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Original Article

Meta-genomic Analysis of Parasites of *Hyalomma* Ticks of Cattle

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Abstract

Introduction: Ticks are the obligate blood-feeding parasites responsible for transmitting various pathogens to domestic animals and humans. Ticks and tick-borne diseases are a continuous threat to livestock development in tropical and subtropical areas of the globe. To the best of our knowledge, this was the first study on the microbiome population of *Hyalomma* spp. infesting livestock in Pakistan.

Methods: Ticks were collected from hemo-parasite-positive cattle and were identified using standard entomological keys. Then, selected ticks were kept in the biochemical oxygen demand (BOD) incubator for egg laying and hatching at optimum temperature and humidity. A total of 16 (saliva and gut) samples were amplified through the reverse line blotting (RLB) primer of which 9 out of 16 were positive. Out of 9 positive samples, 4 samples of saliva and 5 samples of gut showed a polymerase chain reaction (PCR) product of 390 bp on 1.5% agarose gel stained with cyber green safe gel dye. Similarly, all 16 samples were amplified through the PCR by applying primers specific to the genus *Anaplasma*. Out of 16 amplified samples (8 saliva and 8 gut), 9 samples exhibited a positive amplification of 345 bp on 1.5% Agarose gel. After the identification of the genus, all 16 samples were subjected to species identification through PCR by applying primers specific to *Theileria annulata, Babesia bovis,* and *Babesia bigemina*.

Results: The results indicated that 9 out of 16 samples are positive for *Theileria,* showing a PCR product of 460 bp on Agarose gel, while none of the single samples was positive for *B. bovis* and *B. bigemina*, respectively. Overall, 16 samples were subjected to metagenomics analysis within the saliva and gut of *Hyalomma* ticks, and consequently, *Anaplasma* and *T. annulata* were identified. Hence, it was found that the *Hyalomma* tick reported in Pakistan is responsible for the transmission of *T. annulata* and *Anaplasma* spp., and both parasites are responsible for causing theileriosis and anaplasmosis in cattle.

Conclusion: It can be concluded that *Hyalomma anatolicum* transmits both *T. annulata and Anaplasma* species but not *Babesia* species in cattle. Thus, *H. anatolicum* ticks are potent vectors for *T. annulata and Anaplasma* species.

Keywords: *Hyalomma anatolicum*, *Babesia bigemina*, Babesiosis, BOD incubator, Cattle

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Introduction

In Pakistan, smallholder dairy farmers depend upon livestock to fulfill their needs for proteins and nutrients. In the financial year of 2016-2017, the agriculture sector contributed 19.5% to the total gross domestic product (GDP) in Pakistan, while the livestock sector contributed 58.3% to the agriculture value and 11.4% to the overall GDP. In Pakistan, livestock population is about 238.1 million heads, including buffalo, camel, sheep, goat, cattle, mules, horses, and asses. Cattle stand in second (44.4 million) in terms of number following goats (72.2 million), producing 56 080 million tones of milk (1).

Ticks are the second largest group of arthropods that infect mammals, reptiles, amphibians, and birds after mosquitoes. They are classified into two major families: the *Ixodidae* or hard ticks and *Argasidae* or soft ticks (2). *Rhipicephalus, Haemaphysalis, Hyalomma,* and *Ornithodoros* are the main tick genera infesting humans and animals and are widely distributed throughout the world (3). Ticks can transmit a wide range of pathogens, including protozoans, viruses, and bacteria such as the spirochetes and rickettsiae which transmit various diseases into their hosts such as theileriosis, babesiosis, anaplasmosis, borreliosis, rocky mountain spotted fever, cowdriosis of ruminants, Crimean-Congo hemorrhagic fever, rickettsiosis, East Coast fever, and ehrlichiosis (4).

Rhipicephalus microplus is prevalent in half of the area of Mexico and is the most common threat to livestock in Mexico causing US$ 573.6 M losses annually (5). In Brazil, *R. microplus* causes an estimated loss of US$ 3.2.4 billion per annum (6). In Australia, tick control cost (chemical, labor, and others) was US$ 41 million according to the Resource Economics and Australian Bureau of Agriculture during the year 1994 (7). *Theileria orientalis* causes acute and subclinical theileriosis. In Australia, from a herd of 662 dairy cattle, the reduction

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in milk yield due to *T. orientalis* was 288 ± 90.4 and 153 ± 95.9 liters per cattle per 100 days, respectively (8).

