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MORPHOMOLECULAR IDENTIFICATION AND SPATIOTEMPORAL DISTRIBUTION OF HARD TICKS INFESTING CATTLE: A LIVESTOCK AND PUBLIC HEALTH CONCERN IN SELECTED LOCALITIES OF DISTRICT SWAT, KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract
In Pakistan, ixodid ticks have a variety of distribution patterns. Globally, ticks are significant vectors of numerous diseases affecting people, livestock, and other animals. Tick infestations cause significant problems in Pakistan's livestock industry, the country's principal source of rural income. They significantly harm the cattle business by causing hide loss and spreading vector-borne diseases. The distribution and way of life of ticks are significantly influenced by factors like the climate, host accessibility, and the environment. From March 2022 to October 2022, this study was carried out in selected localities of the district Swat. By using a practical sample technique, 2217 ticks were collected from 561 infected cattle of diverse ages and genders. With the help of molecular techniques and morphological characteristics, tick identification was done down to the genus and species level. Three species were identified in which the most prevalent species was Rhipicephalus microplus (40.78%) followed by Hyalomma anatolicum (31.93%) and Hyalomma marginatum (27.29%). Several risk variables were significantly correlated with the prevalence of all ixodid tick species (age, gender, living conditions, area, etc.). The distribution of tick infestation by body region showed that external genitalia (34.93%) was the most popular location, followed by the udder (26.02%), neck and head regions (20.68%), ear and tail (9.63%), shoulder (6.78%), and rear legs (1.96%). Additionally, mature, frail, and female animals are more susceptible to a tick infection. In Swat, the cattle population was primarily infested by the cattle tick Rhipicephalus microplus, and the summer was the tick season with the highest tick load records. In the current investigation, genetic markers (16S rRNA, ITS2, and COX1) were used to successfully amplify the targeted genes of the three tick species. The study highlighted the importance of molecular methods in epidemiological, medical entomological, and parasitological research, as well as the control of tick-borne diseases, which will ultimately improve public safety and livestock health.

INTRODUCTION

Ticks are thrilling blood-sucking ectoparasitic arthropods of the order *Acari* that infest both people and animals (Nasirian, 2022). They can infest mammals, birds, and occasionally reptiles (Jaffar *et al*., 2022). Three families, scattered over the globe, make up their composition. Many species are members of the Ixodidae family, popularly known as "hard ticks". Argasinae and Ornithodorinae, collectively known as "soft ticks" are two subfamilies of the Argasidae family. There is only one species in the Nuttalliellidae family (Defaye *et al.,* 2022). *Dermacentor*, *Haemaphysalis*, *Rhipicephalus*, *Hyalomma*, and *Amblyomma* are all important veterinary genera (Hurtado & Giraldo, 2018). Ticks are deadly dermal parasites that can either directly or indirectly ruin blood loss, milk supply, and the development of numerous diseases. Diseases caused by protozoans, viruses, and bacteria that are spread by ticks within the animal in dairy and meat animals include theileriosis, babesiosis, and hemorrhagic fever (Jaffar *et al*., 2022). The tick families Ixodidae, Argasidae, and Nuttalliellidae have been classed as economically significant. There are 949 recognized species in the Ixodidae, 200 in the Argasidae, and only one in the Nuttalliellidae (Hussain *et al*., 2021). All around the world, terrestrial animals are obligately infected by ticks (Acari: Ixodida), which feed on blood. They can directly harm cattle by inducing itchiness, sensitivities, and immobilization, or they can indirectly harm livestock by spreading infections like protozoa, viruses, and bacteria (Ghafar *et al*., 2020). Through the direct consequences of their feeding on humans and as carriers of numerous disease agents in both humans and cattle, ticks are vital to human health. There are currently known descriptions of over 19 tick-borne diseases in cattle and companion animals, in addition to over 16 human diseases caused by ticks or transmitted by ticks (Zhang *et al*., 2019). Ticks and tick-borne disease (TBD) are thought to be a threat to 80% of the world's livestock population, which is primarily found in the subtropics and tropics. The re-emergence and dissemination of TBDs in animals and humans are also thought to be influenced by continuing seasonal and climatic changes (Theron & Magano, 2022).

Over 17% of illnesses and over 700,000 fatalities worldwide are a result of vector-borne diseases (VBDs) each year. Ticks and mosquitoes are the two most common vectors, in order of significance. In terms of human pathogens, ticks (Ixodida) rank second to mosquitoes in terms of veterinary vector-borne pathogens (Defaye *et al.,* 2021). Ticks are significant contributors to the emergence and reemergence of tick-borne illnesses such as babesiosis, rickettsiosis, anaplasmosis, ehrlichiosis, Lyme disease, relapsing and Q-fever diseases, and lethal arboviruses (Nasirian, 2022).

Animal husbandry provides 53.2% of the agricultural sector of Pakistan's economy and 11.4% of Pakistan's gross domestic product, demonstrating the country's importance as a livestock-raising nation in Asia (Aziz *et al*., 2022). The four most common illnesses spread by ticks are anaplasmosis, theileriosis, babesiosis, and cowdriosis (TBDs). The economy of Pakistan is most affected by the first three of these diseases. In Pakistan, the tick genera *Rhipicephalus*, *Hyalomma*, Haemaphysalis, Ixodes, Ornithodoros, and Argas are reported to transmit a variety of TBPs. The Khyber Pakhtunkhwa (KPK) province of Pakistan is a hotspot for emerging and recurrent TBDs with importance to veterinary and public health (Khan *et al*., 2022). Several studies from Pakistan have found that more than 80% of bovines were infested with ticks of the *Hyalomma* and *Rhipicephalus* species (Hussain *et al*., 2021).

Climate change-induced changes in plant cover and other habitat characteristics may provide new possibilities for tick larvae survival and possible northward translocation of these and other key tick species (Osbrink *et al*., 2022). Although Pakistan has recently done a variety of ecological and genomic studies on ticks and diseases transmitted by ticks, but all eco-epidemiological and the taxonomic aspects have not been covered. For example, an assessment of tick distribution across different biological zones indicated that *Hyalomma anatolicum* and *Rhipicephalus microplus* are the two most common tick species infesting ruminants in Pakistan (Zeb *et al*., 2019).

Ticks of all stages and species can be identified by morphological characteristics of the capitulum (including the hypostome), leg coxae, and scutum (Coley, 2015). Scutum, which covers the entire dorsal surface of the male but only a portion of the female, is the main physical characteristic of the hard tick. While it is absent from the Argasidae family and has a leathery body (Ismael & Omer, 2021). When distinguishing between related species complexes, morphological identification is insufficient, especially when the specimens are engorged, physically injured, or in immature stages. Thus, tick species can be characterized using molecular methods. Molecular characterization is the alternative method of differentiating between the closely related taxa and other *Rhipicephalus* ticks (Low *et al.*, 2015).

