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RESEARCH ARTICLE

Prevalence, Risk Factors, and Molecular Characterization of Hard Ticks in Two Diverse Agro-Ecological Zones of Punjab, Pakistan

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ABSTRACT

Hyalomma anatolicum (*H. anatolicum*) and *Rhipicephalus microplus* (*R. microplus*) are the major tick species affecting cattle populations around the world, especially in Pakistan. Climate change has led to an increase in their prevalence worldwide. Field veterinarians reported a high prevalence of ticks during winter in district Chakwal of Pakistan. The present study was conducted in two different ecological zones to see the variation in tick infestation among different ecological zones. A total of 740 samples of ticks were collected from two selected districts. Tick identification was done under the stereomicroscope. Polymerase chain reaction (PCR) was performed using selected cytochrome C oxidase subunit 1 (COI) gene marker and phylogenetic analysis was also performed after sequencing. The overall prevalence of tick infestation in cattle was 69.46%. A significantly higher prevalence ($P<0.05$) in district Chakwal (80.54%) was observed than in district Faisalabad (58.38%). There was a significant difference in tick species between the two regions. In Faisalabad, *H. anatolicum* was the dominant species, with a prevalence of 72.69% whereas in Chakwal, *R. microplus* was more common, with a prevalence of 66.10%. Despite the colder winter temperatures in Chakwal, ticks were still found to have a prevalence of 19.38%, whereas in Faisalabad, their prevalence was nearly zero during winter. The association of different factors like gender, breed, and housing (walls, floor, grazing, and acaricide) was found to be statistically significant ($P<0.05$) for tick prevalence. In phylogenetic analysis, no variation in sub-species level was noted, ticks in both districts are 99.4% identical. This study will help in developing regional tick control programs and finding alternate tick control methods like immunological tick control. These results could be useful in the development of integrated tick and tick-born disease control strategies in Pakistan.

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INTRODUCTION

Various parasites and parasite-borne diseases impede profitable livestock farming (Aslam *et al.*, 2023; Mehnaz *et al.*, 2023; Nadeem *et al.*, 2023, 2024; Niaz *et al.*, 2023; Qamar & Alkheraije, 2023). Among these, ticks are the most significant hematophagous ectoparasites, affecting animal health and decreasing productivity. Their heavy infestations lead to anemia, irritation, and diminished hide quality due to scarring. In addition to direct damage, ticks act as vectors for various pathogens, including viruses, bacteria, and protozoa (Perumalsamy *et al.*, 2024). Tick-borne diseases rank as the fourth most significant infectious diseases in livestock and the highest among arthropod-borne diseases, following mosquitoes, in livestock, humans and companion animals (de la Fuente *et al.*, 2023). The prevalence of ticks in cattle varies from 6.63 to 79.3% in Pakistan (Ramzan *et al.*, 2020). The prevalence of specific tick-borne diseases closely aligns with the distribution of tick species (Asante *et al.*, 2019). Furthermore, tick bites may lead to toxicosis and tick paralysis, which are significant causes of death in large ruminants especially cattle (Al-Hoshani *et al.*, 2023; Kandil *et al.*, 2024). Approximately 80% of the global cattle population is affected by ticks, highlighting their considerable economic impact (Jabbar *et al.*, 2015; Sindhu *et al.*, 2022).

In tropical and subtropical regions, the most important genera of hard ticks infesting cattle include *Rhipicephalus* (Boophilus), *Hyalomma*, and *Amblyomma* (Parveen *et al.*, 2021; Abbas *et al.*, 2024). The cattle tick *R. microplus* alone causes significant economic losses, with estimates ranging from 13.9 to 18.7 billion USD dollars worldwide (Betancourt, 2017). Various reports from different countries highlight these losses e.g. in Australia, the annual direct and indirect losses due to ticks are estimated as 245 million AUD (Jonsson *et al.*, 2008). In India, economic losses due to ticks range from 0.9 to 1.6 billion USD dollars, while Mexico suffers a substantial 82 million USD dollar loss solely from bovine babesiosis. Meanwhile, Brazil faces an even greater financial burden, with losses soaring to 2 billion USD dollars (Rodríguez-Vivas *et al.*, 2017). In Pakistan, losses are estimated at 50-75 million USD dollars due to babesiosis, with overall losses due to ticks reaching around 1.5 billion USD dollars. These losses are attributed to decreased milk production, weight loss, and the death of infected animals (Ghafar *et al.*, 2020a). Pakistan, a subtropical country, offers a favorable environment for tick propagation due to its geographical location. The most common tick species in the country include *H. anatolicum*, *H. dromedarii*, *R. microplus*, *R. annulatus*, *Dermacentor*, and *Haemaphysalis bispinosa* (Kariam *et al.*, 2017; Ramzan *et al.*, 2020; Ghafar *et al.*, 2020a). Among these species the *H. anatolicum*, *R. microplus* are most prevalent in cattle with variable prevalence ranging from 6.63-79.3% depending upon season and location (Ramzan *et al.*, 2020).