The losses due to haemoparasitic diseases are divided into various categories such as direct losses due to lower production and death of the animal and indirect losses such as vaccination, tick control, treatment losses, and restriction in cattle movements. In the USA, up to US$ 500 M losses may occur annually if ticks and tick-borne pathogens (TBDs) are again re-established in the cattle industry. Queensland and New South Wales spend US $7.8 M per annum to control the tick and tick-borne diseases, while in Tanzania, 10 000 cases of *Babesia bigemina* were reported annually, and 10% of the animals died. In Sweden, US$ 2.5 M per annum losses were reported due to *Babesia divergens* (9).

Hyalomma tick species are responsible for transmitting the protozoan parasite *Theileria annulata* in cattle which poses a major threat to livestock production in Pakistan (10-14). *Hyalomma* spp. of tick simultaneously harbor a variety of pathogenic species in its gut and saliva, and such communities of organisms are known as microbiomes (15). Microbiomes are virulent mammalian either pathogenic commensal or symbiotic microorganisms that occupy various niches of the body and are also responsible for various diseases (16-18). Different molecular biological techniques detect the valuable processes occurring within the tick vector such as stages of a pathogen's life cycle and also detect the mixed pathogen population in the saliva or gut of infected ticks (19).

Such types of studies are termed meta-genomics, and little information is available on the meta-genomics of ticks and the associated vector capacity of ticks in Pakistan.

Meta-genomics is an emerging and essential tool to study parasitic diversity within tick microbiota. The meta-genomic approach offers a convenient alternative pathway for obtaining parasitic profiles within ticks (20). Briefly, meta-genomics is a term that is used to describe information from various meta-genomes by creating shotgun sequence libraries. These libraries may be sequenced or, if cloned in an expression system, may be screened for desired functional activities.

Therefore, this study aimed to evaluate the possible role of ticks in the transmission of pathogen species and protozoan diversity in these ticks to facilitate a better understanding of these species in Pakistan. It further aimed to obtain information on co-infections in ticks. To the best of our knowledge, this was the first detailed molecular study on *Hyalomma* spp. for detecting its haemoparasites in Pakistan.

Materials and Methods

Identification of Tick Samples

Tick samples were collected to identify the type of tick responsible for the inoculation of *T. annulata* in cattle, and the collected tick samples were brought to the laboratory at the Department of Parasitology, University of Veterinary and Animal Sciences, Lahore. Tick specimens were identified to belong to various genera based on their morphological characteristics using the identification key of Entomology by Taylor and Urquhart (21).

Rearing of Ticks

Following the morphological identification of ticks, they were incubated in a biochemical oxygen demand (BOD) incubator (company, machine, or model) in the presence of 80% ≥ humidity and under a constant temperature of 27 ± 1° C in 2 mL flat bottom centrifuge tube with a perforated cape (22). Approximately, after 7 days, almost all female ticks laid eggs followed by egg hatching within 7-9 days, respectively. All ticks were then allowed to rest under the condition stated earlier until further use.

Feeding of Hyalomma Ticks on Theileria Positive Cattle

Hyalomma ticks larvae were placed in a feeding chamber consisting of a stocky net cloth sac (16 cm diameter and 24 cm height) glued on the previous day with a toxic and non-adhesive preparation (Cementing bond CR 2000) to the shaved ear of the experimentally infected male cross-bred calf.

Dissection of Ticks for Collection of Salivary Gland and Gut

Multiple ticks were dissected under a stereomicroscope (model) for the collection of the salivary gland and tick gut, respectively. Salivary glands of dissected ticks were analyzed for various pathogens existing in the saliva of the dissected tick. Furthermore, all the salivary glands were carefully removed by tweezers, specially to avoid mixing of salivary contents and gut contents. After collection, the salivary gland was washed with 1X phosphate buffer saline (Bio World, Catalog No.41620016-2) and was stored at 4 °C (23-25).