MATERIALS AND METHODS

2.1: Study area

The study was conducted in Northern Khyber Pakhtunkhwa's Swat Valley, which is encircled by soaring mountains (Figure 1). Based on physical characteristics, Swat is separated into two geographical zones: mountain ranges and plains. The seven tehsils of Swat are Babuzai, Barikot, Kabal, Matta, Charbagh, Khwazakhela, and Bahrain (Rasool *et al.*, 2018). The 5,337 km^2 Swat Valley is situated between 34°-40′ and 35° N latitude and 72′ to 74°-6′ E longitude (Ullah & Zahid, 2022).

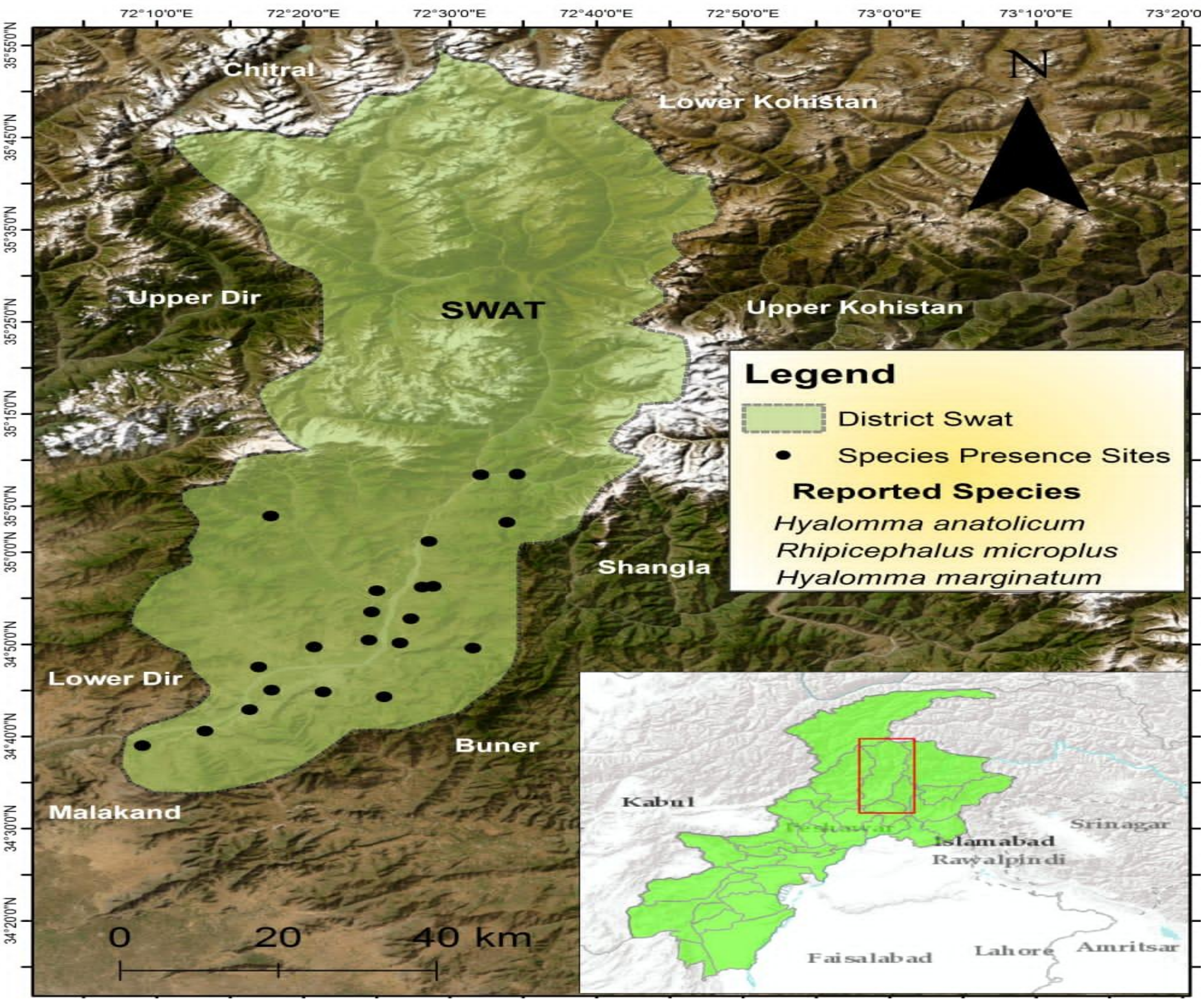

Figure 1: The map of Swat shows the selected regions of data collection

2.2: Ticks Collection and Preservation

A total of 770 cattle were observed randomly from different villages in seven tehsils of District Swat for tick infestation for a period of eight months with effect from March to October 2022. The animals' entire body, including their skin, as well as their head, belly, back, udder/scrotum, genital regions, leg, and tail, were carefully studied. With the aid of forceps and hand-picking, adult ticks and ticks in various phases of development were removed from infected animals and placed in separate glass bottles containing 70% alcohol for preservation. Each tube was labeled with the date, location, body parts, sex, season, living and health condition, and age of the animal.

2.3: Morphological Identification of Ticks

Tick specimens were identified using established taxonomic keys based on morphological traits using a stereo-zoom microscope (SZ61, Olympus, Tokyo, Japan). Tick species were recognised morphologically using hard tick morpho-taxonomic features.

2.4: Molecular identification

The genomic DNA of ticks was extracted using the phenol-chloroform method. The samples were run in 1% agarose gel for confirmation. In PCR, the following 20µl of the reaction mixture were used, including forward primer (1µl), reverse primer (1µl), master mix (12µl), PCR water (4µl), DNA template (2µl). The PCR reaction mixture was prepared in an ice container in a biosafety cabinet to avoid contamination. The primer sequences were generated using the Primer-Blast NCBI program, and the parameters of the primers (GC content and melting temperature) were evaluated using the PCR-Primer Stats tool.

The PCR was performed using a manual thermocycler (kyratec SC300). The primers of the 460-bp fragment (16S+1=CCGGTCTGAAACTCAGTCAAGT),(16S-1=GCTCAATGATTTTTAAATTGCTGT) of tick's species were used for amplification of the 16s rRNA gene under the following conditions: Initial denaturation was carried out at 94.0°C for 2:30 minutes, annealing at 54.0°C for 0.30s, extension at 72.0°C for 0.45s, post cycling extension at 72.0°C for 7 minutes, and final hold at 4°C. The thermocycler's top heater was set to 105 (deg C) for this reaction, and the reaction was completed in 34 cycles. For the amplification

of the ITS2 region, primers targeting an approximately 800-bp segment were employed: ITS2+1 (5′-CCATCGATGTGAATGCAGGACA-3′) and ITS2−1 (5′-GTGAATTCTATGCTTAAATCAGGGGGT-3′). The total PCR reaction for ITS2 took 35 cycles, with the following conditions for such primers: initial denaturation at 95.0°C for 2:45 minutes, annealing temperature and time at 55.0°C for 1 minute, extension at 72.0°C for 1.5 minutes, and post cycling extension at 72.0°C for 7.00 minutes. For the amplification of the COX1 region, primers targeting an approximately 800-bp segment were used: COX1+ (5′-CCGGTCTGAACTCAGATCAAG-3′) and COX1− (5′-TCAATGATTTTTAAATTGCTGT-3′). The complete PCR process for COX1 took 34 cycles, with initial denaturation at 95.0°C for 5 minutes, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final elongation at 72°C for 5 minutes. After the PCR reaction was completed, the sample tubes were removed and placed in an ice container to protect the product from destruction during the following phase (gel electrophoresis). To check the PCR product, 2.5µl was taken from each sample and combined with 2.5µl loading dye before loading directly into agarose gel wells. 5µl of DNA Gene Ruler was loaded into one well for measuring amplicon size in base pairs. For 40 minutes, a 400-ampere current was applied to the gel electrophoresis equipment at 120v. The dye front was used to track the movement of samples. The samples were run in a 2% agarose gel for confirmation.