The prevalence of hard ticks is influenced by factors such as macro and micro-climate, geography, and host availability. Moreover, recent climate change is reshaping tick habitats by altering temperature and precipitation patterns, which can influence tick biology, host distribution, and pathogen transmission (Gilbert, 2021). Some tick species, like *R. microplus* and *H. anatolicum*, may expand their range due to rising temperatures and changing rainfall patterns, which will potentially increase the incidence of tick-borne diseases in Pakistan (Ghafar *et al.*, 2020b). Changing temperatures in certain areas of Pakistan may also lead to the expansion of tick habitats, increasing tick populations compared to other regions. Recent climate changes have significantly impacted the population dynamics of ticks in Pakistan. *Hyalomma* spp. has become the dominant tick species in leveled plain areas with harsher temperatures, while *R. microplus* is more common in hilly or moderate areas and can survive in lower temperatures. Field observations by many veterinarians working in the semi-hilly area of district Chakwal have reported a higher prevalence of ticks during the winter season, unlike the plain area of district Faisalabad where almost zero prevalence is observed in winter. According to Mansoor Ahmad and Javed Ahmad, veterinary officers working in the area, this trend has been a persistent challenge for livestock farmers [Ahmad & Ahmad, 2022 (personal communication)]. However, limited data make it challenging to assess the exact impact of climate change on tick prevalence and disease transmission. To understand these complex interactions, it is important to conduct detailed studies over time and across different locations. This will help identify changes in tick distribution and develop effective strategies for their control (Ghafar *et al.*, 2020b; Younas *et al.,* 2023). This study has been planned to identify and determine the prevalence of the *R. microplus* and *H. anatolicum* ticks in ecologically discrete zones of Punjab (Faisalabad and Chakwal). This study also helps to understand the possible factors either genetic or managemental, that influence higher prevalence during winter in Chakwal.

MATERIALS AND METHODS

Study design and sample collection: This study was conducted on cattle of two districts viz, Faisalabad (31.4504°N, 73.1350°E) having five tehsils (Faisalabad Sadar, Samundri, Jaranwala, Chak Jhumra, Tandlianwala) and Chakwal (32.9328°N, 72.8630°E) having 5 tehsils (Chakwal, Kalar Kahar, Talakang, Choa Sadan Shan and Lawa) of Punjab province, Pakistan (Fig. 1). Two different data sets were collected; one measured the point prevalence in both zones, while the other tracked monthly prevalence throughout the year to evaluate the seasonal variations. Cattle of different breeds were the target population in both districts. For point prevalence, a total of 740 (370 from each district) cattle were screened randomly. The sample size was calculated by considering the expected prevalence of 40% at a 95% confidence interval and 5% precision level by using the following formula as described by Thrusfield *et al.* (2018).

$$\mathbf{n} = \frac{\mathbf{1.96^2} P_{exp}\left(\mathbf{1} - P_{exp}\right)}{d^2}$$

The seasonal effects on tick infestation were checked through monthly (August 2020 to July 2021) screening on selected ten farms as shown in Fig. 1. A questionnaire-based survey was also completed to collect data regarding the association of different risk factors (Thrusfield *et al.*, 2018) like ecological (temperature, rainfall, humidity), biological (age, sex, species, and breed) and managemental (housing, feeding, and treatment). Data was collected from a total of 50 farms in each district through in-person interviews with farmers. The ecological parameters were confirmed by the Metrological Department of Punjab, Pakistan.

Ticks were collected through forceps and samples were stored in a plastic bottle having holes in the lid for proper aeration for transportation to the Department of Parasitology, University of Agriculture, Faisalabad.

Identification and Molecular characterization of ticks: Identification of ticks was done with the help of a stereomicroscope (Leica Wild M3C), based on their morphological points described by Walker *et al.* (2003). For DNA extraction the ticks were dissected for slivery glands DNA was extracted using WizPrep™ gDNA Mini Kit (Cell/Tissue, Ref # W71060-100) according to manufacturer instructions. Extracted DNA was quantified by using NanoDrop™ (Thermo Scientific USA).