DNA Extraction

DNA was extracted from all the collected salivary glands through the Exgene (Cat. 106-101 GeneAll Biotechnology. Co. Ltd) kit method following manufacturer instructions. Briefly, the salivary glands of ticks were minced as small as possible and then put into a 1.5 mL tube. Afterward, 200 µL of buffer TL was added, and it was then vortexed for 15 seconds. Then, 20 µL of proteinase K solution was mixed with the salivary glands in the tube, and after mixing the sample, it was incubated at 56 °C until the sample was completely lysed. Further, 400 µL of TB buffer was added to the tube, and it was immediately vortexed to mix thoroughly. The mixture was transferred to the SV column provided with the kit, centrifuged for 1 minute

at 8000 rpm, and the collection tube was replaced with a new one. Afterward, 600 µL of buffer BW was added and centrifuged for 30 seconds at 8000 rpm; moreover, 700 µL of buffer TW was added, and the mixture was centrifuged for 30 seconds at 8000 rpm. The sample was centrifuged at full speed for 1 minute to remove residual wash buffer. SV column was placed in a fresh 1.5 mL tube, and 200µL of buffer AE was then added to it. After providing incubation at room temperature for one to two minutes, the mixture was rotated at high speed for maximum collection of DNA, and the collected DNA was stored at -20°C for further processing.

Polymerase Chain Reaction Amplification of DNA from Saliva and Gut of Hyalomma Tick

Collected DNA was amplified through polymerase chain reaction (PCR) using various pairs of primers for both species-specific as well as genus-specific primers for each *Babesia* spp., *Theileria* spp., and *Anaplasma* spp., and details of each primer are given in Table 1.

Molecular Detection of Theileria spp.

For each PCR amplification of *Theileria*, 2 µL of extracted DNA was used as the template in a 20 µL reaction mixture containing 10 µL 2 × PCR master buffer (Cat No. W1401-2, Wizbiosolutions Inc, Korea), 2 µL of each primer, and 4 µL diethylpyrocarbonate (DEPC) treated water (Catalog NO. 750023 Invitrogen™, USA). The reactions were conducted in a GS482 thermocycler (G-STORM, UK) at 95°C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 1 minute, followed by an extension step at 72 °C for 10 minutes.

Molecular Detection of Babesia spp. from Gut and Saliva

Babesia Bigemina

For each PCR amplification of *Babesia*, 2 µL of extracted DNA was used as the template in a 20 µL reaction mixture containing 10 µL 2 × PCR master buffer (Catalog NO. W1401-2 Wizbiosolutions Inc, Korea), 2 µL of each primer, and 4 µL DEPC treated water (Cat No. 750023 Invitrogen™, USA). The reactions were conducted in a GS482 thermocycler (G-STORM, UK) at 95 °C for 1 minute followed by 37 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, followed by an extension step at 72 °C for 5 minutes.

Babesia Bovis

For each PCR amplification, 2 µL of extracted DNA was used as the template in a 20 µL reaction mixture containing 10 µL 2 × PCR master buffer (Catalog No. W1401-2 Wizbiosolutions Inc, Korea), 2 µL of each primer, and 4µL DEPC treated water (Cat No. 750023 Invitrogen™, USA). The reactions were conducted in a GS482 thermocycler (G-STORM, UK) at 94 °C for 5 minutes followed by 40 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds, and 72 °C for 45 seconds, followed by an extension step at 72 °C for 10 minutes.

Molecular Detection of Anaplasma spp. from Gut and Saliva

For each PCR amplification, 2 µL of extracted DNA was used as the template in a 20 µL reaction mixture containing 10 µL 2 × PCR master buffer (Catalog NO.W1401-2 Wizbiosolutions Inc, Korea), 2 µL of each primer, and 4 µL DEPC treated water (Cat No. 750023 Invitrogen™, USA). The reactions were conducted in a GS482 thermocycler (G-STORM, UK) at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute, followed by an extension step at 72 °C for 5 minutes.