2.5: Statistical analysis

SPSS and Microsoft Excel 2019 were used to analyze and compile the data. Through SPSS Chi-square test was used to find relations. The prevalence (P) was calculated using the following formula:

$$P= \frac{\textit{Number of infested cattle during specific time period}}{\textit{total number of cattles surveid}} x100$$

RESULTS

In the current study, n=2217 ticks were collected from seven tehsils: Babuzai, Barikot, Kabal, Matta, Charbagh, Khwazakhela, and Bahrain of the district Swat. A high number of ticks (n=421) were collected from tehsil Bahrain. Among all the tehsils, two genera of *Hyalomma* and *Rhipicephalus* were identified. Among two genera, three species; *H. anatolicum*, *Hy. marginatum* and *Rh. microplus* were identified. Although all three species were collected from all the tehsils, but the most occurring specie was *Rh. microplus* (40.78%) followed by *H. anatolicum* (31.93%) and *H. marginatum* (27.29%) respectively as shown in table 3.1 and figure 2.

Table 3.1: Ticks' species overall abundance in seven tehsils of Swat.

Tehsils	Villages	*Hy. anatolicum*	*Rh. microplus*	*Hy. marginatum*	Total
Babuzai	Saidu	17	16	14	47
	Odigram	31	18	22	71
	Kokarai	20	28	18	70
Barikot	Kota	42	33	27	99
	Manyar	48	29	33	110
	Barikot	24	36	26	86
Kabal	Kanju	20	20	29	69
	Kabal	19	27	25	71
	Ningolai	23	26	29	78
Matta	Sherpalam	30	36	36	102
	Matta	49	58	26	133
	Biha	10	136	28	174
Charbagh	Charbagh	29	27	23	79
	Gulibagh	21	65	56	142
	Taligram	34	36	20	90
Khwazakhela	Khwazakhela	51	52	52	155
	Shin	23	41	13	77
	Shalpin	46	68	30	144
Bahrain	Madyan	43	65	25	133
	Miandam	67	55	46	168
	Chail	61	32	27	120
Total		**708**	**904**	**605**	**2217**
Percentage		**31.93%**	**40.78%**	**27.29%**	**100%**

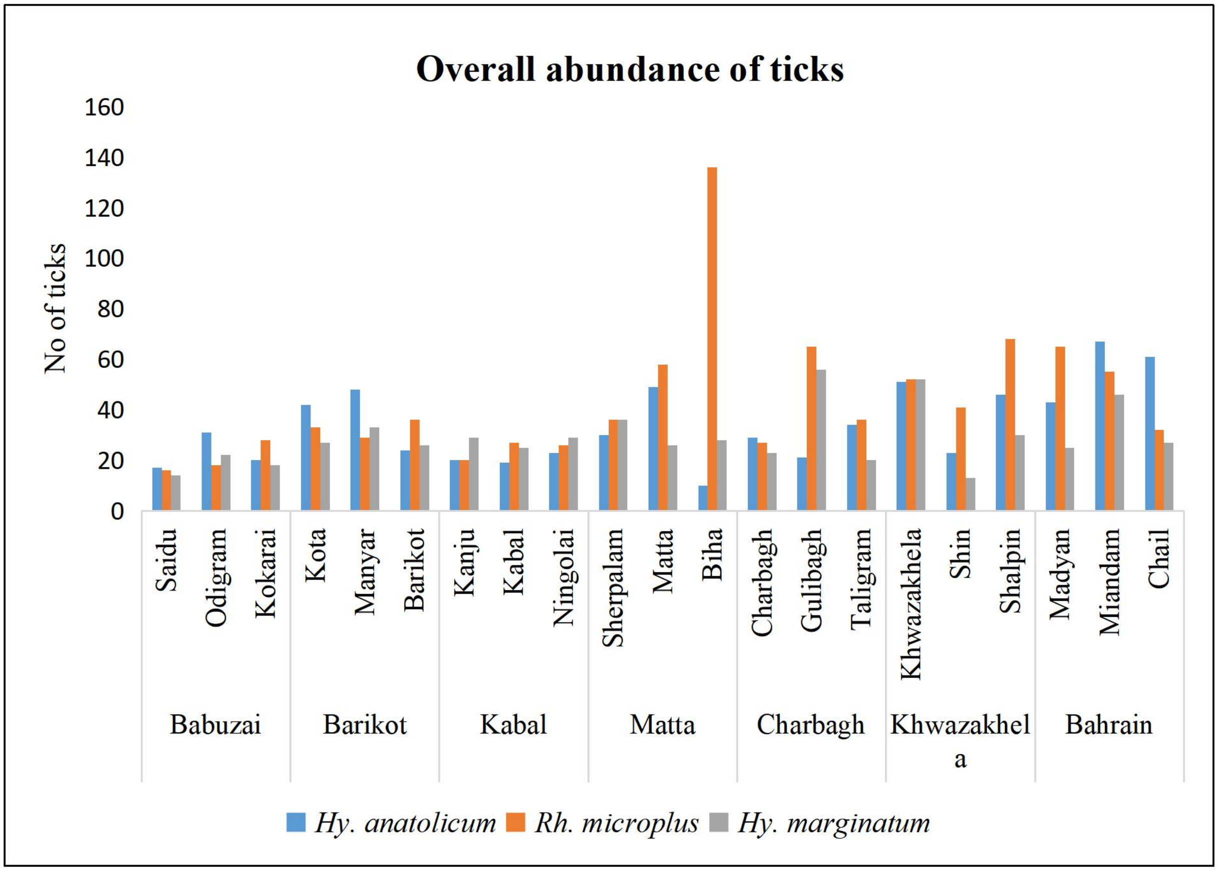

Figure 2: Overall abundance of ticks in seven tehsils

3.2: Gender-wise distribution of tick species

In this study, a total of 2217 ticks were collected, belonging to two genera, *Hyalomma* and *Rhipicephalus*, of which three species were identified. Among 2217 ticks, *H. anatolicum* were 708 (31.95), *Rh. microplus* were 904 (40.78%) and *H. marginatum* were 605 (27.28%). The adults (male and female) and nymphs were also studied, as shown in Table 3.2 and Figure 3.

