RESEARCH ARTICLE

A cross-sectional survey of hard ticks and molecular characterization of *Rhipicephalus microplus* parasitizing domestic animals of Khyber Pakhtunkhwa, Pakistan

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Abstract

Background

In tropical and subtropical countries, tick infestation causes major public health problems and considerable financial losses to the livestock industry. This study was aimed to assess the species composition of richness and analyze the phylogeny of *Rhipicephalus microplus* in the District Bannu of Khyber Pakhtunkhwa, Pakistan.

Methods

Collected ticks were identified morphologically and DNA extracted from *R. microplus* was amplified and subjected to sequencing.

Results

A total of 3,600 animals were examined among them 1,494 animals were found to be infested with ticks, including 669 cows, 476 buffaloes, 163 goats, and 186 sheep ($p = 0.001$). Tick infestation was significantly high (43.58%) in animals of age group (<1 year) (p-value = 0.027). Female animals were more (44.05%) infested with ticks than males (34.43%) ($p = 0.001$). The intensity of infestation was significantly higher in summer (77.49%) ($p = 0.001$). A total of 5,557 ticks were collected comprising three genera and six species. *R. microplus* was predominantly prevalent (n = 1,474; 26.52%), followed by *Rhipicephalus annulatus* (n = 1,215; 21.86%), *Hyalomma anatolicum* (n = 1,139; 20.49%), *Hyalomma marginatum* (n = 1,086; 19.54%), and *Rhipicephalus turanicus* (n = 761; 13.69%), while the least common was *Haemaphysalis aciculifer* (n = 80; 1.43%) ($p = 0.001$). Morphologically identified *R. microplus* species were also analyzed genetically by using two genetic

markers 16S ribosomal RNA (*16S rRNA*) and internal transcribed spacer 2 (*ITS2*) genes. The phylogenetic study revealed that *R. microplus* is genetically diversified and clustered in clade B with *R. microplus* species from China, India, and Pakistan.

Conclusion

Ticks infestation was significantly correlated with various factors including age, sex, season, and animal type. *R. microplus* genetically resembled species reported from India and China. However, major knowledge gaps concerning various species of ticks exist and many areas are still unexplored in Pakistan. Therefore, it is necessary to explore the epidemiological and molecular aspects of various tick species in other regions of southern Khyber Pakhtunkhwa.

Introduction

Ticks are the blood-sucking arthropod parasites and a major source of emerging economic and public health concern in the regions of the tropics and subtropics [1]. Ticks act as a reservoir for several contagious pathogens of medical and veterinary importance and can transmit a wide variety of bacteria, protozoans, spirochetes, and arboviruses more than any other blood-sucking parasites [2]. Approximately, 10% of all tick species are known to transmit various pathogens [3, 4] while 80% of the cattle population is affected by ticks and tick-borne diseases [4, 5]. Besides, the transmission of infectious diseases, the parasites can also cause great loss in milk, meat production, and harm to the skin as well as hide quality [6]. The economic impact on the livestock industry due to the common cattle tick *Rhipicephalus microplus* in different regions was estimated to be 22–30 billion US$ annually [4, 5].

Various environmental factors affect the prevalence and adaptation of ticks in different regions of the world [7]. Pakistan is an agricultural country and the livestock sector is an essential part of the national budget as the 2nd larger sector, which contributes 21.2% to the GDP (Gross Domestic Product) by paying 45.0% of the employees. In Pakistan, 70.0% of the population lives in rural areas, where most of them depend on livestock as the main source of income and food to survive. The population of cattle (*Bos indicus* and *Bos taurus*) 41.2 million, buffaloes (*Bubalus bubalis*) 35.6 million, goats (*Capra hircus*) 68.4 million, and 29.4 million sheep (*Ovis aries*) was estimated (based on Livestock Census 1996 & 2006) [8]. The climatic conditions of Pakistan are tremendously satisfying for the growth and survival of different tick species and other animal disease-causing parasites [9–14]. Ticks harm livestock holders, especially low-income farmers and the majority of them are unaware of different tick species and consider all ticks as a single species. The farmers also do not have any knowledge about the role of ticks in the transmission of various pathogens to human [5, 8, 15].

To date, several studies have reported the prevalence of different tick species in different provinces of Pakistan, for instance, Punjab [8, 16–18], Sindh [19], Baluchistan [20], and Khyber Pakhtunkhwa [21–23]. However, only a limited number of studies described ticks on the species level in Pakistan [8, 24, 25]. Major knowledge gaps concerning various species of ticks still exist and many areas are still unexplored in Pakistan. Therefore, this study aimed to evaluate the presence of ticks in the District Bannu of Khyber Pakhtunkhwa, Pakistan.

Materials and method

Study area

The present study was carried out in District Bannu located in the southern belt of Khyber Pakhtunkhwa, Pakistan. Its borders are attached with North Waziristan to the northwest, Karak to the northeast, Lakki Marwat to the southeast, and South Waziristan to the southwest. The total region of the district is 1,227 km² and out of the total, 74,196 hectares area is under cultivation (**Fig 1**). In summer, the temperature range is about 48°C, while in winter it remains about 6°C.

Ethical approval and consent to participation

The study was approved by the Ethical Committee of Hazara University Mansehra, Khyber Pakhtunkhwa, Pakistan. A brief purpose and aim of the study were explained and written informed consent was taken from the owner of animals before data collection.

Sampling and morpho-taxonomic identification of ticks

The cross-sectional survey was carried out from September 2018 to August 2019. Tick specimens were collected from domestic animals (e.g. cows, buffaloes, goats, and sheep) by performing regular visits to the grazing fields and farms of the study area. The detailed history of sex, age, place, date, type of animal was recorded on a prescribed proforma. Different body parts (e.g. shoulders, dewlap, belly, head, ears, neck, back, legs, perineum, and tail) of animals
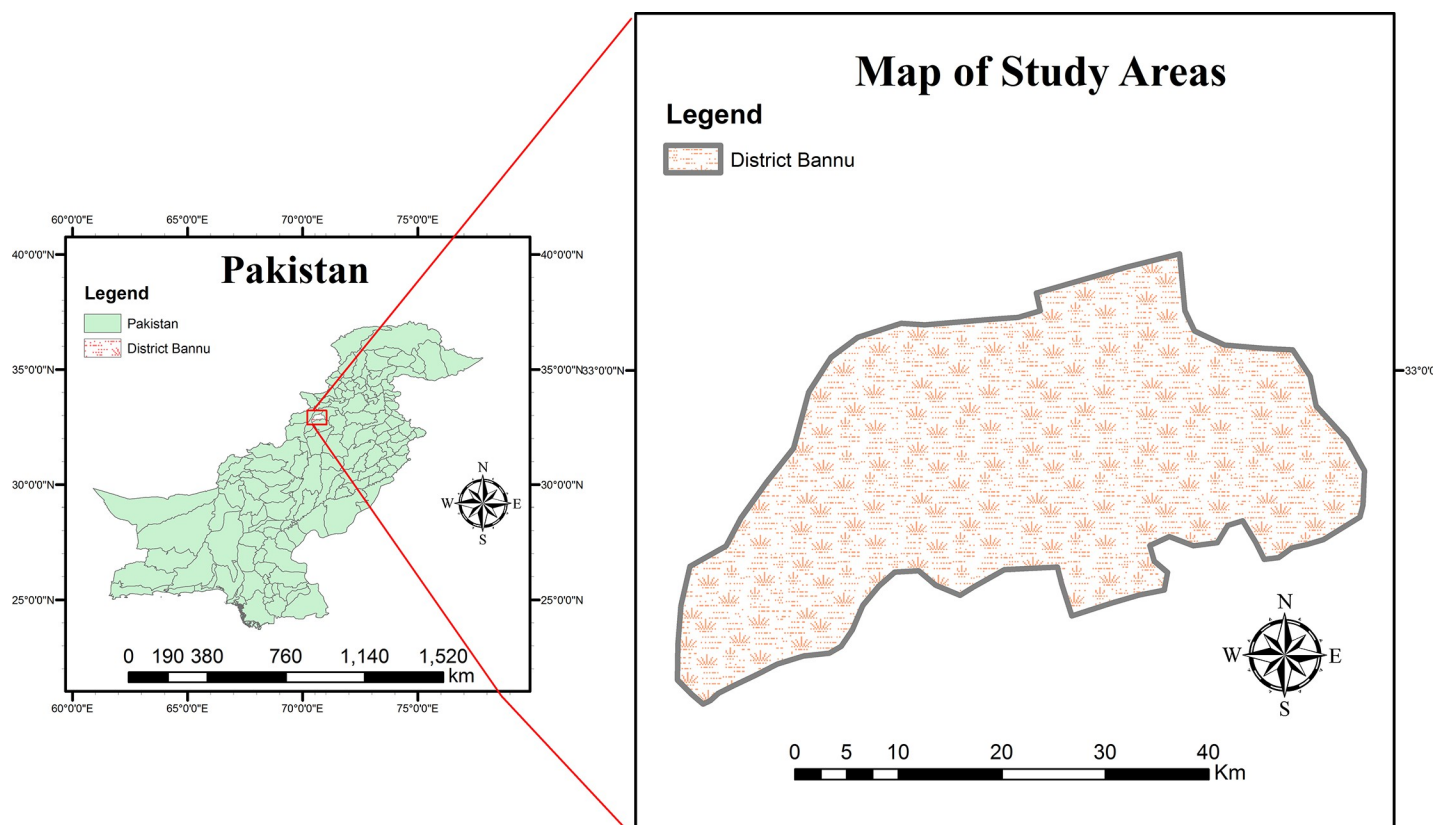

Fig 1. Map of the study area showing District Bannu.

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were examined and forceps were used to pluck/separate ticks from the animal's skin safely. Ticks after collection were stored in small plastic bottles to ensure safe transportation to the Parasitology Laboratory, Department of Zoology, Hazara University of Mansehra for morphological identification. The collected specimens were washed gently with phosphate buffer saline (PBS) and preserved in a separate container containing 70% ethanol. Adult ticks (both male and female) were morphologically identified with the help of a Stereomicroscope (100–200-fold magnification) and standard taxonomic keys [26–29]. The identified specimens of *R. microplus* were transported to the College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University Garden Campus Mardan (AWKUM), for further molecular analysis.

DNA extraction

Individual ticks were washed gently with ethanol and then snap-frozen in liquid nitrogen. The frozen specimens were cut into small pieces using a scalpel and ground with Genogrinder (SPEX Sample Prep). The ground tissues were placed in a sterile Eppendorf tube containing PBS for future use. Genomic DNA was extracted by using a Gene Jet Genomic DNA purification kit (Thermo Fisher Scientific) according to the standard protocol as recommended by the manufacturer. The Nanodrop ND-100 (Thermo Fisher Scientific) was used to quantify the concentration of DNA samples and placed at –20˚C for further processing.

Polymerase Chain Reaction (PCR)

Morphologically identified *R. microplus* species were further subjected to a polymerase chain reaction (PCR). To confirm the identity consigned to *R. microplus*, two genes i.e. *16S rRNA* and *ITS2* were analyzed. For the *16S rRNA* gene, a 450 base pair (bp) fragment was amplified by PCR in a thermocycler (HT, ILF, UK) using the forward primer *16S rRNA*-F (5′–AATT GCTGTAGTATTTTGAC–3′) and reverse primer *16S rRNA*-R (5′CTCCGCCTGAAGGGTCA AA–3′) as described by [30]. In the case of *ITS2*, a 750 bp fragment was amplified using primers *ITS2*-F (5′CGGATCACATATCAAGAGAG–3′) and *ITS2*-R (5′–CCCAACTGGAGTGGCCC AGTTT–3′) [31]. The total volume of the PCR reaction was 25 μL, comprising of 12.5 μL master mix [2x] (Thermo Fisher Scientific), 1.5 μL of each forward and reverse primers, 4 μL DNA template, and then 5.5 μL nuclease-free water was added to complete the final reaction. The general PCR conditions for *16S rRNA/ITS2* were followed as; an initial denaturation step at 94˚C for 5 min; followed by 25 cycles of 94˚C for 30 s, annealing temperature 54˚C for *16S rRNA* and 57˚C for *ITS2* for 30 s, initial extension temperature at 72˚C for 90 s; and a final extension step at 72˚C for 10 min. For validation, a negative control (distilled water) was added in each amplification reaction. The PCR products were confirmed through 2% agarose gel with ethidium bromide. DL 2,000 DNA marker (Takara) was used to find the length and concentration of the amplified products and DNA was visualized using the GeneDoc (UVP BioDoc-It Imaging System) **(S1 and S2 Figs)**. The amplicons were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing. The obtained sequences were trimmed and edited using BioEdit (V. 7.0.) [32]. Consensus sequences were BLAST in NCBI and sequences of *R. microplus* and related species were retrieved from the gene bank for the downstream construction of the phylogenetic tree.

Phylogenetic analysis

The trimmed sequences were aligned using ClustalW and Maximum-likelihood (ML) algorithm was employed to construct the phylogenetic tree in MEGA-X with bootstrapping at 1000 replicates [33]. The evolutionary distances were computed using the Tamura-Nei method

[34] and are in the units of the number of base substitutions per site. Gaps and missing data were eliminated by using the partial deletion option. *Hyalomma detritum* (KC203349) was used as an outgroup for the *16S rRNA* gene, while *Ixodes scapularis* (GU319067) for the *ITS2* gene phylogenetic tree construction.

Data analysis

The epidemiological data of different variables were analyzed using the statistical tool IBM SPSS Statistics (version 23). To determine the association between ticks and several risk factors (e.g. age, season, animal type, and sex) Chi-square Pearson's test (x^2) was used. The *p*-value (0.05) was considered to be statistically significant.

Results

A total of 3,600 animals were studied randomly including cows, buffaloes, sheep, and goats (n = 900 each). The overall prevalence of infested animals was 41.5%. Among the studied animals, the infestation rate was higher in cows (74.3%) followed by buffalos (53.0%), sheep (21.0%), and goats (18.1%) (**Table 1**). This rate of infestation was highly significant among the studied animals (P<0.001).

Age-wise comparison revealed a high prevalence in calves/lams/kid (43.58%) than younger (41.0%) and adult (40.2%) animals (*p* = 0.027). Similarly, the majority of infested animals were females (44.0%) than males (34.4%). This difference was also statistically highly significant (*p* = 0.001) (**Table 2**). Season-wise prevalence depicted highest percentage in summer (77.5%), followed by spring (65.1%), autumn (30.0%), and winter (11.4%). While month-wise the highest prevalence was recorded in July (83.2%) and the lowest prevalence (5.9%) in December (**Table 3**).

All ruminants including cows, buffalos, sheep, and goats, regardless of their geographic position were examined for hard tick species. A total of 5,557 adult ticks were collected from various body parts of infested animals. Three genera comprising of *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis*, and 6 species i.e. *R. microplus*, *Rhipicephalus turanicus*, *Rhipicephalus annulatus*, *Hyalomma marginatum*, *Hyalomma anatolicum*, and *Haemaphysalis aciculifer* were identified morphologically. *R. microplus* (n = 1,474; 26.0%) was observed to be the most dominant species, followed by *R. annulatus* (n = 1,215; 21.0%), *Hya. anatolicum* (n = 1,139; 20.0%), *Hya. marginatum* (n = 1,086; 19.0%), and *R. turanicus* (n = 761; 13.0%), while the least common was *Hae. aciculifer* (n = 80; 1.4%) (*p* = 0.001) (**Table 4**).

BLAST analysis showed a remarkable (100%) identity of *16S rRNA* gene nucleotide sequences with species from India (accession no. MG811555; MF946459; KY458969), Pakistan (accession no. MN726558; MT799952), and China (accession no. KU664521). Similarly, *ITS2* nucleotide sequence also showed high resemblance (100%) with species reported from China (accession no. MK224585; MK224584; KX450289), India (accession no. MK621182; MH598985; MF946462), and Pakistan (accession no. MW580928; MW580866). The

Table 1. Overall prevalence of hard ticks in District Bannu.

Animal host	Infested/ Animals examined (%)	Pearson's Chi-square test (x^2)	*p*-value
Cows	669/900 (74.3)		
Buffaloes	476/900 (53.0)		
Sheep	186/900 (21.0)	811.419	0.001
Goats	163/900 (18.1)		
Total	**1,494/3,600 (41.5)**		

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Table 2. Age and sex-wise prevalence of hard ticks in District Bannu.

Variables	Cows Infested/ Examined (%)	Buffaloes Infested/Examined (%)	Sheep Infested/Examined (%)	Goats Infested/Examined (%)	Prevalence (%)	Pearson's Chi-square (x^2)	*p*-value
Age (year)							
Calf/lamb/kid (<1)	219/299 (84.0)	177/349 (65.2)	62/262 (26.5)	58/274 (24.0)	44.0		
Younger (2–3)	219/294 (79.0)	151/301 (47.0)	61/292 (20.0)	45/280 (17.0)	41.0	7.195	.027
Adult (>5)	231/307 (52.0)	148/250 (45.0)	63/346 (13.5)	60/346 (13.0)	40.2		
Sex							
Male	154/193 (62.0)	82/229 (36.0)	45/229 (20.0)	53/316 (17.0)	34.4		
Female	515/707 (79.1)	394/671 (59.0)	141/671 (21.0)	110/584 (19.0)	44.0	14.145	0.001

nucleotide sequences of *16S rRNA* (MZ540266, MZ540267, MZ540268) and *ITS2* (MZ542565) of the current study were deposited to the NCBI GenBank.

The *16S rRNA* ML phylogenetic tree has strong bootstrap support for monophyly of *R. microplus* and its division into clade A and clade B. The clade A was comprised of *R. microplus* from South Africa (Mozambique), Malaysia, and America (Argentina, Brazil). While the clade B was comprised of *R. microplus* from China, India, and Pakistan. *R. microplus* from India, China, and Pakistan collectively depicted a moderate bootstrap value (77%), and *R. microplus* from Malaysia, America, and Africa have shown (80%). The tree also revealed a well-supported clade (94%) of *R. australis* from Australia, Caledonia, and Indonesia (**Fig 2**). On the other hand, *ITS2* analysis depicted that all species of *R. microplus* complex were clustered together and well-supported monophyly (**Fig 3**). However, this tree revealed a little structure within the *R. microplus* complex other than the average support for monophyly of *R. annulatus* (73%) and the two *R. australis* from Australia (59%). *R. microplus* specimens from China have a similar *ITS2* sequence to several other *R. microplus* specimens. The *ITS2* tree has also strong support for the placement of most other *Rhipicephalus* species and places a clade consisting of *R. bursa*, and sister clades of *R. zambeziensis*, *R. appedniculatus*, *R. turanicus*, *R. sanguineus*, and *R. guilhoni*.

Table 3. Season-wise prevalence of hard ticks in District Bannu.

Variables Seasons	Months	Cows Infested/ Examined	Buffaloes Infested/ Examined	Sheep Infested/ Examined	Goats Infested/ Examined	Overall prevalence% Month-wise %	Overall prevalence% Season-wise %	Pearson's Chi-square test (x^2)	*p*-value
Spring	Sep	72/83	54/88	18/41	20/41	64.82	65.1		
	Oct	43/55	39/64	10/52	12/54	46.22			
	Nov	19/59	27/74	8/79	13/86	22.48			
	Dec	7/56	12/82	4/140	2/143	5.93			
	Jan	17/60	14/91	4/141	4/141	9.00			
	Feb	29/64	21/65	1/105	7/115	16.61			
Winter	Mar	46/64	33/64	7/91	7/93	29.80	11.4		
	Apr	64/74	37/71	19/68	8/59	47.05			
	May	68/72	41/60	15/34	13/34	68.50		1224.196	0.001
	Jun	88/90	59/69	22/40	20/41	78.09			
	Jul	128/140	71/83	40/56	29/49	83.22			
Autumn	Aug	88/89	68/89	38/53	28/44	80.72	30.0		
Summer							77.5		

Table 4. Genus and species-wise prevalence of hard ticks in District Bannu.

Variables	No. of ticks	No. of infested animals	Percentage (%)	Pearson's Chi-square test (x^2)	*p*-value
Genera					
Haemaphysalis	80	20	1.4	3600.000	
Hyalomma	2,225	587	39		0.001
Rhipicephalus	3,450	887	60		
Species					
Haemaphysalis aciculifer	80	20	1.4		
Hyalomma anatolicum	1,139	332	20		
Hyalomma marginatum	1,086	255	19		
Rhipicephalus annulatus	1,215	309	21	3600.000	0.001
Rhipicephalus turanicus	761	225	13		
Rhipicephalus microplus	1,474	353	26		
Total	**5,755**	**1,494**	**100**		

Discussion

In the current cross-sectional study, the overall prevalence of ticks was reported to be 41.47%. In different regions of Pakistan, other investigators [16, 20, 21] also reported the prevalence of tick infestation where the percentage was 75.0, 11.3, 13.4, and 35.0%, respectively. The difference in the current and previous studies might be due to differences in species, breed, sex, age, host [35, 36], sample size, sample period, different management systems, climatic conditions, humidity, and environmental conditions [37]. Higher tick prevalence can also be justified by a systematic rainy season that makes the environment suitable for tick propagation [38]. Among animals, the highest prevalence was reported in cows (74.3%) and buffalos (52.7%). This study was consistent with the study conducted by [17] where the overall prevalence was 36.5%, while cows and buffaloes were highly infested at 37.5 and 42.4%, respectively. In large ruminants, the higher prevalence of ticks infestation in the cow population may be due to the thinner skin of cows as compared to buffaloes [39]. Moreover, the cows adapt to a dried habitat, while buffaloes prefer marshy places [40]. The reason for the higher prevalence in small ruminants may not be evident however, genetics may play a vital role [41].

Females were more affected in the current study which coincided with the previous study conducted in ruminants [42, 43]. In Pakistani society, male animals are mostly used for breeding purposes throughout the year. Moreover, in Muslim countries male animals are sacrificed at "Eid-ul-Adha" (holy festival of Muslims) therefore, they take more consideration as compared to female animals. Hence, the male would have a low ticks burden and physically groom well. Besides, pregnancy and lactation period may also be one of the factors that decrease the resistance in females [44].

Tick infestation varied throughout the year and the highest was recorded during June, July, and August. Similar results were reported by [18, 45] from Punjab, where the infestation rate was higher in June and July. It was observed that infestation was higher in the summer season because in summer the weather becomes warm, humid, and makes the environment suitable for tick growth and multiplication. These results were following the previous studies [46–48]. Various ecological factors like different study areas, climatic conditions, rainfall, temperature, and humidity may also affect tick infestation rates [37]. Some other factors like farming practices [49], host availability, and nutrition status might have a vital role [36]. Similarly, Mondal et al. (2011) also reported a higher prevalence in the summer season (41.6%) followed by winter (31.5%) [50].
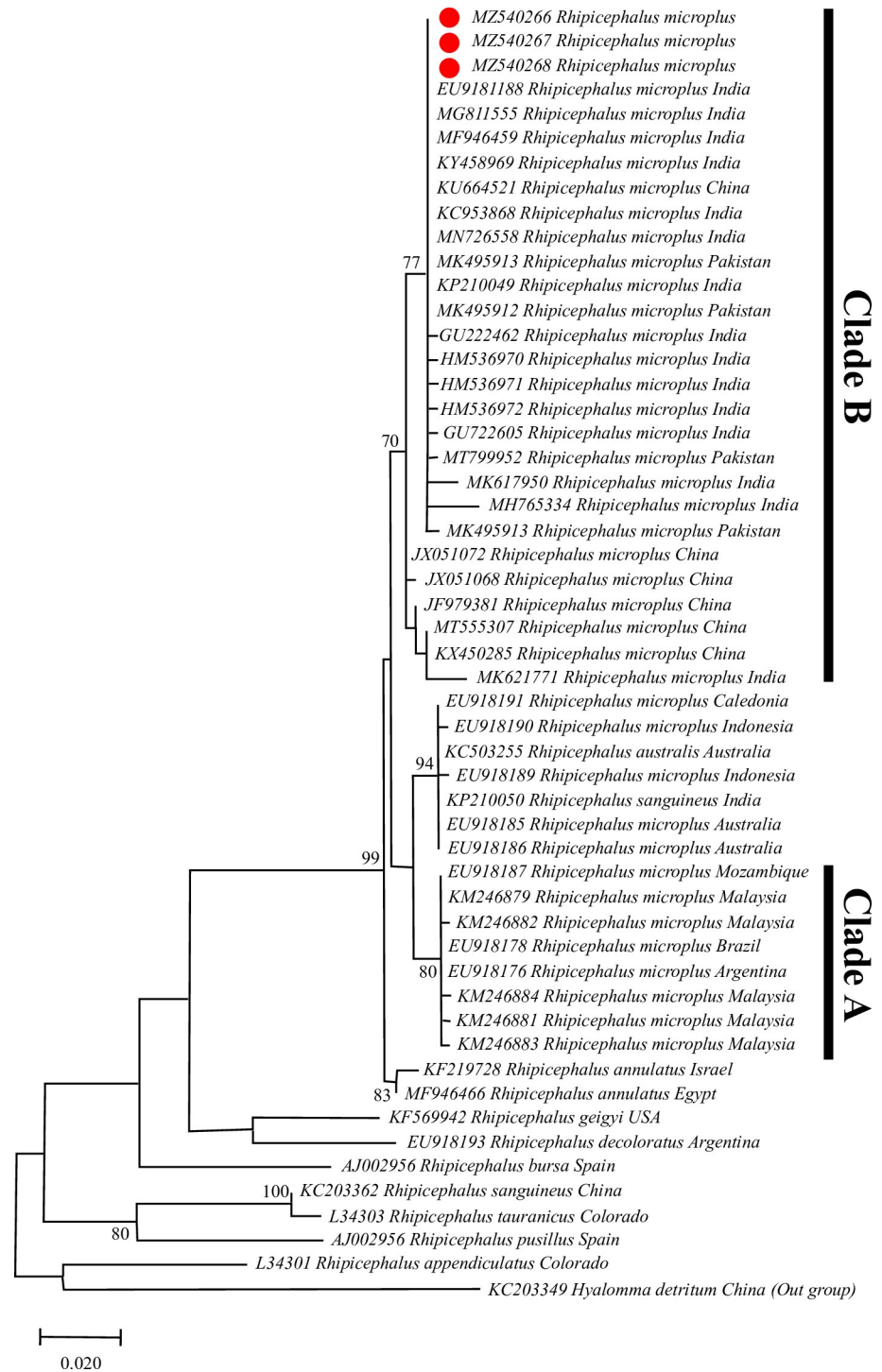
Fig 2. Phylogenetic tree of *16S rRNA* sequences of the genus *Rhipicephalus* and using *Hyalomma detritum* sequence as outgroup. The tree was inferred using the Maximum likelihood method and evolutionary distances were computed using the Tamura-Nei model. The bootstraps values (1000 replicates) are shown next to the taxa. The sequences of the present study are marked in red.

Fig 3. Phylogenetic tree of *ITS2* sequences of the genus *Rhipicephalus* and using *Ixodes scapularis sequence* as outgroup. The tree was inferred using the Maximum likelihood method and evolutionary distances were computed using the Tamura-Nei model. The bootstraps values (1000 replicates) are shown next to the taxa. The sequences of the present study are marked in red.

The age of the animal has an important role in the prevalence of tick infestation [21]. It was observed, that calves were more susceptible to tick infestation than other age group animals. It might be since adults are productive animals, therefore, adults are carefully looked after to increase production. Besides, adult animals may also acquire resistance to tick infestation due to repeated exposure over time [51]. Similar trends of ticks infestation were observed in the past by other investigators [21, 52].

In our finding, it was observed that the genus *Rhipicephalus* was more prevalent 62.0% than other tick genera. This finding was in accordance with previous studies [22, 53]. The higher infestation rate of the predominant genera might be due to the rainfall ranging from high to temperate [53]. The decline configuration of the genus *Hyalomma* was due to the rainy to semi-arid features of temporal areas, as stated by [53, 54]. Similarly, at the species level, the *R. microplus* was the most prevalent species in our study which was present in all infected animals throughout the study area. These results were in parity with the study carried out by [22] where the author observed *R. microplus* in a massive number. Retaining water capability beneath the layer of the soil and increase in humidity of the study area sponsors the distribution and propagation of this tick species. In the near past, it was discovered that *R. microplus* is a complex, comprising of five taxa: *R. microplus* clade A, *R. microplus* clade B, and *R. microplus* clade C, *R. australis*, and *R. annulatus* [55, 56]. Two *Hyalomma* species (*Hya. anatolicum* and *Hya. marginatum*) and one *Haemaphysalis* species (*Hae. aciculifer*) were also reported in the present study which was previously documented from Pakistan [22]. The *Hyalomma* species act as a vector and transmit various diseases like *Theileria annulata* and *Theileria lestoquardi* in Pakistan [57]. Also, *Hyalomma* species have long mouthparts that can damage the cattle hides extremely and mostly target the teat of the cattle that may cause difficulty in calves suckling [58]. *Heamaphysalis* species also transmit various diseases like theileriosis and babesiosis in sheep and goats [46]. Furthermore, the causative agents of bovine anaplasmosis in Pakistan are mainly transmitted by *R. microplus* and *Hyalomma* species [59]. By comparing the studies in Pakistan to other countries the data on tick epidemiology and genetic diversity is limited and insufficient. However, the tick infestation rate was almost same among Khyber Pakhtunkhwa and other provinces of the country [42, 47]. It may be due to the free movement of animals across the province, districts, and keeping of mixed species in the same area, particularly in Khyber Pakhtunkhwa [22].

The genetic markers i.e. *16S rRNA* and *ITS2* define the intraspecific genetic diversity and phylogeographical connections of the important global pest of livestock, *R. microplus*. Previously, both genetic markers have been used to investigate the precise relationship among hard ticks such as *R. microplus* complex [25, 55, 56, 60, 61]. In phylogenetic analysis *ITS2* gene has shown to be a useful genetic marker however, it contains little intraspecific variation but significant interspecific inconsistency [62]. On the other hand, *16S rRNA* has been also used for the investigation of the phylogenetic relationships of various important tick species across the globe [63–66]. For good identification of the cryptic species of *R. microplus* in different geographical regions, *16S rRNA* sequences were used [30]. Phylogenetic analysis of the *16S rRNA* gene revealed two different genetic clades of *R. microplus*. Similar studies were also reported elsewhere, where two genetic clades were confirmed [8, 25, 31, 56, 60, 67]. However, the genetic marker *ITS2* was clustered together in a single clade on the ML phylogenetic tree. It means that this marker cannot differentiate within the same species and support the

monophyly of the *R. microplus* complex. This finding was similar to a previous study reported from Pakistan [25]. To indicate the evolutionary connections of *R. microplus*, the *16S rRNA* gene provides sufficient power as compared to the *ITS2* because mitochondrial DNA sequences evolve rapidly and are inherited maternally [66, 68]. From the current findings, it is clear that the *16S rRNA* marker has a well-resolving power as compared to the ITS2 gene.

Conclusion

This is the first attempt to explore the prevalence of hard tick fauna as well as molecular characterization of *R. microplus* in the Bannu district. We reported three genera and six species i.e. *R. microplus*, *R. turanicus*, *R. annulatus*, *Hya. marginatum*, *Hya. anatolicum*, and *Hae. aciculifer*, where *R. microplus* was reported as one of the most prevalent species. Various factors like age, sex, season and animal type significantly affected the tick infestation rate. It was also concluded that genetically *R. microplus* showed more similarity with that of India and China. However, major knowledge gaps concerning various species of ticks exist and many areas are still unexplored in Pakistan. Therefore, it is necessary to explore the epidemiological and molecular aspects of various tick species in other regions of southern Khyber Pakhtunkhwa. This study will be useful in the investigation and designing control strategies for ticks control in Pakistan.

Supporting information

S1 Fig. *16S rRNA* amplified product. A 2000 ladder was used. 1–4 represent samples of the present study. N represents negative control and P represents the positive control. (DOCX)

S2 Fig. *ITS2* amplified product. A 2000 ladder was used. 1–3 represent samples of the present study. N represents negative control and P represents the positive control. (DOCX)

S1 Raw images. (PDF)

Acknowledgments

Author Contributions

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