Agarose Gel Electrophoresis

The amplified products were analyzed using 1.5% agarose gel electrophoresis. Furthermore, 10 µL of each PCR product was gel electrophoresed at 120 volts for 40 minutes, and the size of the PCR products was compared against a DNA ladder marker.

Gel Visualization

The gel was visualized under ultraviolet light.

Table 1. The List of Genus-Specific Primers Used for Amplification of DNA Extracted from Salivary Gland

Sr. No.	Gene Targeted	Name	Primer Sequence	Length (bp)
1	18S SSUrRNA	RLB-F	5′-GAG GTAGTGACAAGAAATAACAATA-3′	390-460
		RLB-R	5-TCTTCGATCCCCTAACTTTC-3	
2	16SrRNA HER	EHR 16 SD	5-GGTACCYACAGAAGAAGTCC-3	345
		EHR 16 SR	5-TAGCACTCATCGTTTACAGC-3	
3	Cytochrome b	Tann F	5′-TGGTCAAATGAGCTTCTGGGG-3′	460
		Tann R	5′-TCCTGCCATTGCCAAAAGTC-3′	
4	16SrRNA	*Babesia bigemina* F	5′-AGAGGGACTCCTGTGCTTCA-3′	321
		Babesia bigemina R	5′-GACGAATCGGAAAAGCCACG-3′	
5	16SrRNA	*Babesia bovis* F	5′-AATATGGGTTGGGCAATGCG-3′	268
		Babesia bovis R	5′-CCACCCAAAACAAGAGCAACT-3′	

Note. RLB: Reverse line blotting; SSU: *S*mall subunit.

This study was designed for the detection of metagenome in the saliva of *Hyalomma* ticks fed on experimentally infected *T. annulata*-positive animals through PCR.

Collection of Ticks

Ticks were collected from different field cattle and brought back to the Parasitology Laboratory of the University of Veterinary and Animal Sciences, where they were studied under a stereomicroscope for their identification.

Results

Identification of Ticks

A total of 50 ticks were identified under stereomicroscope based on their morphological features using the standard key of Entomology by Urquhart and Taylor (21), as depicted in Figure 1. Out of 50 ticks, 20 *Hyalomma* ticks were selected based on morphological features.

Rearing of Hyalomma Ticks in Biochemical Oxygen Demand Incubator

Ticks were incubated in a BOD incubator (company, machine, or model) in the presence of ≥ 80% humidity and under the temperature of 27 ± 2°C in a 2 mL flat bottom centrifuge tube with a perforated cape (22). Approximately, after 7 days, almost all female ticks laid eggs followed by egg hatching within 7-9 days, respectively. All ticks were then allowed to rest under the condition stated earlier, until further use as depicted in Figure 2.

Feeding of Hyalomma ticks on Theileria-Positive Cattle

All ticks were allowed to feed on the infected animal, and they were observed on an hourly basis and were collected when fully engorged. *Hyalomma* ticks approximately completed their feeding within 3 days (± 1) under the natural environmental conditions. Figure 3 shows the attachment of the tick to the host for feeding along with the feeding sac to avoid the escape of the tick.

Figure 1. Stereomicroscopic Image of *Hyalomma* Tick: (a) The scutum is colored brown, (b) Its mouth parts are anterior, (c) The second palp is longer than the first and third, (d) Banded legs