Table 3.2: Distribution of ticks gender-wise

Species	Adults (N)		Nymphs (N)	Total (N)	Percentage (%)
	Male	Female			
Hy. anatolicum	182	334	192	708	31.95%
Rh. microplus	252	389	263	904	40.77%
Hy. marginatum	207	215	183	605	27.28%
Total	**641**	**938**	**638**	**2217**	**100%**

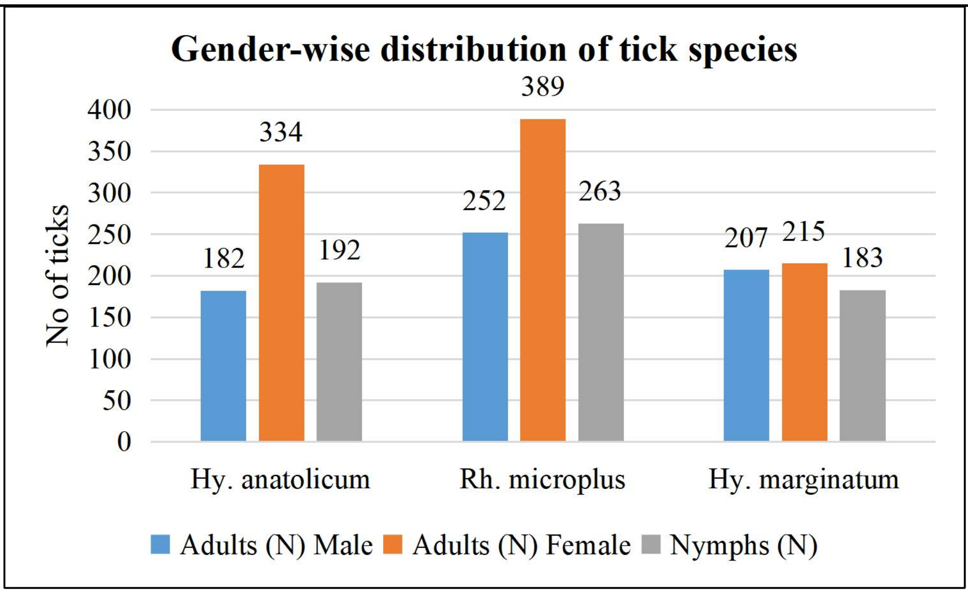

Figure 3: Distribution of ticks gender-wise.

3.3: Cattle Gender-wise prevalence of tick infestation.

A total of 770 cattle were observed for tick infestation, of which 415 (53.89%) were female and 355 were male (46.10%). A higher prevalence of tick infestation was recorded in female cattle than in males, as shown in Table 3.3.

Table 3.3: Cattle Gender-wise prevalence of tick infestation

Gender	Cattle examined (N)	Cattle Infested (N)	Cattle Non-infested (N)	Ticks Number	*(χ2)*	*P-value*
Male	355	189	166	909	128.186	0.0001
Female	415	372	43	1308		
Total	**770**	**561**	**209**	**2217**		

*Statistical analysis. The difference in the prevalence of tick infestation in the gender groups was statistically significant (P<0.05).

3.4: Age-related differences in tick prevalence on cattle.

A tick infestation was observed in 561 out of 770 cattle. During the study, the overall prevalence (72.85%) was reported. Younger female cattle of age <1 year had the lowest tick infestation (30.35%) than 1-5 years (45.45%), 6-10 years (68.47%), and 11-15 years (93.89%). Similarly, younger male cattle of age <1 year had the lowest (21.74%) tick infestation than 1-5 years (29.73%), 6-10 years (56.82%), and 11-15 years (74.12%) as shown in Table 3.4. The statistical analysis revealed a difference in tick infestation prevalence (P<0.05) between age groups of cattle.

Table 3.4: Age-wise prevalence of tick infestation in female cattle.

Female								
Female Age Group		**Cattle examined (N)**	**Infested Cattle (N)**	**Non-infested Cattle (N)**	**Prevalence (%)**	**Ticks number (N)**	X_2	***p*-value**
Young cattle	<1	56	17	39	30.35	86	99.719	0.001
Adult cattle	1-5	77	35	42	45.45	112		
	6-10	92	63	29	68.47	498		
	11-15	147	138	9	93.88	612		
Male								
Male Age Group		**Cattle examined (N)**	**Cattle positive (N)**	**Healthy cattle (N)**	**Prevalence (%)**	**Ticks number (N)**	X_2	***P*-value**
Young cattle	<1	23	5	18	21.74	72	32.441	0.001
Adult cattle	1-5	37	11	26	29.73	92		
	6-10	44	25	19	56.82	339		
	11-15	85	63	22	74.12	407		
*Statistical analysis. A significant difference (P<0.05) in the prevalence of tick infestation in different age groups of female and male cattle.								

3.5: Identification of the most prevalent tick species and their preferred places on cattle bodies.

The present study showed that the most commonly occurring tick *Rh. microplus* (40.78%) with predilection sites shoulder, external genitalia, abdominal area, neck, and udder, followed by *H. anatolicum* (31.94%) with predilection sites external genitalia, udder, ear, and tail, while *H. marginatum* (27.29%) was noted with predilection sites, external genitalia, udder, hind legs, head, and especially neck regions as shown in table 3.5. Among these external genitalia and udder were observed in most tick-infested regions of the body.

Table 3.5: Most prevalent tick species along their preferred sites

Ticks identified	Preference sites	No of ticks	Percentage (%)
Rh. microplus	Shoulder, external genitalia, udder, neck, and abdomen	904	40.78 %
H. anatolicum	External genitalia, udder, ear and tail	708	31.94 %
H. marginatum	Head regions, hind legs, and external genitalia	605	27.29%

3.6: Distribution percentage (%) of hard ticks on cattle various body parts

The present study revealed that among the 561 infested cattle, the most infested body region was external genitalia (34.93%) followed by udder (26.02%), neck and head region (20.68%), ear and tail (9.63%), shoulder (6.78%) and hind legs (1.96%), ear and tail (9.63%), shoulder (6.78%) and hind legs (1.96%) as shown in figure 4.

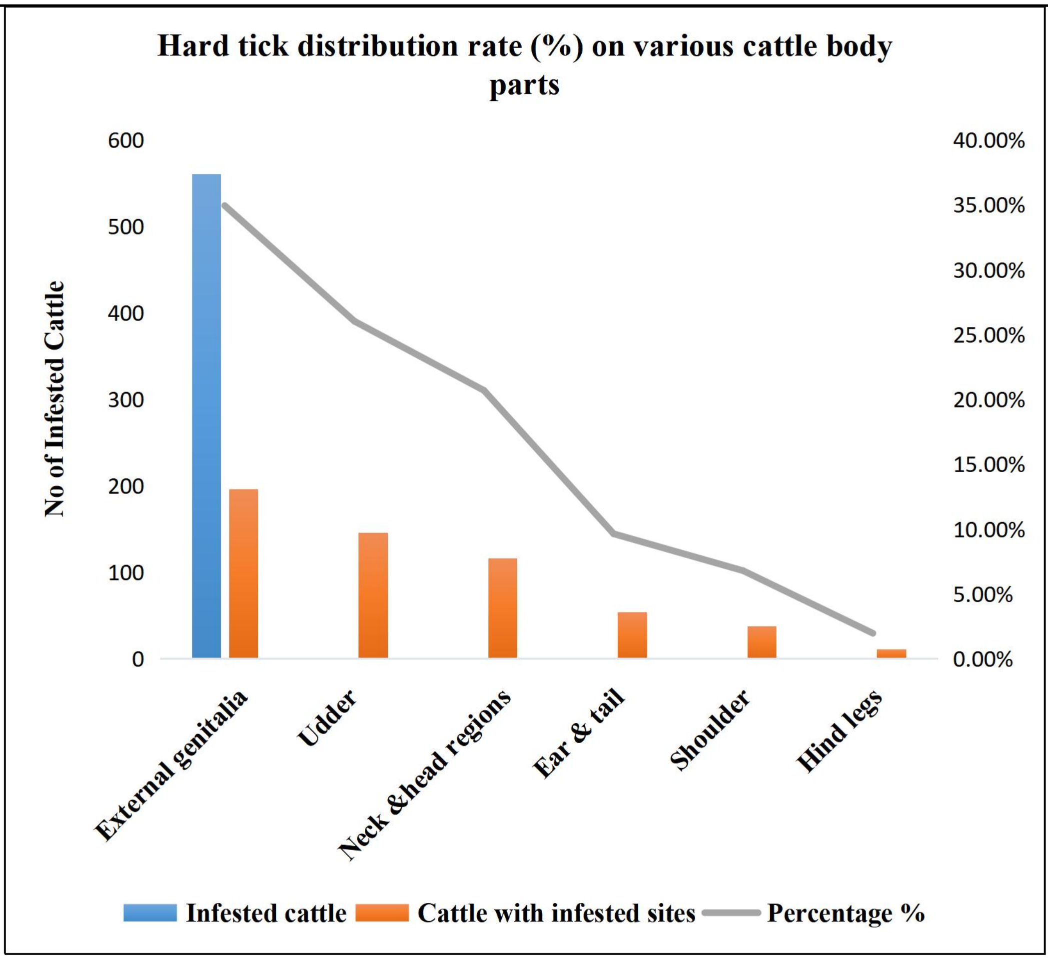

Figure 4: Hard tick distribution rate (%) on various cattle body parts

3.7: Tick prevalence based on certain factors associated with cattle.

The highest tick infestation was recorded in those cattle whose body condition was poor, In the present study out of 770 observed cattle, the poor health condition cattle had the highest tick infestation (96.46%), then those who were in good health (70.13%) followed by excellent body condition (40.22%). Compared to the animals kept in cement concrete houses, animals kept in standard-type houses had significantly greater tick infestations (87.28%). The animal maintained on an earthen floor had the highest ($p<0.05$) tick incidence (89.10%), followed by cement concrete (54.06%). The cattle feeding in group were significantly more tick infested (62.65%) than feeding individually (8.92%). In the case of the effect of environmental factors high tick infestation was observed in free-grazing cattle (97.20%) than in semi-grazing cattle (61.42%) followed by non-grazing cattle. Tick infestation was statistically substantially greater ($P < 0.05$) in free-grazing cattle compared to semi-grazing and non-grazing cattle in terms of the management approach. In terms of acaricide use tick infestation was lower in those cattle who were regularly treated (43.71%) than in irregularly treated cattle (76.81%) and the highest tick infestation was recorded in those cattle who were not treated with acaricide (88.81%) respectively. The cattle living in hilly areas were more tick-infested (90.43%) than those living in plain areas (51.99%) as shown in table 3.7. In this study, it was found that cattle raised in mountainous regions had significantly higher tick prevalence ($p < 0.05$) than cattle raised in plain areas.

Table 3.7: Tick prevalence based on certain factors associated with cattle.

Factors		Cattle observed (N)	Infested cattle (N)	Non- infected cattle (N)	Ticks (N)	Percentage (%)	X_2	*P*- value
Body condition	Excellent	179	72	107	375	40.22%	177.32	0.001
	Good	308	216	92	786	70.13%		
	Poor	283	273	10	1056	96.46%		
House type	Muddy	456	398	58	1539	87.28%	117.637	0.001
	Cement	314	163	151	678	51.91%		
Floor type	Earthen	413	368	45	1398	89.10%	118.902	0.001
	Cement	357	193	164	819	54.06%		
Feeding	Group	557	349	208	1667	62.65%	178.319	0.001
	Individual	213	19	194	550	8.92%		
Grazing mode	Free	286	278	8	994	97.20%	138.997	0.001
	Semi	267	164	103	756	61.42%		
	Non	217	119	98	467	54.84%		
Acaricides	No use	295	262	33	314	88.81%	125.603	0.001
	Irregular	276	212	64	892	76.81%		
	Regular	199	87	112	1011	43.71%		
Living Area	Hilly Area	418	378	40	1421	90.43%	142.797	0.001
	Plain Area	352	183	169	796	51.99%		

3.8: Seasonal prevalence of tick infestation

The result of the present study showed the highest tick infestation in summer, followed by Spring and Autumn. In a month-wise analysis, the highest infestation was recorded in June (89.10%) and July (93.37%). Ticks started appearing in March, and their number increased till the end of August, and then gradually decreased in number. Seasonal variations in the proportions of non-infested and tick-infested animals were found to be significant ($p < 0.05$) as shown in Table 3.8 and Figure 5.

Table 3.8: Seasonal fluctuation in tick prevalence

Seasons	Months	Cattle examined (N)	Cattle infested (N)	Cattle not infested (N)	Infestation rate	X_2	*p*-value
Spring	March	87	43	44	49.43	130.610	0.001
	April	70	54	16	71.14		
	May	97	71	26	73.19		
Summer	June	101	90	11	89.10		
	July	134	125	9	93.37		
	August	124	105	19	84.68		
Autumn	September	69	33	36	47.83		
	October	88	40	48	45.45		

*Statistical analysis: Highly significant difference.

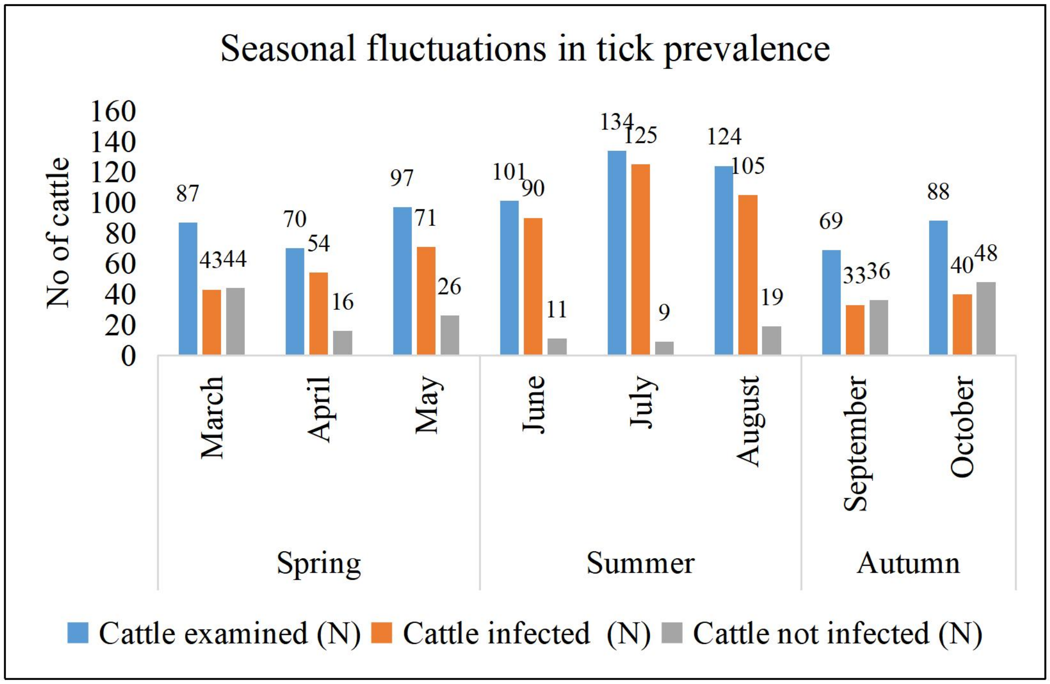

Figure 5: Prevalence of tick infestation based on seasonal fluctuations.

3.9. Molecular confirmation of ticks

3.9.1 Extracted DNA bands and PCR Results of ticks by Gel electrophoresis

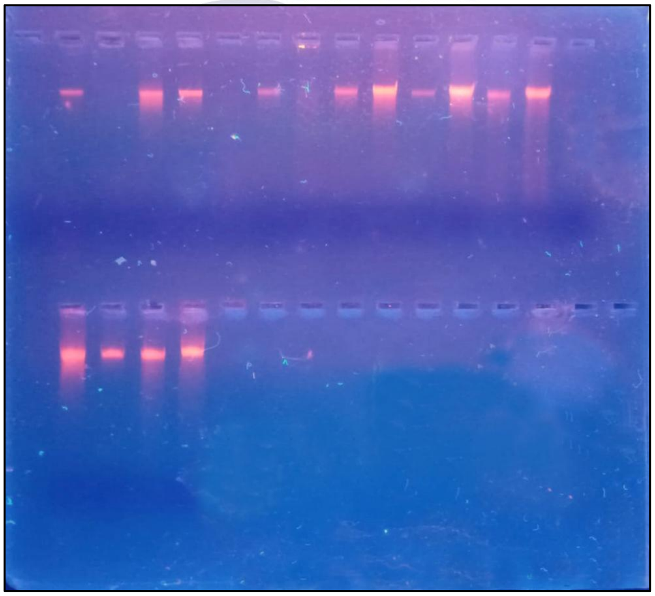

Figure 6: Extracted DNA of ticks

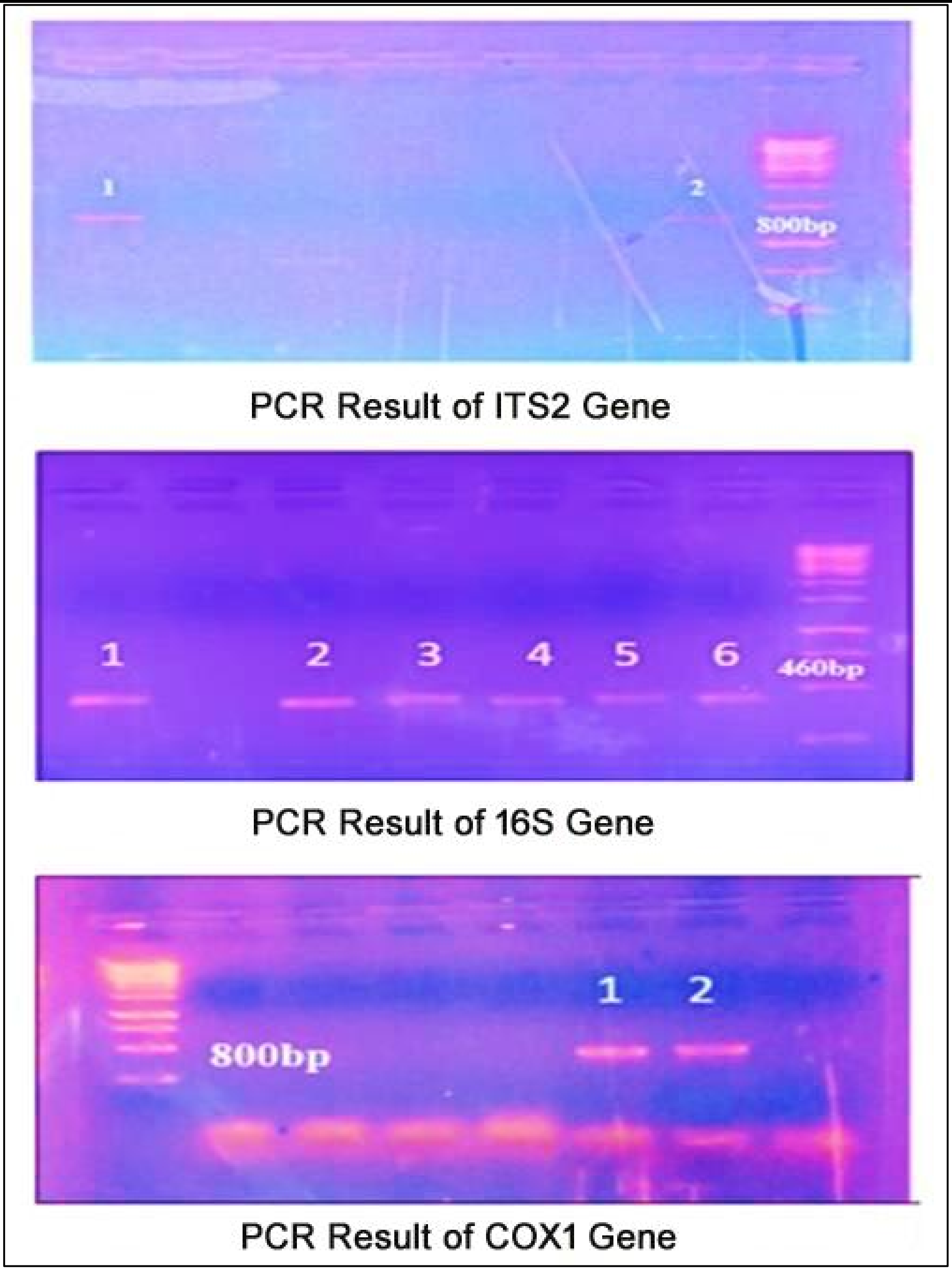

Figure 7: Showing molecular confirmation of the ticks

DISCUSSION

Livestock is important to the economy of Pakistan, a predominantly agricultural nation. A variety of tick species can grow and survive in Pakistan due to the country's favourable climate (Ali *et al.,* 2019). A hotspot for new and recurring TBDs that are significant to veterinary and public health is the Pakistani province of Khyber Pakhtunkhwa (Khan *et al.,* 2022). The livestock industry experiences considerable losses due to diseases spread by ticks on a global scale. They have a significant economic impact since they result in decreased productivity, decreased worker effectiveness, and fatalities (Shoaib *et al.*, 2022).

The primary goal of the current study was to examine the geographical distribution of ticks, molecular confirmation of ticks, their infestation in cattle and to evaluate the risk of diseases for livestock and public health of the area. Tick and TBDs distribution have greatly impacted by environmental fluctuations (temperature, animals' interaction, habitat changes), as the evidence shows that the mean temperature of the world will likely increase by 1.5°C (2.7°F), such that a rise in temperature has a long-term impact on tick distribution (Leger *et al.*, 2013). The highest tick species infection during this study was recorded from June to August (summer) due to rainfall and vegetation availability, which creates a suitable environment for ticks' life stages. Mean temperatures were detected, which demonstrate a strong correlation. The lowest infestation was observed from September to October, and like earlier findings, the same reports were made during that time (Kamran *et al.,* 2021 and Ali *et al.*, 2021).

Seven tehsils of district Swat were studied in which Bahrain district has highest tick burden (18.98%) followed by Matta (18.55%), Khwazakhela (16.96%), Charbagh (14.04%), Barikot (13.44%), Kabal (9.83%) and Babuzai (8.29%). Among seven tehsils, three medically important ticks' species; *Rh. microplus, H. anatolicum* and *H. marginatum* were reported from 770 cattle hosts.

This study revealed that *Rhipicephalus* is the most prevalent genus followed by *Hyalomma*, hence our results correlate with (Shoaib *et al.,* 2022; Farooqi *et al.*, 2017 and Haque *et al.*, 2011). The species *Rh. microplus* was found to be the most prevalent tick species in the current study. A similar study was conducted in Taiwan and India and reported to have greater *Rh. microplus* prevalence rates (Tsai *et al.*, 2011; and Rath, 2013). *Rh. microplus*, however, was the second-most prevalent species after *H. anatolicum*, according to studies from other Pakistani provinces; Punjab and Baluchistan (Sajid *et al.*, 2009; Ali *et al.*, 2013; Sultana *et al.*, 2015; Rafiq *et al.*, 2017 and Rehman *et al.*, 2022). The varying ecological conditions in the several provinces may be the cause of this variance in species distribution. For instance, higher tick prevalence is favored by the temperature in arid and semi-arid regions (Estrada-Pena *et al.*, 2006; Kapur *et al.*, 2008; Jabbar *et al.*, 2015 and Ali *et al.*, 2019). In the studied area, hard tick prevalence in cow populations is demonstrated by the current study. Due to the existence of a rich host species, dense vegetation, and favorable geoclimatic conditions, cattle had a higher tick infestation (72.85%) (Teel *et al.*, 1996; Gray, 2002; Bianchi *et al.*, 2003; Jouda *et al.*, 2004 and Greenfield, 2011).

The prevalence of tick infestation is also influenced by the gender of the cow. Compared to male animals, female animals in this study had a higher prevalence of tick infection. Similar results were confirmed by (Kabir *et al.*, 2011), who discovered that female cattle had a significantly higher prevalence of tick infection (59.37%) than male cattle (35.83%). Female animals were discovered to be 2.61 times more vulnerable than males. Although the precise reason for the higher incidence of tick infestation in females is unclear but the higher prevalence might be due to hormonal effects, immunosuppression during pregnancy and lactation, and stress (Kakar *et al.*, 2017). The findings, however, do not support (Atif *et al.*, 2012) conclusion, who found that males (56.46%) had a higher frequency of tick infestation than females (54.17%). The current findings also disagreed with (Musa *et al.*, 2014 and Hitcheock, 1993), who argued that male cattle in the tropics become more tick-infested than female cattle because they are used for most farming duties and moved around in search of food and female tick infestations are less common in the tropics because the females are primarily restricted for reproductive purposes. Even still, the precise reason for the greater frequency of tick infestation in female cattle remains a mystery, although it is conceivable that this phenomenon may be linked to some hormonal factors like the body's prolactin and progesterone levels increase an individual's susceptibility to infections.

Tick development and growth are facilitated by these favorable environmental variables, which results in greater prevalence rates. The lack of knowledge among farmers on tick control and host susceptibility may also contribute to the high prevalence of ticks (Pinheiro *et al.*, 2010). The study revealed that male ticks had a lower ratio than female ticks. Our results mismatch with (Telmadarraiy *et al.*, 2010). Male ticks stay attached to the host for a longer period than female ticks do because they feed on the host for a longer period and spend more time mating with other female ticks before dropping off to the ground after finishing their blood meal, this characteristic may be the reason that male ticks are more dominant than females (Gebre *et al*., 2001).

Age and grazing were identified as two potential risk factors for greater tick infestation after analysis of host and environmental factors related to tick infestation. Similar outcomes have also been recorded in the past from several regions of Pakistan (Durrani and Kamal, 2008; Khan *et al*., 2013; Karim *et al*., 2017 and Rafiq *et al*., 2017). The host animal's age significantly affects the pattern of tick infection (Manan *et al*., 2007). Current study showed that younger animals of age <1 year had the lowest infestation than adult animals of age group 1-5 years, 6-10 years age group and age group of 11-15 years. Similar outcomes were also reported by (Patel *et al*., 2013 and Kaur *et al*., 2015). Among old animals, the tick infestation recorded than other age groups. The findings of the current study are reliable with those of (Khan *et al*., 2022 and Kakar *et al*., 2017), who found that adult animals older than 5 years had the highest prevalence of tick infestation (71.61%). whereas the youngest animals (20.80%) had the lowest percentage. Kakar *et al.,* (2008) acknowledged the significance of colostrum feeding in calf production of antibodies against illnesses. Strong innate immunity in calves is thought to be advantageous for reducing tick exposure.

The results of the current investigation demonstrated that ticks can be found in various body parts in varying numbers. Tick infestations were observed in abundance throughout the udder, including the teats, perineum, and external genitalia. The most prevalent tick species found in the current study was *Rhipicephalus microplus* (40.78%) with preference sites including the neck, udder, shoulder area, and external genitalia, fondness sites for *Hyalomma anatolicum* (31.94%) include the external genitalia, udder, ear, and tail. Similarly, the external genitalia, udder, rear legs, and head regions are preferred locations for *Hyalomma marginatum* (27.29%). These findings are in line with those made by Moges *et al*., (2012), who discovered tick infection in 169 local cattle in the Chilga area of Ethiopia. Our results are reliable with (Kakar *et al*., 2017), whose study found that Boophilus (35.5%) was the most common tick species, with preference areas including the shoulder area, dewlap, external genitalia, udder region, legs, especially the rear legs, abdominal area, and in some cases head regions, particularly the neck. Likely predilection areas for *Hyalomma* (26.5%) were the perineum region, udder, and external genitalia. They noted nearly the same tick preference locations as those found in the current study. The fact that ticks favor warm, moist, concealed areas with a good vascular supply and thin skin could potentially be a contributing factor to the increased tick infestations on the external genitalia and udder (Muchenje *et al*., 2008). For infestation, ticks typically favor skin with shorter hair and thinner layers. This facilitates feeding by allowing mouth parts to easily enter a highly vascular location. Like our findings, Atif *et al*., (2012) found that the udder and external genitalia (98%) were the most often infested areas, followed in decreasing order by dewlap (92%), inner thighs (90%), neck and back (54%), tail (26%), ears (13%), around eyes (10%), flanks (4%) and legs (2%).

In the current study, there was a strong correlation between bodily condition and tick prevalence among the variables considered. Animals with poor body conditions had higher tick infection rates than the other groups, according to body condition. This might be because unfit animals had little resistance to tick infection and insufficient bodily capacity to develop resistance. although animals in good physical condition fought the parasite reasonably well, according to (Manan *et al.,* 2007). The present study showed that cattle had the highest tick infestation (96.46%) with poor body condition followed by good (70.13%) and excellent body condition (40.22%). A major management risk factor for tick prevalence is the host's poor physical condition (Patel *et al* 2019). Cattle who were reared in ordinary-type houses recorded with high tick infestation (87.28%) in comparison to those who were kept in concrete houses (51.91%). The animal maintained on an earthen floor had the highest tick incidence (89.10%), preceded by cement concrete (54.06%). In keeping with the current finding, Farooqi *et al*., (2017), Patel *et al*., (2019), and Rehman *et al*., (2017) also found that animals raised in wooden/traditional rural houses had considerably higher tick prevalence than animals kept in concrete-style houses. The earthen/wooden homes' flaws and crevices give ticks a place to hide and promote their growth. The current study's findings revealed that the prevalence of ticks was lowest (8.92%) in the animals who were fed alone and highest (62.65%) in the animals that were allowed to feed in groups. Our findings were consistent with those of Patel *et al.*, (2019), who found that tick prevalence was lowest (8.92%) in animals fed singly and highest (62.65%) in those allowed to eat in groups.

Tick infestation was found to be higher in cattle kept in free-grazing (93.28%) and semi-grazing (83.17%) regions in the grazing system than it was in cattle kept in non-grazing areas (44.94%). Likewise, compared to farms where cattle were managed with stall feeding, tick infection was noticeably greater in roaming animals. Our results were found similar with (Zeb *et al.*, 2020), in which cattle housed in non-grazing regions are less infested by ticks (59.5%) from cattle kept in semi-grazing (86.0%) and free-grazing (85.7%) areas. It is possible to speculate that routine barn cleanings and acaricide treatments will lessen the likelihood of a tick infection in stall-feeding animals while grazing cattle can graze everywhere, this increases their susceptibility to tick infestation (Kabir *et al.*, 2011).

Likewise, when it came to the usage of acaricides, the prior research is supported by the observation that cattle not frequently treated with acaricides were substantially more likely to experience a tick infestation than were cattle that were (Rehman *et al.*, 2017). The present investigation revealed that the prevalence of infestation was greater in cattle that were not routinely treated with acaricides (88.81%) compared to those who were (43.71%). Our results match with (Zeb *et al.*, 2020) who discovered that the prevalence of tick infestation was significantly ($P < 0.001$) lower (25.2%) in cattle that received frequent acaricide treatment compared to those that did not receive treatment (80.1%) and received treatment sometimes (75.3%). Additionally, farms that used acaricides inconsistently had greater rates of tick infestation, which may be a sign of acaricide resistance. The incidence of acaricide resistance in cattle ticks in Pakistan, however, is not well known. In other regions of the world, reports of widespread acaricide resistance in cattle ticks have been made (Abbas *et al.*, 2014). According to these results, a nationwide survey should be carried out to investigate acaricide resistance in cattle ticks in Pakistan.

According to the current study, cattle raised in mountainous areas had considerably higher tick prevalence (90.43%) than animals raised in plain areas (51.99%). Kabir *et al.*, (2011) reported similar findings, that tick prevalence was considerably greater in cattle raised in hilly areas (44.44%) than in cattle raised in plain area (30.27%). Because of the existence of various types of imperata grass, shrubs, and herbs, which provided a favorable environment for all ticks to lay their eggs and hatch throughout the year, the intensity of infestations in mountainous and flat zones varied.

Tick population dynamics are greatly influenced by season, and there is a discernible shift in prevalence rates between seasons. Seasonal temperature fluctuations have an impact on annual patterns of tick activity, which affects tick and TBD dynamics. Variations in tick occurrence in the same area may be caused by changes in the seasons. Since all stages of ticks hibernate in cold climates, the winter season hinders tick infestations. These findings corroborate earlier observations from the area of Ali *et al.*, (2019); Khan *et al.*, (2022), and Ali *et al.*, (2021). The present study revealed the highest tick infestation in the summer followed by spring and autumn. July and August are the wettest months because of the rising temperatures and rising humidity. Similar results were published by Patel *et al.*, (2013), and Kaur *et al.*, (2015). The Study findings were also matched with Rony *et al.*, (2010); Sajid *et al.*, (2009) and Mohanta *et al.*, (2011) revealed a rise in infection rates in the summer. The monsoon season's hot and muggy weather is ideal for the development of ticks in all their developmental phases. However, due to the harsh winter weather that makes it difficult for them to survive, ticks spend the season lurking in cracks and crevices as engorged females, nymphs, larvae, and unfed adults Singh and Rath, (2013). The increasing prevalence of ticks during the monsoon season shows that humidity may be a macroclimatic element that affects the rate of tick infestation (Vatsya *et al.*, 2008). According to research by Rony *et al.*, (2010), the summer season (78.46%) had a

significantly ($p<0.001$) higher seasonal prevalence than the winter (62.85%) and rainy season (52.11%). Typically, during droughts, tick populations remain low (Urquhart, 1996). Higher temperatures and humidity during the summer months contributed to a rise in tick infestation (Khan *et al.*, 1993).
To distinguish closely related species of ticks, many types of genetic markers, including COX1, ITS, 12S rRNA, and 16S rRNA, have been utilized to identify ticks accurately (Abdullah *et al.*, 2016). Three genetic markers, 16s rRNA, ITS2, and COX1 were used in the present research and successfully amplified the crucial genes. All three primers amplified *Rh. microplus, H. anatolicum, and H. marginatum* under the circumstances. Molecular identification is crucial for determining disease risk and implementing targeted control strategies as ticks are the vectors of many pathogens and are the major public health and livestock health problems. To reduce the risk of disease to humans and livestock requires effective tick management strategies and education campaigns.

CONCLUSION

This study concludes that *Rhipicephalus* (*Boophilus*) was the predominant tick genus of the cattle population in the district Swat, followed by *Hyalomma*. There was a significant association between tick infestation and certain factors like age, gender, living conditions, and health factors. Tick prevalence was highest in the tehsil Bahrain, female and adult cattle, mountainous areas, diseased, and those cattle who were kept in concrete houses. There was a higher female tick ratio than male. The prevalence of tick species was at its peak during the summer and rainy seasons and remained low during the winter season. In the current investigation, genetic markers (16S rRNA, ITS2, and COX1) were used to successfully amplify the targeted genes of the three tick species. The study highlighted the importance of molecular methods in epidemiological research and the control of tick-borne diseases, which will ultimately improve public safety and livestock health.

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