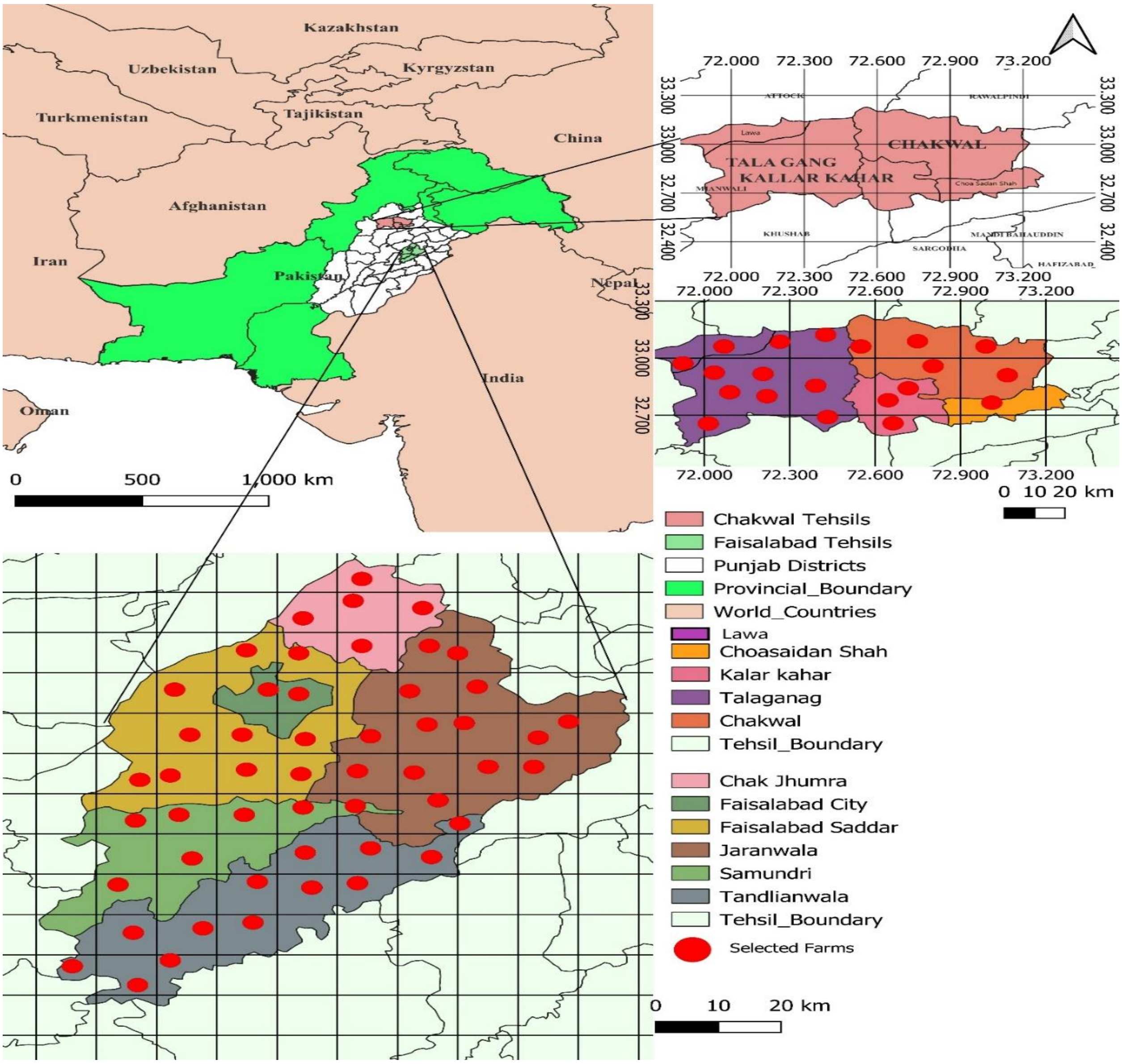


Fig. 1: Sample collection areas in district Faisalabad and Chakwal, Punjab, Pakistan.

PCR amplification, DNA sequencing, and phylogenetic analysis: PCR was performed using selected cytochrome C oxidase subunit 1 (COI) gene marker (Vrijenhoek, 1994) forward primer LCO1490 (5'- GGTCAACAAATCATAA AGATATTGG-3') and reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). Briefly, 50µl of the master mixture was used with the Taq polymerase enzyme. The amplification process began with an initial denaturation at 95°C for 5 minutes, followed by 40 cycles consisting of denaturing at 95°C for 15 seconds, annealing at 53°C for 15 seconds, and extension at 72°C for 45 seconds. The process concluded with a final extension at 72°C for five minutes (Ghafar *et al.,* 2020a). The final product was run on 2% agarose gel and amplicons were visualized using UV light. Sanger sequence (STABVida, Portugal) in both directions was done, and the generated sequence was compared using the Basic Local Alignment Search Tool (BLAST). All the unique sequences with more than 90% similarity were selected for onward submission to the NCBI for accession numbers. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

Statistical analysis: Prevalence was estimated as a percentage and the association of risk factors was analyzed using the Chi-Square test and T-test with statistical significance set at $P<0.05$. The regression model was used to correlate the effect of weather on tick prevalence.

RESULTS

Prevalence of ticks: In both districts two species of ticks were identified in cattle *i.e.*, *R. microplus* and *H. anatolicum.* The overall prevalence in both study areas was calculated at 69.46% (514/740). There was a significantly higher prevalence of 80.54% (298/370) in Chakwal than in Faisalabad 58.38% (216/370) as the Chi-square value was 42.83 ($P<0.05$). Specie-wise, a higher prevalence of *H. anatolicum* 72.69% in Faisalabad was recorded than in Chakwal (24.83%). The prevalence of *R. microplus* was significantly higher in Chakwal (66.11%) compared to Faisalabad (19.91%), with the difference being statistically significant (P<0.05). In Faisalabad, the highest prevalence was in tehsil Jaranwala (67.14%) while least in Jhumra (63.16%). Similarly in Chakwal the highest prevalence was noted in Choa Sadan Shah (94.37%) and lowest in Kalar Kahar tehsil (68.65%).

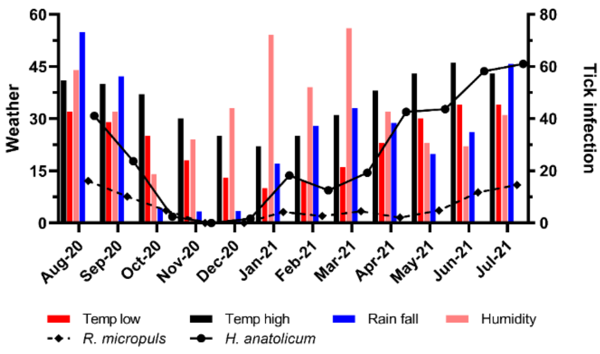
The tick infestation was higher during the hot and humid months (May and August) in both districts. The tick prevalence in both districts followed an almost similar trend throughout the year although the tick burden was statistically higher in Chakwal as compared to Faisalabad (P<0.05). The difference in trend was observed from October to January when the tick infestation was low (1.67 to 4.8 %) in Faisalabad. During the same period in Chakwal high tick prevalence was noted that was statistically significant (P<0.05). The effect of temperature and rainfall on tick infestation in both districts can be predicted by using the equations (Fig. 2; Fig. 3). The regression model summary calculated S= 1.598 R-sq 94.65%, R-sq (adj) 91.59% and R-sq (pred) 83.67%.

Associated risk factors: Higher infestation was found in young animals *i.e.*, 67.06 and 84.25% in Faisalabad and Chakwal, respectively. The least infestation was determined in adult animals although the age difference of animals was statistically non-significant (P>0.05). Gender was also checked for tick infestation and found a positive relationship in the case of animals in Faisalabad as the chi-square value was 8.29. Male animals had a higher prevalence (71.59%) than female animals (54.26%) (P<0.05). Whereas it was found non-significant in Chakwal where females have a higher rate of infestation (83.11%) than males (76.82%) (P>0.05). The breed of animals was found to be the most significant intrinsic factor. The highest infestation rate was in exotic animals (62.41%) followed by crossbreed (61.21%) and local (44.44%), which was statistically significant (P<0.05).

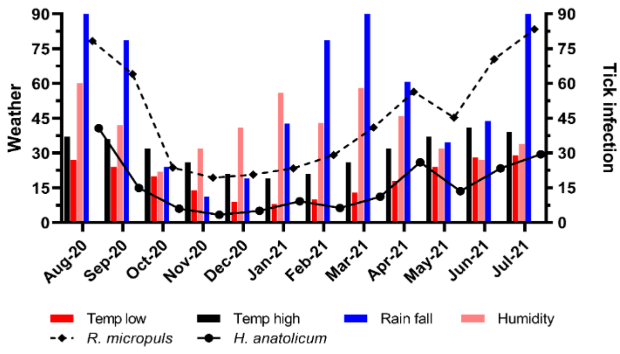
The housing of the animals is the most important factor that influences the prevalence of ticks in both districts. The minimum tick infestation was seen in close farms (53.02% and 78.16%) where control measures can easily be adopted in both districts. Followed by mixed farms (68.42% and 82.48%) and the highest in open farms (64.29% and 80.14%) in open animal farming. The structure of farm walls played a crucial role, showing a high Chi-square value of 60.03%. Tick infestation was highest in farms with non-cemented walls, reaching 76.35% in Faisalabad and 82.06% in Chakwal. Cemented walls in both districts had the lowest infestation rate. Similar results were seen in floor structure effect significantly (P<0.05), least in cemented floors (29.03%) and highest in non-cemented (83.4%). Management of the farm had a key role in limiting disease and keeping them profitable. Farms with good management had the least infestation of ticks (41.86 and 54.95%) in both districts followed by poorly managed farms. The feeding pattern of animals was another factor that influenced tick infestation and tick prevalence was higher in grazing animals (79.17 and 85.16%) than in semi-grazed (68.75 and 79.87%) and zero-grazed (54.58 and 74.7%) animals in Faisalabad and Chakwal. Farms where acaricides were used regularly had less infestation (42.4%) in Faisalabad while 70.63% in Chakwal. The most significant factors among all discussed factors are walls and floor structure, acaricide use, and overall management. The Chi-square values for these factors were above 50. Other factors like gender, breed, housing, and feeding pattern also affect tick infestation but their maximum Chi-square values were 11.06.

The questionnaire response from the farmers along with their degree of association is shown in Table 1. Questions with their close-ended answers and tick prevalence at the farms in both districts show a statistically significant relation to these practices (P<0.05).

Phylogenetic analysis: A uni-locus analysis of *H. anatolicum* and *R. microplus* was achieved with COI sequence and compared with COI sequences available in NCBI (GenBank). The phylogram studied identified the *H. anatolicum* as a monophyletic group, with the sequence generated in this study clustering within this clade C. The phylogram based on the COI gene analysis identified the subgenus *H. anatolicum excavatum* as a monophyletic group, with the sequences generated (Fig. 4). The studied phylogram identified the *R. microplus* as a monophyletic group, with the sequence generated in this study clustering within clade C. The phylogram based on the COI gene analysis identified the subgenus *Boophilus* as a monophyletic group, with the sequences generated in this study clustering within clade C (Fig. 5).



$$Tick\ infestation = 2.18 - 1.04\ \text{t high} + 1.48\ \text{t low} + 0.16\ \text{humidity} + 0.04\ \text{rain fall}$$



$$Tick\ infestation = 4.37 + 3.40\ \text{t high} - 1.26\ t\ low + 0.26\ \text{humidity} + 0.21\ \text{rain fall}$$

Fig. 2: Month-wise prevalence of *Hyalomma anatolicum* and *Rhipheceplus microplus* with climatic variation in Faisalabad

Fig. 3: Month-wise prevalence of *Hyalomma anatolicum* and *Rhipheceplus microplus* with climatic variation in Chakwal

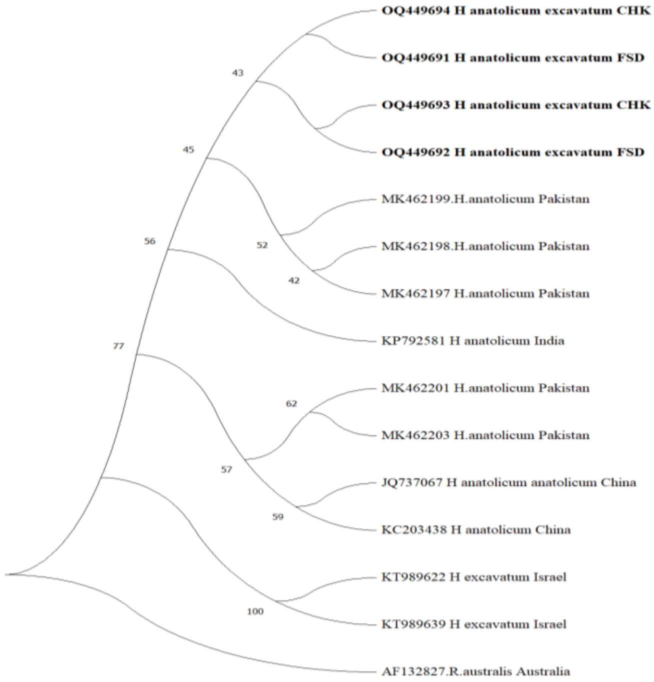


Fig. 4: A Maximum Likelihood tree was generated using CIO sequences, with the sequence formed in this study for *H. anatolicum* highlighted in bold. Bootstrap values are shown at each node.

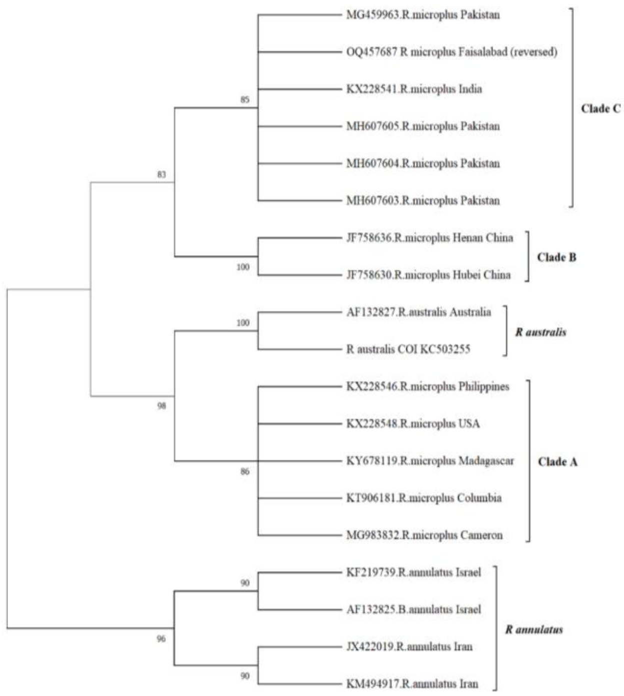


Fig. 5: A Maximum Likelihood tree was generated using CIO sequences, with the sequence formed in this study for *R. microplus* highlighted in bold. Bootstrap values are shown at each node.

Table 1: Questionnaire responses of farmers for tick control and point prevalence of tick infestation in Faisalabad and Chakwal

Sr. No.	Question	Options	Farmer Response		Chi-square	P-value	Tick Prevalence %		Chi-square	P-value
			Faisalabad	Chakwal			Faisalabad	Chakwal		
1	Animal keeping	Lose	18	37	14.59	0.000	26.7	54.28	0.94	0.009
		Tie	32	13			18.41	38.5		0.027
2	Feeding	Stall feeding	28	6	24.32	0.000	12.83	24.6	5.84	0.043
		Grazing	13	35			34.28	67.4		0.023
		Mix	9	9			29.49	56.25		0.104
3	Watering	Fresh	41	16	29.49	0.000	16.72	13.9	1.24	0.024
		Pounds	2	23			18.63	26.4		0.022
		Mix	7	11			20.5	23.2		0.033
4	Walls	Solid bricks	27	18	30.93	0.000	34.67	32.6	2.74	0.019
		Stones	0	23			NIL	46.03		0.040
		Mud/Chapar	23	9			48.76	64.25		0.026
5	Floor	Cemented	17	6	15.08	0.001	19.2	13.7	2.96	0.018
		Non cemented	19	11			57.6	42.93		0.008
		Sandy	14	33			63.4	54.06		0.051
6	Housing	Open	11	2	10.10	0.006	24.8	22.65	1.48	0.036
		Close	18	31			13.25	40.8		0.016
		Both	21	17			16.53	36.2		0.028
7	Management	Very poor	9	13	2.60	0.458	74.8	84.7	7.17	0.044
		Poor	24	19			63.41	72.05		0.021
		Good	14	17			37.6	46.3		0.041
		Excellent	3	1			12.23	14.6		0.044
8	Treatment	Vet	16	23	2.66	0.448	14.9	16.35	9.33	0.032
		Herbal	11	9			38.41	40.02		0.046
		Acaricide	21	15			26.7	36.93		0.011
		No treatment	2	3			67.6	86.8		0.032
9	Frequency of treatment	2 months ago	26	31	15.66	0.004	13.9	19.5	15.851	0.012
		4 months ago	16	2			43.2	57.06		0.027
		6 months ago	7	14			59.63	72.9		0.032
		Year ago	1	1			80	92.33		0.029
		Never	0	2			NIL	94.65		0.040
10	Method of treatment	Injection	14	35	21.89	0.000	36.4	42.6	3.52	0.017
		Spray	23	9			28.25	36.4		0.037
		Both	13	4			23.4	32.95		0.040
		Nothing uses	0	2			NIL	92.33		0.028
11	Farmer education/awareness	Aware	18	6	9.56	0.023	16.8	24.06	6.28	0.018
		Moderate	13	15			24.9	32.45		0.026
		Slight	17	22			46.2	58.6		0.009
		Not aware	2	7			78.2	64.9		0.046

12	Animal density	Congested	9	17	4.68	0.096	62.7	78.9	3.67	0.011
		Moderately	18	19			41.05	56.33		0.032
		Spacious	23	14			32.5	39.6		0.012

DISCUSSION

The overall prevalence of ticks in both study areas was 69.46%, with a significantly higher prevalence in Chakwal compared to Faisalabad, which could be due to the wide host range of ticks, auspicious geo-climate and vegetation index. These factors have also been reported previously as conceivable risk factors for higher tick infestation (Gharbi *et al.*, 2013; Rehman *et al.*, 2017; Ali *et al.*, 2019; Parveen *et al.*, 2021; Tantrawatpan *et al.*, 2022; Ergunay *et al.*, 2024). The suitable micro and macro environmental factors help in the development and propagation of ticks that lead to higher prevalence. One most prominent leading factor is the lack of farmer awareness about host susceptibility and tick (Kariam *et al.*, 2017; Tesfaye and Abate 2023), which appears to be a significant factor in the study area because of the lack of technical education and inadequate extension services. This was also reported in previous studies conducted by researchers in Pakistan and worldwide (Kabir *et al.*, 2011; Mustafa *et al.*, 2014; Rothen *et al.*, 2016; Khan *et al.*, 2022; Maqbool *et al.*, 2022). Among positive animals of Faisalabad, the *Hyalomma* spp. was 72.68% while *Rhipicephalus spp*. 19.91% and mixed infestation in 7.4%. Maqbool *et al.* (2022) reported tick prevalence in Faisalabad at 85 and 12% of *Hyalomma* spp. and *Rhipicephalus* spp. respectively. The higher prevalence of *H. anatolicum* (52.20 %) was also reported in Indian Punjab by Haque *et al.* (2011) while 20.45% of *R. microplus.* Chakwal there was a higher prevalence of *Rhipicephalus* spp. 66.10% than *Hyalomma* spp. (24.83%) and mixed infestation was 9.06%. A similar finding was reported by Batool *et al.* (2019) that central Punjab had a higher prevalence of *R. microplus* due to a higher population of cattle than buffalo, and other climatic variations between these two zones. Rehman *et al.* (2017) reported that there was a higher prevalence of *Hyalomma* spp. (94.3%) than *Rhipicephalus spp.* (3.1%) ticks in irrigated districts like Okara, Khanewal, Multan, Vehari and Bahawalpur. While semi-arid districts Attock, Gujrat and Khushab) had more prevalence of *Rhipicephalus spp.* (64.1%) than *Hyalomma* spp. (35.9%). Islam *et al.* (2006) reported similar findings in Bangladesh where a higher prevalence of *R. microplus* (56.3%) than *H. anatolicum* (15.0%). Gosh *et al.* (2007) also reported this species distribution in Pakistan, Bangladesh and India which shared almost similar climates and topography. In Chakwal the prevalence of *R. microplus* (66.10%) was high, this variation was due to host difference. There was a seasonal variation in tick prevalence, the highest infestation was observed during the summer season, June to August (58.26 to 41.11%) in Faisalabad, while June to September (80.47 to 64.05%) in Chakwal. Higher infestation during these months was due to the moon soon season when the climate was hot and humid. The lowest infestation was during the winter season 4.8% in Faisalabad and 23.42% in Chakwal, these findings were in line with those reported earlier (Ali *et al.*, 2013; Batool *et al.*, 2019; Rehman *et al.*, 2019; Kumar *et al.*, 2020; Zeb *et al.*, 2020). In Chakwal the infestation rate for both above mentioned ticks was higher than in Faisalabad even during the winter when there was infestation went to zero these findings were aligned with Kabir *et al.* (2011) who reported a high prevalence in the winter of 31.5% in Bangladesh. This was due to other factors like temperature, humidity, vegetation index, and more rainfall than in Faisalabad. The microenvironment was also a crucial factor in the internal farm environment (Gharbi *et al.*, 2013). Chakwal is a semi-hilly area with moderate temperatures but due to global warming in the past decade. A study conducted in Pakistan and reported that *R. microplus* is the most abundant species in northern areas (cold) of Pakistan (Sultan *et al.*, 2022). Other studies conducted in Taiwan and India reported that *H. anatolicum* is the most abundant species and the second most abundant species is *R. microplus* (Singh and Rath, 2013; van Oort *et al.*, 2020). Similarly, other studies conducted in other provinces of Punjab and concluded that *R. microplus* is the most abundant species in these regions (Parveen *et al.*, 2021; Rehman *et al.*, 2017). The high prevalence of *R. microplus* and *H. anatolicum* in these regions may be due to variations in climatic conditions (Ali *et al.*, 2019). Tick infestation is also associated with the age and grazing of the animals in different seasons and different regions. Similar kinds of results have also been shown in various previous studies (Kariam *et al.*, 2017; Jamil *et al.*, 2021; Rafiq *et al.*, 2022). In our study high tick prevalence was observed in young cattle and it may be because young animals get less attention as compared to adult or productive animals. Another major reason is that adult animals are much stronger in immunity and have less chance of resistance as compared to young cattle. In calves, the tick infestation is lower and the reason for this low infestation may be due to the grooming and smaller body size. Smaller body size provides less surface area for the attachment of the ticks, hence low infestation (Mooring *et al.*, 2000). Furthermore, grazing cattle have more chances of tick infestation as compared to farm animals where cattle are restricted to one specific area and boundary and there are no chances of exposure to heavily infested animals (Rehman *et al.*, 2017; Jamil *et al.*, 2021). Similarly, animals without acaricide treatment are more prone to tick infestations compared to the animals that are treated with chemical acaricides, which correlates with a previous study in which regular treatment of acaricides has low infestation as compared to non-treated animals (Rehman *et al.*, 2017; Batool *et al.*, 2019). Furthermore, the breed of the animal was also the main limiting factor of tick infestation, in the current study cross-bred were less prone to infections as compared to local breeds. This study was in contrast with the previous studies where more tick infestation was observed in cross-bred animals as compared to local breeds (Kakar *et al.*, 2017; Kumar *et al.*, 2020; Zeb *et al.*, 2020). This variation could be due to more care from the farmers for high producer animals. Gender and sex also play an important role in tick infestations. In a recent study males in different areas of Faisalabad showed heavy tick infestation but when a similar study was conducted in Chakwal it was confirmed that female cattle in Chakwal showed heavy tick infestation as compared to males. This may be due to the fact that during the milking period, the nutrient level and immunity in females is decreased and they are more prone to infection (Santos *et al.*, 2024). Housing and management are two other factors that affect the prevalence of the ticks. The farmed animals having cemented floors have less chance of infestation while the open-housed animals have more chance of tick infestation. This may be because open-housed animals provide greater area and space for the ticks to grow and survive in multiple places (Quintero *et al.*, 2021). In our study, it was concluded that open-housed animals have shown heavy tick infestation as compared to farmed and well-managed animals.

A few research studies have tried to genetically characterize the data to find the resistant species but due to the limited availability of genetic markers like COI and ITS-2 scientists are not able to determine the intensities and frequencies of different species. The phylogenetic analysis of *H. anatolicum* tick showed subgenus *H. anatolicum excavatum* that there was more than 99% match with the previously reported sequence in Pakistan, India, and China, whereas the ticks had a genetic similarity of 82.7% with Australian ticks 96.9% in Israel (Burger *et al.*, 2014; Roy *et al.*, 2018). The COI gene is sufficient to identify variability between various genetic assemblages of *R. microplus* and *H. anatolicum*. The *R. microplus* clade C sequence was 100% matched with the previously reported sequence in Pakistan and India, whereas it was 95% matched with a different subspecies *R. annulatus* from Israel, Iran, and 88% with *R. australis* Australia (Tantrawatpan *et al.*, 2022). The species in our study had also 91.7% similarity with the *R. microplus* clade A of the USA, Philippines, Columbia, Madagascar, and Cameron. While the *R. microplus* clade B was reported in China with the same result. This dispersion of species might be due to human activities and interaction with the globe. These two factors will lead to more genetic flow into different areas of Pakistan and around the globe.

Conclusions: Our result concluded that *R. microplus* and *H. anatolicum* are dominant species in cattle of Faisalabad and Chakwal regions. Extrinsic and intrinsic factors are the dominant risk factors that play an important role in heavy tick infestations in both regions. It was confirmed that the COI genetic marker could be used as a standard marker in finding *R. microplus* and *H. anatolicum.* These findings give greater distribution of both species in different regions of Faisalabad and Chakwal. Furthermore, it was concluded that variations in management practices across different agroecological regions of Pakistan can influence tick prevalence. The presence of ticks during winter increases the risk of economic losses to the livestock industry. Therefore, conducting further studies in neighboring regions of Punjab is crucial to developing improved husbandry practices that mitigate the risk of ticks and tick-borne diseases, especially in the context of a changing climate.

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Authors contribution: MA Executed the study and wrote the initial draft; ZuDS Planned and supervised the study; MAZ Supervised the study and wrote the initial draft; MS Reviewed and finalized the manuscript.

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