Dissection of Hyalomma Tick

The dissection of the tick has been done as shown in

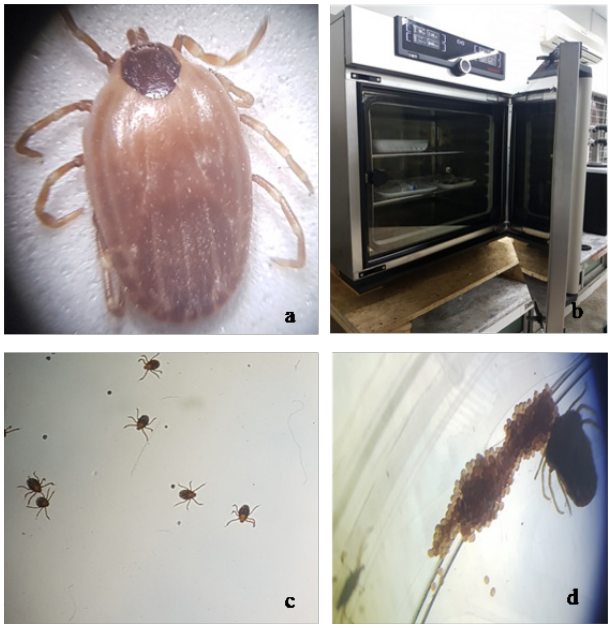

Figure 2. (a) Collected adult ticks were identified as *Hyalomma* ticks, (b) BOD incubator for tick rearing, (c) Eggs laid by *Hyalomma* tick, and (d) Larvae of *Hyalomma* tick

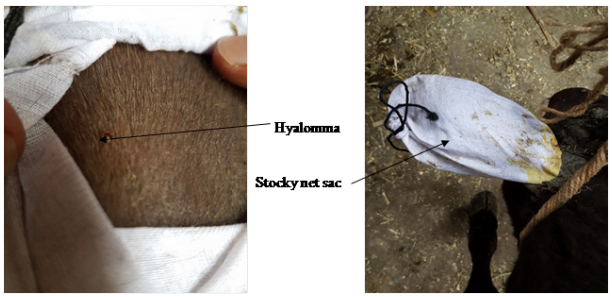

Figure 3. Feeding Process of *Hyalomma* Tick along with Stocky Net Sac on Experimental Animal

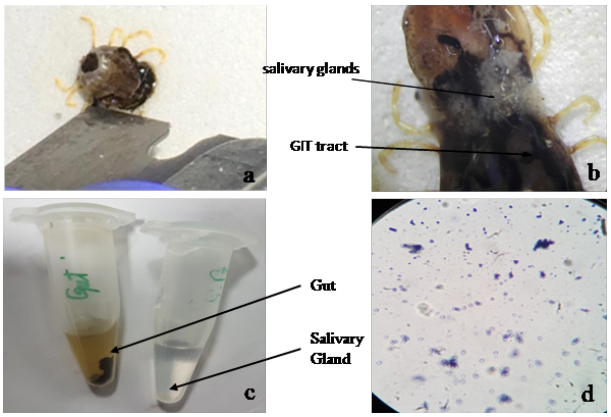

Figure 4. (a) Dissection of a *Hyalomma* tick using a sterile sharp blade, (b) Salivary glands and GIT tract are exposed, (c) Collection of salivary glands and GIT in phosphate buffer saline solution, (d) Impression smear of salivary glands in which sporozoites are clearly visible. *Note*. GIT: Gastrointestinal

Figure 4.

DNA Extraction

DNA was extracted from the salivary glands or gut of the *Hyalomma* ticks using the Exgene (Cat. 106-101 GeneAll Biotechnology. Co. Ltd).

Polymerase Chain Reaction Confirmation of Pathogen from Saliva or Gut of Hyalomma Tick

A Total of 16 samples of salivary glands of *Hyalomma* ticks were amplified by targeting the 18S small subunit rRNA gene yielding an amplicon size of 460 bp (Figure 5). Out of these 16 samples, 9 (56%) were positive for piroplasms, and from these 9 samples, 5 from saliva and 4 from gut were positive.

Polymerase Chain Reaction Confirmation of Theileria annulata

A total of 16 samples of salivary glands of *Hyalomma* ticks were amplified by targeting the 18S rRNA gene yielding an amplicon size of 460 bp (Figure 6). Out of these 16 samples, 9 (56%) were positive for *T. annulata,* and of these 9 samples, 3 from the saliva and 6 from the gut were positive.

Polymerase Chain Reaction Confirmation of Babesia bigemina

A total of 16 samples of salivary glands of *Hyalomma* ticks were amplified by targeting the 18S rRNA gene yielding an amplicon size of 321 bp (Figure 7). All 16 samples were negative for *B. bigemina.*

Polymerase Chain Reaction Confirmation of Anaplasma

A total of 16 samples of salivary glands of *Hyalomma* ticks were amplified by targeting the 16S rRNA gene yielding an amplicon size of 345 bp (Figure 8). Out of these 16 samples, 9 (56%) were positive for *Anaplasma,* and of these 9 samples, 5 from saliva and 4 from gut were positive.

Co-infection of Parasites

Table 2 shows the occurrence of the protozoan parasites.

Discussion

Hyalomma tick species are responsible for transmitting the protozoan parasite *T. annulata* in cattle which poses a major threat to livestock production in Pakistan (10-14). This *Hyalomma* tick simultaneously harbors a variety of microbial organisms in its gut and saliva, and such

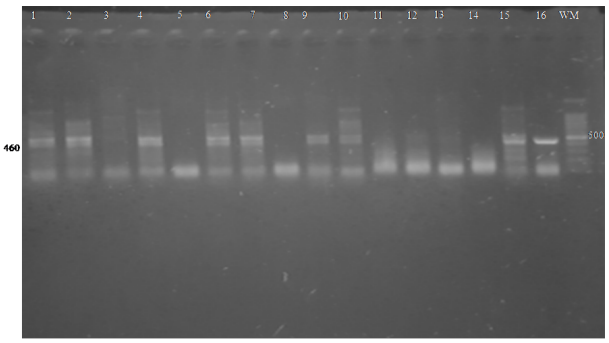

Figure 5. Agarose Gel Electrophoresis Showing the Clear Band on 460 bp length of PCR Product. Primers targeting the 18s SSU rRNA gene. MW: 100 bp molecular weight. DNA marker. Lanes 1, 2, 4, 5, 6, and 9 are negative samples. Lanes 3, 7, 8, 10, 11, 12, 13, 14, and 15 are positive samples showing a specific amplicon of 460 bp. Lanes 1-8 represent samples of the salivary gland, while lanes 9-16 represent samples of the gut. Note. PRC: Polymerase chain reaction; SSU: Small subunit

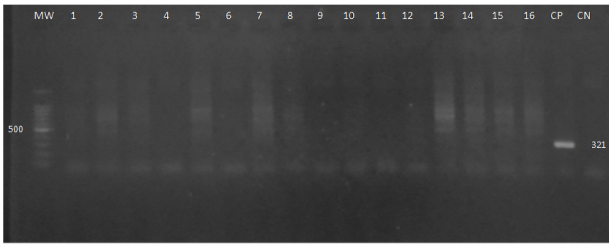

Figure 7. Agarose gel electrophoresis showing the clear band on 390 bp length of PCR product. Primers targeting the 18s ribosomal gene of *Theileria annulata.* MW: 100 bp molecular weight. DNA marker. Lanes 1-16 in all samples are negative. Lane 17 is positive control, lane 18 is negative control, and lanes 1-8 represent samples of the salivary gland, while lanes 9-16 represent samples of the gut

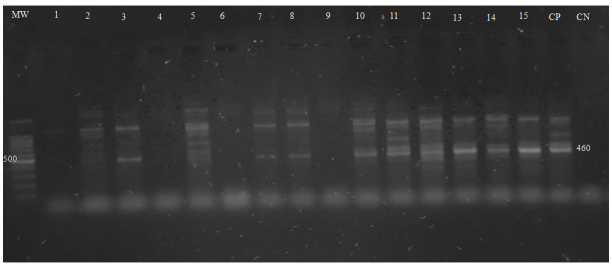

Figure 6. Agarose gel electrophoresis showing the clear band on the 460 bp length of the PCR product. Primers targeting the 18S ribosomal gene of *Theileria annulata.* MW: 100 bp molecular weight. DNA marker. Lanes 1, 2, 4, 5, 6, and 9 are negative samples. Lanes 3, 7, 8, 10, 11, 12, 13, 14, and 15 are positive samples showing a specific amplicon of 460 bp. Lane 16 is positive control, lane 17 is negative control, and lanes 1-8 represent samples of the salivary gland, while lanes 9-16 represent samples of the gut. *Note.* PRC: Polymerase chain reaction

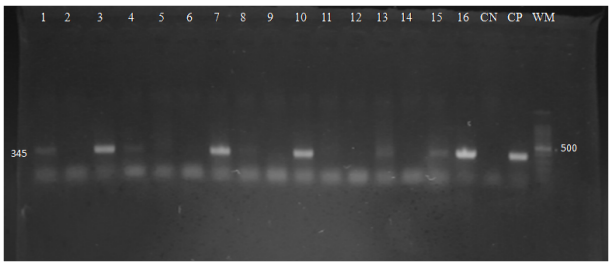

Figure 8. Agarose gel electrophoresis showing the clear band on 345 bp length of PCR product. Primers targeting the 16SrRNA HER gene of *Anaplasma.* MW: 100 bp molecular weight. DNA marker. Lanes 1, 3, 4, 7, 10, 13, 15, and 16, positive samples showing specific amplicon of 345 bp. Lane 1-8 represents samples of the salivary gland, while lanes 9-16 represent samples of the gut. The lane 17 sample is negative, and lane 18 is positive control

Table 2. The Occurrence of Hemoparasites in the Saliva and Gut

Samples	Saliva			Gut		
	T	B	A	T	B	A
1	-ve	-ve	+ve	-ve	-ve	-ve
2	-ve	-ve	-ve	+ve	-ve	+ve
3	+ve	-ve	+ve	+ve	-ve	-ve
4	-ve	-ve	+ve	+ve	-ve	-ve
5	-ve	-ve	-ve	+ve	-ve	-ve
6	-ve	-ve	-ve	+ve	-ve	-ve
7	+ve	-ve	+ve	+ve	-ve	+ve
8	+ve	-ve	-ve	-ve	-ve	+ve
Total -ve	5	8	4	2	8	5
Total +ve	3	0	4	6	0	3

communities of organisms are known as Microbiomes (15). Microbiomes are either pathogenic commensals or symbiotic microorganisms that occupy various niches of the body and are also responsible for various diseases (16-18). Different molecular biological techniques detect the valuable processes occurring within the tick vector such as stages of a pathogen's life cycle and also detect the mixed pathogen population in the saliva or gut of infected ticks (19).

To verify the hypothesis that "*Hyalomma* ticks prevalent in Pakistan are responsible for the transmission of multiple pathogens", we designed the present study to evaluate and identify the pathogen within the saliva of ticks. For this purpose, a total of 16 samples (saliva and gut) were amplified through the reverse line blotting (RLB) primer in which 9 out of 16 were positive. Out of 9 positive samples, 4 samples of saliva and 5 samples of guts exhibited a PCR product of 390 bp on 1.5% agarose gel stained with cyber green safe gel dye (Figure 7). Similarly, all 16 samples were amplified through the PCR by applying primers specific to the genus *Anaplasma*. Out of 16 samples, 8 saliva and 8 gut were amplified, and 9 samples showed a positive amplification of 345 bp on 1.5% Agarose gel (Figure 8). After the identification of the genus, all 16 samples were subjected to species identification through PCR by applying primers specific to *T. annulata, B. bovis*, and *B. bigemina.* The results revealed that 9 out of 16 samples were positive for *Theileria,* showing a PCR product of 460 bp on Agarose gel (Figure 6), while none of the samples was positive for *B. bovis* and *B. bigemina,* respectively.

Our results are in agreement with those of Berggoetz et al who collected 13 tick species from wild and domestic ruminants (26). A total of 129 *Hyalomma rufipes* were analyzed by RLB hybridization using probes allowing the identification of TBDs at the genus and species level. Out of 129 samples, 20 ticks (15.5%) were infected. Multiple pathogens were detected in the saliva of *Hyalomma* spp., which included *T. annulata* (1), *Theileria buffeli* (1), *Erica ovina* (1) *Anaplasma,* and *Ehrlichia* (6), while the present study revealed that 9 out of 16 samples are positive for *T. annulata,* and 8 out of 16 samples are positive for *Anaplasma* or *Ehrlichia* (Table 2). Similarly, our results are in agreement with the study by Karim et al who reported that none of the *Babesia* spp, infecting cattle is detected in the saliva or gut of *Hyalomma* tick (Figure 5), but co-infection is reported (27). The present study reported that the *Hyalomma* spp. carries the protozoan parasites, while Karim et al (27) reported multiple infections within *Hyalomma* (Figures 6, 7, and 8).

In sum, we concluded that *Hyalomma anatolicum* transmits both *T. annulata and Anaplasma species* but not *Babesia* species in cattle. Thus, *H. anatolicum* ticks are potent vectors for *T. annulata and Anaplasma* species.

Conclusion

In sum, we concluded that *H. anatolicum* transmits both *T. annulata and Anaplasma species* but not *Babesia* species in cattle. Thus, *H. anatolicum* ticks are potent vectors for *T. annulata and Anaplasma species.*

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Authors' Contribution

Conceptualization: Zia Ul Rehman, Muhammad Imran Rashid.
Data curation: Zia Ul Rehman, Irtaza Hussain.
Investigation: Sarfraz Ur Rahman.
Methodology: Abdul Jabar.
Resources: Abdul Jabar, Muhammad Zubair Munir.
Supervision: Zia Ul Rehman.
Validation: Zia Ul Rehman.
Visualization: Sarfraz Ur Rahman, Muhammad Imran Rashid.
Writing–original draft: Zia Ul Rehman.
Writing–review & editing: Sarfraz Ur Rahman.

Competing Interests

There is no conflict of interests.

Ethical Approval

The study design was approved by the Ethical Committee, University of Veterinary and Animal Sciences (UVAS), Lahore.

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References

1. Usman M. Contribution of agriculture sector in the GDP growth rate of Pakistan. J Glob Econ. 2016;4(2):184. doi: 10.4172/2375-4389.1000184.
2. Sauer JR, McSwain JL, Bowman AS, Essenberg RC. Tick salivary gland physiology. Annu Rev Entomol. 1995;40:245-67. doi: 10.1146/annurev.en.40.010195.001333.
3. Robertson RG, Wiseman CL Jr, Traub R. Tick-borne rickttsiae of the spotted fever group in West Pakistan. I. Isolation of strains from ticks in different habitats. Am J Epidemiol. 1970;92(6):382-94. doi: 10.1093/oxfordjournals.aje.a121221.
4. de Castro JJ, James AD, Minjauw B, Di Giulio GU, Permin A, Pegram RG, et al. Long-term studies on the economic impact of ticks on Sanga cattle in Zambia. Exp Appl Acarol. 1997;21(1):3-19.

5. Rodríguez-Vivas RI, Grisi L, Pérez de León AA, Silva Villela H, de Jesús Torres-Acosta JF, Fragoso Sánchez H, et al. Potential economic impact assessment for cattle parasites in Mexico. Review. Rev Mex Cienc Pecu. 2017;8(1):61-74. doi: 10.22319/rmcp.v8i1.4305.
6. Grisi L, Leite RC, de Souza Martins JR, de Barros AT, Andreotti R, Cançado PH, et al. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev Bras Parasitol Vet. 2014;23(2):150-6. doi: 10.1590/s1984-29612014042.
7. McLeod RS. Costs of major parasites to the Australian livestock industries. Int J Parasitol. 1995;25(11):1363-7. doi: 10.1016/0020-7519(95)00071-9.
8. Perera PK, Gasser RB, Firestone SM, Anderson GA, Malmo J, Davis G, et al. Oriental theileriosis in dairy cows causes a significant milk production loss. Parasit Vectors. 2014;7:73. doi: 10.1186/1756-3305-7-73.
9. Zwart D. Haemoparasitic diseases of bovines. Rev Sci Tech. 1985;4(3):447-78. doi: 10.20506/rst.4.3.208.
10. Perveen F. Distribution and identification of ixodid tick species on livestock in northern Pakistan. J Agric Sci Technol. 2011;1:73-81.
11. Jabbar A, Abbas T, Sandhu ZU, Saddiqi HA, Qamar MF, Gasser RB. Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. Parasit Vectors. 2015;8:283. doi: 10.1186/s13071-015-0894-2.
12. Durrani AZ, Kamal N. