birds	Rumer et al. (2011)
		Hy. marginatum, Hy. rufipes	Sheep, horse, and cat	Chitimia-Dobler et al. (2019)
	Georgia	*H. sulcata, Hy. scupense, Hy. marginatum, H. punctata,*	Cows and dogs	Sukhiashvili et al. (2020)
	Greece	*Hy. aegyptium*	Wild birds	Diakou et al. (2016)
		Hy. anatolicum	Sheep	Psaroulaki et al. (2006)
	Greece and Italy	*Hy. marginatum, Hy. rufipes, I. frontalis, Haemaphysalis* sp.	Migratory birds	Wallmenius et al. (2014)
	Serbia	*I. ricinus*	Humans	Banović et al. (2022)
	Italy	*Hy. lusitanicum*	Humans	Otranto et al. (2014)
		Hy. lusitanicum, Hy. marginatum, D. marginatus, I. ricinus	Humans	Blanda et al. (2017)
		I. ricinus, Rh. turanicus	Insugherata Natural Reserve	Mancini et al. (2015)
		Hy. marginatum, Hy. lusitanicum	Humans, domestic mammals, and wildlife	Chisu et al. (2018)
		Hy. marginatum	Herbivores (donkeys, cattle, and sheep)	Beninati et al. (2005)
		D. marginatus	Dogs	Sgroi et al. (2022)
		Hy. marginatum	Vegetation	Tomassone et al. (2013)
		Hy. marginatum, Hy. truncatum, Hy. rufipes, Hyalomma sp.	Great reed warbler, Reed warbler, Icterine warbler, Western yellow wagtail, Whinchat, Garden warbler, European pied flycatcher, Sedge warbler, Spotted flycatcher, Common whitethroat, Common redstart, Common nightingale, Collared flycatcher, Subalpine warbler, and Northern wheatear	Pascucci et al. (2019a,b)
		Hy. rufipes, A. marmoreum, Hyalomma sp.	Black redstart, Pied flycatcher, Redstart, Tree pipit, Whinchat, Whitethroat, Collared flycatcher, European robin, Northern weathear, Redstart, Song thrush, Whinchat, Whitethroat, and Wood warbler	Battisti et al. (2020)

(Continued)

TABLE 2 (Continued)

Continents	Country	Tick specie/Source	Host	Reference
		Hy. rufipes	Whitethroat tree pipit, Northern, Wheatear, and Whinchat	Rollins et al. (2021)
		Human	Human	Tosoni et al. (2016)
		Hy. marginatum	Wild boar	Maioli et al. (2009)
		Hy. m. marginatum, Hyalomma spp., *Amblyomma* sp.	Migratory birds	Toma et al. (2014)
		Hy. truncatum, Hy. rufipes	Cattle	Tomassone et al. (2016)
		Hy. marginatum rufipes	Cattle	Ehounoud et al. (2016)
		Hy. marginatum	Free living	Scarpulla et al. (2016)
		Hy. m. marginatum	Mouflon	Chisu et al. (2018)
		Hy. marginatum	*C. elaphus*	Pascucci et al. (2019a,b)
	Poland	*D. reticulatus*	Human	Koczwarska et al. (2023)
	Portugal	*Hy. marginatum*	Wild birds	Santos-Silva et al. (2006)
	Russia (western Russia)	*Hy. marginatum*	Cattle and vegetation	Shpynov et al. (2009)
	Romania	*Hy. marginatum*	Cattle	Andersson et al. (2018)
		H. concinna	Hedgehogs	Borşan et al. (2021)
	Spain	*H. inermis*	Vegetation	Portillo et al. (2008)
		Hy. marginatum	Humans	Fernández-Soto et al. (2009)
		Hy. marginatum, H. punctata, I. ricinus, Rh. bursa, Rh. turanicus, Rh. sanguineus	Humans	Fernández-Soto et al. (2003)
		Hy. marginatum	Humans and cows	Oteo et al. (2005)
	Spain and Morocco	*Hy. marginatum*	Humans and birds	Palomar et al. (2016)
	Sweden	*Hy. rufipes, Hy. marginatum*	Humans, Horse	Grandi et al. (2020)
	Turkey	*Hy. marginatum*	Humans	Keskin et al. (2016)
		Hy. marginatum	Humans	Keskin and Bursali, 2016
		Rh. bursa, Hy. marginatum, Hyalomma sp.	Humans	Gargili et al. (2012)
		Hy. marginatum	Humans	Bursalı et al. (2017)
		Hy. marginatum, Hy. aegyptium, Rh. turanicus, H. parva, H. punctata, H. sulcata, D. marginatus, I. ricinus, Hyalomma spp.	Humans	Karasartova et al. (2018)
		Hy. scupense, Hy. aegyptium, Hy. marginatum, Hy. excavatum	Humans	Orkun et al. (2014)
		Rh. turanicus, Hy. marginatum	Wild boar	Orkun and Çakmak (2019)
		Hy. aegyptium	Hedgehogs	Orkun and Çakmak (2019)
		Hy. marginatum, Rh. bursa	Cattle, goat	Demir et al. (2020)
		Hy. aegyptium	*Testudo graeca*	Akveran et al. (2020)
	United Kingdom	*Hy. rufipes*	Horse	Hansford et al. (2019)
South America	Bolivia	*A. tigrinum*	Dogs	Tomassone et al. (2010)
North America	Panama	*A. dissimile*	*Boa constrictor, Dasypus novemcinctus*	Bermudez et al. (2021)

Detection of *Rickettsia* spp. in ticks

DNA of a subset of 223 ticks was used for the detection of *Rickettsia* spp. by the amplification of three rickettsial markers, namely, *gltA*, *ompA*, and *ompB* gene fragments. Positive ticks for rickettsial *gltA* were also positive when tested with the primers of *ompA* and *ompB*. In total, 8 out of 223 (3.58%) ticks including 5 of 28 (17.85%) from Mohmand, 2 of 30 (6.6%) from Dir Lower, and 1 of 27 (3.7%) from Bajaur were found positive for *Rickettsia* spp. (*P*, 0.1621). Rickettsial DNA was found in three tick species, namely, *H. montgomeryi*, *H. bispinosa*, and *H. turanicum,* which was collected from sheep and goats. The overall prevalence of *Rickettsia* spp. was 3.58% (8 of 223). No rickettsial DNA was detected in *Rh. microplus* (27), *Hy. isaaci* (4), *Hy. scupense* (11), *Rh. turanicus* (33), *Hy. anatolicum* (33), *Rh. haemaphysaloides* (12), *Rh. sanguineus* (23), *H. cornupunctata* (7), and *H. sulcata* (23). Information regarding the prevalence of *Rickettsia* spp. in various ticks is shown in Table 3.

TABLE 3 Occurrence of ticks infesting various domestic animals and detection of rickettsial DNA associated with ticks.

District/Areas	Host	No. of examined host	No. of infested host	Collected Tick species	No. of Nymphs/ Female/male	Total No. of ticks	Molecularly screened Ticks*	*Rickettsia* spp. detected via *gltA, ompA, ompB*
Mohmand/ Rural	Cattle	23	15	*Hy. anatolicum*	8 N/3F/2F*/2M	15	3N,1F	0
				Rh. microplus	8 N/7F/3F*/7M	25	3N,1F	0
	Goats	14	9	*Hy. turanicum*	5N/5F/1F*/3M	14	2N,2F	2N,1F
				H. sulcata	6N/7F/3M	16	2N,1F,1M	0
	Sheep	12	8	*Hy. isaaci*	6N/7F/4M	17	2N,2F	0
				H. bispinosa	6N/7F/3M	16	2N,1F,1M	1N,1F
				H. montgomeryi	3N/8F/2M	13	2N,2F	0
Total		49	32 (8.6%)		42N/44F/6F*/24M	116 (13.3%)	16N,10F,2M	3N,2F
Dir Upper/ Rural	Cattle	20	14	*Rh. haemaphysaloides*	2N/6F/2M	10	2N,2F	0
				Rh. microplus	9N/7F/3F*/8M	27	2N,2F	0
	Goats	15	9	*Rh. turanicus*	4N/5F/2M	11	2N,2F	0
				H. montgomeryi	2N/8F/2M	12	2N,2F	0
	Sheep	10	8	*H. sulcata*	5N/6F/2M	13	2N,2F	0
				Hy. scupense	2N/6F/1M	9	2N,2F	0
				Hy. anatolicum	5N/3F/3F*/4M	15	2N,2F	0
	Dogs	7	3	*Rh. sanguineus*	5N/6F/3M	14	2N,2F	0
Total		52	34 (9.2%)		34N/47F/6F*/24M	111 (12.7%)	16N,16F	0
Dir lower/Rural	Cattle	26	18	*Rh. microplus*	10N/6F/4F*/9M	29	2N,2F,1M	0
	Goats	17	11	*H. bispinosa*	2N/8F/2M	12	3N,2F	0
				H. montgomeryi	2N/10F/3M	15	2N,2F,1M	IN,1F
	Sheep	13	7	*H. sulcata*	2N/8F/3M	13	3N,2F	0
	Dogs	5	2	*Rh. turanicus*	4N/5F/3M	12	3N,2F	0
				Rh. sanguineus	5N/3F/2F*/2M	12	3N,2F	0
Total		61	38 (10.2%)		25N/40F/6F*/22M	93 (10.6%)	16N,12F,2M	1N,1F
Bajaur/Rural	Cattle	19	17	*Rh. microplus*	10N/8F/2F*/8M	28	2N,1F	0
				Hy. anatolicum	6N/5F/3F*/7M	21	2N,1F	0
	Goats	16	14	*H. sulcata*	7N/8F/4M	19	2N,1F	0
				H. montgomeryi	7N/8F/3M	18	2N,1F	0
				Rh. turanicus	5N/4F/2M	11	2N,1F	1N
	Sheep	13	10	*Rh. turanicus*	8N/4F/2F*/3M	17	1N,1F,1M	0
				H. sulcata	7N/8F/3M	18	1N,1F,1M	0
				H. bispinosa	8N/9F/5M	22	2N,1F	0
	Dogs	11	2	*Rh. sanguineus*	5N/5F/2F*/3M	15	1N,1F,1M	0
Total		59	43 (11.65%)		63N/59F/9F*/38M	169 (19.3%)	15N,9F,3M	1N

(Continued)

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TABLE 3 (Continued)

District/Areas	Host	No. of examined host	No. of infested host	Collected Tick species	No. of Nymphs/ Female/male	Total No. of ticks	Molecularly screened Ticks*	*Rickettsia* spp. detected via *gltA, ompA, ompB*
Charsadda/Urban	Cattle	17	9	*Rh. microplus*	8N/4F/4F*/7M	23	2N,2F	0
				Rh. haemaphysaloides	6N/4F/2M	12	3N,1F	0
	Goats	15	12	*Rh. haemaphysaloides*	3N/8F/1M	12	3N,1F	0
				Rh. turanicus	3N/4F/2M	9	2N,2F	0
				Hy. anatolicum	6N/4F/2F*/4M	16	2N,1F,1M	0
	Sheep	13	8	*H. cornupunctata*	2N/5F/1M	8	2N,2F	0
				H. bispinosa	2N/4F/1M	7	2N,2F	0
				Hy. anatolicum	5N/3F/3F*/3M	14	2N,2F	0
	Dogs	5	2	*Rh. turanicus*	4N/5F/2M	11	2N,2F	0
				Rh. sanguineus	4N/4F/2M	10	3N,1F	0
Total		50	31 (8.40%)		43N/45F/9F*/25M	122 (13.9%)	23N,16F,1M	0
Mardan/Urban	Cattle	16	13	*Rh. microplus*	6N/4F/2F*/4M	16	2N,1F	0
				Hy. anatolicum	5N/3F/2F*/4M	14	2N,1F	0
	Goats	13	9	*Rh. turanicus*	4N/3F/2M	10	2N,1F	0
				Hy. anatolicum	4N/5F/2F*/2M	11	2N,1F	0
	Sheep	20	14	*H. cornupunctata*	6N/6F/4M	16	2N,1F	0
				Hy. scupense	5N/8F/3M	18	2N,1F	0
				H. bispinosa	5N/5F/2F*/3M	15	1N,1F,1M	0
				H. montgomeryi	2N/4F/2M	6	1N,1F,1M	0
	Dogs	5	2	*Rh. sanguineus*	5N/6F/3M	14	2N,1F	0
				Rh. turanicus	4N/4F/3M	11	1N,1F,1M	0
Total		54	38 (10.2%)		47N/46F/8F*/30M	131 (15.0%)	17N,10F,3M	0
Swabi/Urban	Cattle	17	13	*Rh. microplus*	6N/5F/3F*/4M	18	3N,1F	0
				Hy. anatolicum	5N/4F/2F*/3M	14	2N,1F,1M	0
				Rh. turanicus	4N/3F/2M	9	2N,1F,1M	0
	Goats	13	10	*H. bispinosa*	5N/5F/4M	14	2N,1F,1M	0
				H. montgomeryi	6N/6F/4M	16	2N,2F	0
	Sheep	10	7	*Hy. scupense*	6N/4F/2F*/4M	16	2N,1F,1M	0
				Hy. anatolicum	7N/4F/2F*/4M	17	2N,2F	0
				H. sulcata	5N/6F/3M	14	2N,2F	0
	Dogs	4	1	*Rh. sanguineus*	5N/5F/2M	12	2N,2F	0
Total		44	31 (8.40%)		49N/42F/9F*/30M	130 (14.90)	19N,13F,4M	0
Overall total		369	(66.65)		303N/323F/53F*/193M	872	122N,86F,15M	5N,3F

N, nymphs; F, Adult females; F*, Engorged females; M, males. *Ticks subjected to PCR to screen for *Rickettsia* spp.

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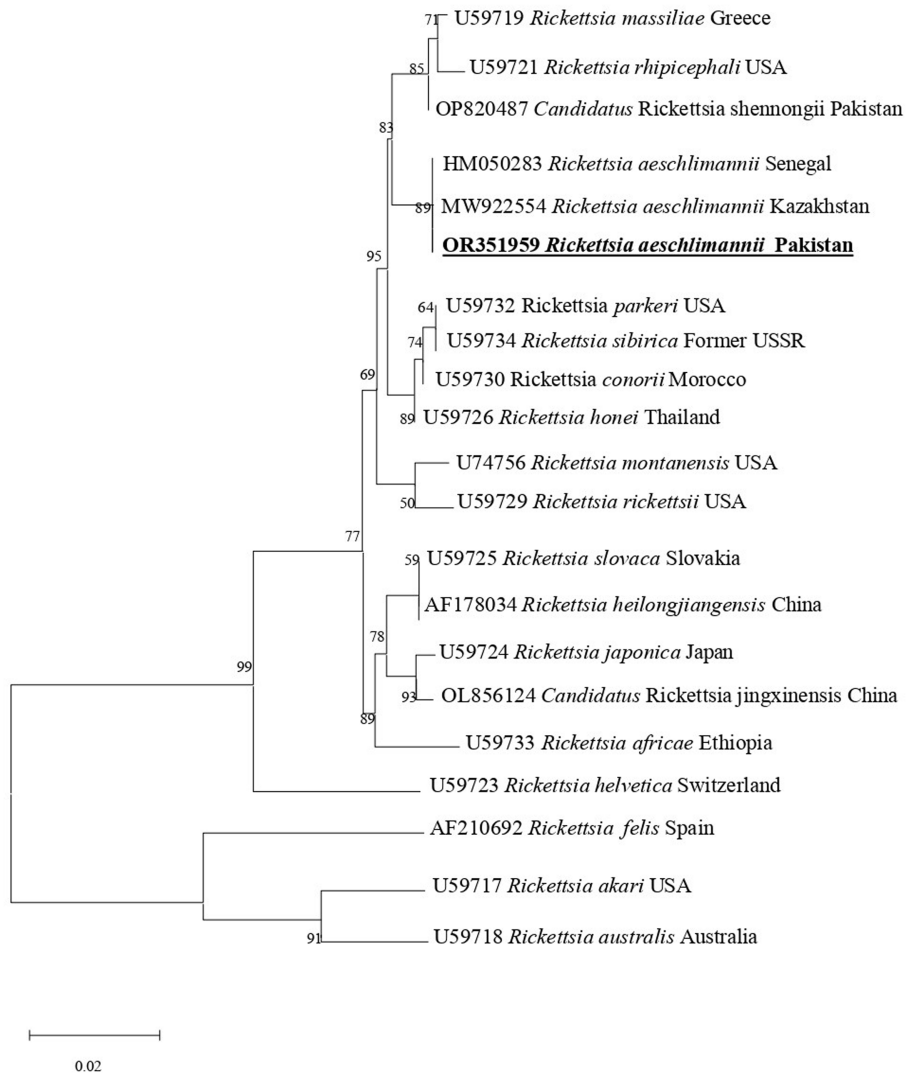

FIGURE 2
Phylogenetic analysis based on the *gltA* sequences of *Rickettsia aeschlimannii*. The obtained sequences of the present study are indicated in bold and underlined fonts. *Rickettsia akari* and *Rickettsia australis* were used as out-group.

Sequence and phylogenetic analysis

Assembled contigs of direct and reverse sequence reads for each PCR-amplified fragment were analyzed. Due to a single haplotype, consensus sequences were generated for each partial gene. The consensus sequence of *gltA* (348 bp) showed 100% identity with *R. aeschlimannii* from Russia, Senegal, and Kazakhstan, followed by 99.71% identity with *R. aeschlimannii*-type strain from Morocco. Similarly, 100% identity was shown by the obtained *ompA* (467 bp) consensus sequence with *R. aeschlimannii* from Russia, Spain, Turkey, and Kazakhstan, followed by 99.79% identity with *R. aeschlimannii*-type strain. Similarly, the *ompB* (764 bp) consensus sequence also showed 100% identity with *R. aeschlimannii* from Kazakhstan, Russia, Italy, and Portugal, followed by 99.74% identity with *R. aeschlimannii*-type strain. In all cases, the query coverage was 100%. The obtained rickettsial *gltA* sequence (accession number: OR351959), *ompA* sequence (accession number: OR351960), and *ompB* sequence (accession number: OR351961) were submitted to GenBank.

In phylogenetic tree based on rickettsial *gltA*, *R. aeschlimannii* clustered with *R. aeschlimannii* reported from Senegal (HM050283) and Kazakhstan (MW922554) (Figure 2). Rickettsial *ompA* clustered with *R. aeschlimannii* reported from Senegal (HM050286), Kazakhstan (MW922585), and Morocco (U43800) (Figure 3), while *ompB* sequence clustered with *R. aeschlimannii* reported from China (MF098413), Senegal (HM050278), and Morocco (AF123705) (Figure 4).

Discussion

Previous studies have recorded the presence of *Rickettsia* spp. in different tick-infesting hosts in Pakistan. However, there was a paucity of information regarding *R. aeschlimannii* in the region. This study presents the first report on molecular detection of *R. aeschlimannii* in *H. bispinosa*, *H. montgomeryi*, and *Hy. turanicum* ticks collected from sheep and goats in Pakistan. The obtained sequences showed maximum

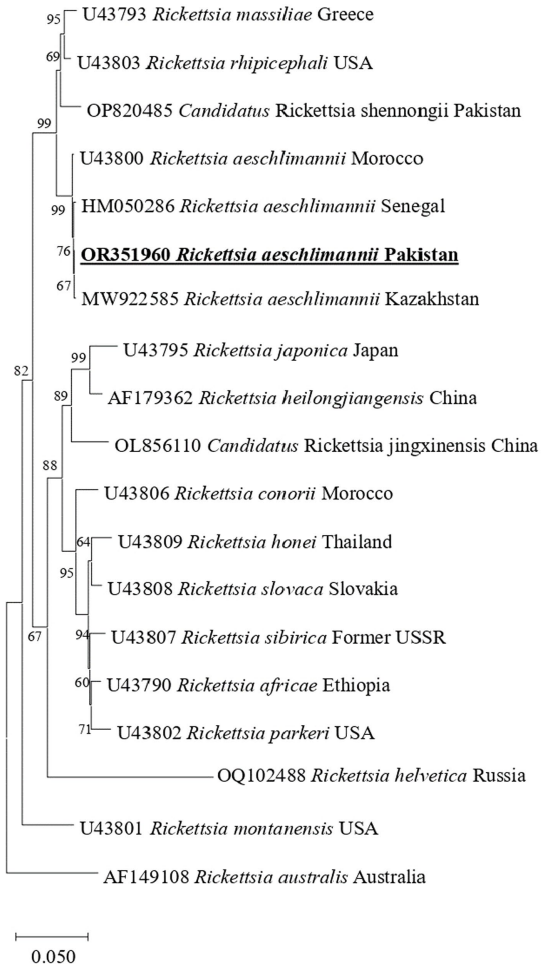

FIGURE 3
Phylogenetic analysis based on the *ompA* sequences of *R. aeschlimannii*. The sequences obtained in this study are indicated in bold and underlined fonts. *Rickettsia australis* is used as an out-group.

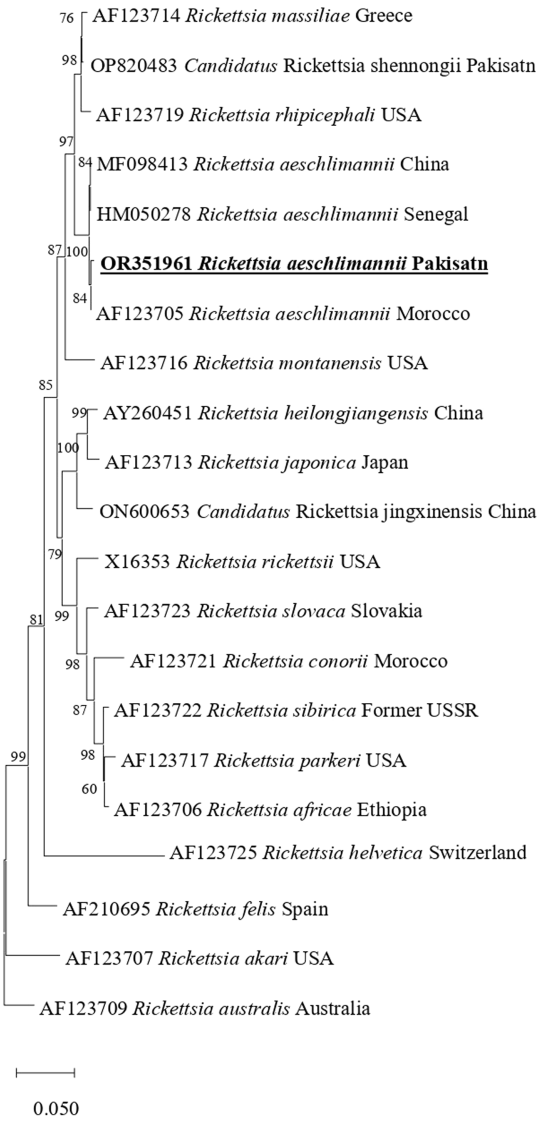

FIGURE 4
Phylogenetic analysis based on the sequences of the *ompB* genes of *R. aeschlimannii*. The obtained sequences in the present study are indicated in bold and underlined fonts. *Rickettsia australis* used as an out-group.

identity and phylogenetically clustered with *Rickettsia aeschlimannii*, which confirms the occurrence of *Rickettsia aeschlimannii* in the regions. *Rickettsia aeschlimannii*, an emerging pathogen with zoonotic potential, has been observed to cause infections in humans across various countries such as Morocco (Raoult et al., 2002), Tunisia (Znazen et al., 2006), Algeria (Mokrani et al., 2008), Greece (Germanakis et al., 2013), and Russia (Igolkina et al., 2022). The detection of *R. aeschlimannii* in tick-parasitizing domestic animals suggests a high exposure of livestock holders to this pathogen.

Small ruminants (goats and sheep), domestic dogs, and cattle were found infested by the ticks of genera *Rhipicephalus, Hyalomma,* and *Haemaphysalis*. Among the collected ticks, *Rh. microplus*, *Hy. anatolicum*, *H. bispinosa* and *H. montgomeryi* were the dominant tick species. These findings mirror the pattern observed in other previous studies conducted in same region (Karim et al., 2017; Ali et al., 2019; Alam et al., 2022; Khan Z. et al., 2022; Tila et al., 2023) underscoring the regional significance of these tick species. Furthermore, it emphasizes the need for further research to comprehensively investigate their prevalence, distribution, and potential implications for public health (Ali et al., 2023).

Ticks of different genera have been reported as carriers for various *Rickettsia* spp. in Pakistan (Ali et al., 2021, 2022; Khan M. et al., 2022; Khan Z. et al., 2022; Numan et al., 2022; Ullah et al., 2023). Herein, *Rickettsia aeschlimannii* was detected in *H. bispinosa*, *H. montgomeryi,* and *Hy. turanicum* ticks using three genetic markers, namely, *gltA*, *ompA*, and *ompB*. To date, there has been a lack of information regarding the detection of *R. aeschlimannii* in *H. bispinosa* and *H. montgomeryi* tick-infesting domestic animals, such as goats and sheep. There is also a possibility that the rickettsial DNA is detected in the ticks may be due to ingesting rickettsemic host blood. Literature search revealed that *R. aeschlimannii* is associated with a variety of tick species belonging to six genera of hard ticks, namely, *Hyalomma*, *Rhipicephalus, Haemaphysalis, Amblyomma, Dermacentor*, and *Ixodes* (Parola et al., 2001; Fernández-Soto et al., 2003; Shpynov et al., 2004; Tomassone et al.,

2010; Karasartova et al., 2018). Furthermore, there are limited reports, which have detected this pathogen in Asia (Wei et al., 2015; Satjanadumrong et al., 2019). This study found its association with two new ticks, expanding its known host and geographical range. The detection of *R. aeschlimannii* in *Hyalomma* and *Haemaphysalis* ticks suggests a potential threat to livestock holders. Additionally, the rate of *R. aeschlimannii* was observed highest in *Haemaphysalis* ticks, which are the primary ticks that infest goats and sheep. This enhances the feasibility of public health risks as these ticks may occasionally infest humans (Guglielmone and Robbins, 2018). *Rickettsia aeschlimannii* has been detected in all life stages of ticks, such as adult females, males, larvae, and nymphs (Raoult et al., 2002; Shpynov et al., 2009; Germanakis et al., 2013; Orkun et al., 2014; Wallmenius et al., 2014; Tosoni et al., 2016). This study presents the first molecular evidence of *R. aeschlimannii* in *H. bispinosa* and *H. montgomeryi* ticks, which suggests that other tick species could also serve as competent vectors for this pathogen in the region.

Molecular methods are considered faster and more accurate for the genetic characterization and phylogenetic analysis of *Rickettsia* spp. (Fournier et al., 1998; Roux and Raoult, 2000). The *gltA*, *ompA, ompB,* and *sca*4 DNA sequences have been used as suitable genetic markers to discriminate different *Rickettsia* spp (Roux and Raoult, 2000; Fournier et al., 2003; Labruna et al., 2004). Herein, these sequences were targeted for molecular characterization and phylogenetic analysis of *R. aeschlimannii* which revealed the close relatedness with corresponding species of the SFG. We assume that human infection caused by rickettsial agents of SFG maybe underreported due to the lack of epidemiological information among health practitioners and laboratory technicians and the lack of diagnostic procedures in Pakistan, given the relevance of the occurrence of these agents in the region. Further studies in the region should be encouraged to obtain information on zoonotic outcomes due to these infectious agents.

Conclusion

This study for the first time contributes to the neglected knowledge and genetic characterization of tick-borne *R. aeschlimannii* in *H. bispinosa*, *H. montgomeryi,* and *Hy. turanicum* ticks in Pakistan. The results of this study also indicated that goats and sheep are exposed to *R. aeschlimannii*. Further molecular studies are important to screen *R. aeschlimannii* in livestock and livestock holders who have close contact with domestic animals.

Data availability statement

We have released your GenBank submissions in OR351959-OR351961. Your sequences will be available for public access within a few days. If you have additional information about your sequences or wish to make further revisions, see: https://www.ncbi.nlm.nih.gov/Genbank/update.html for proper update formats.

Ethics statement

The design of the current work has received an approval from the Advanced Study and Research Board members (Dir/A&R/AWKUM/2023/0014) and the Faculty of Zoology department, Abdul Wali Khan University Mardan, Pakistan. Permissions were obtained from the animal's owner before collecting ticks from their animals. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AM:Datacuration,Investigation,Methodology,Software,Writing–original draft. MA: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. AbdA: Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. TT: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. T-YY: Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. K-HT: Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. AbiA: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing –original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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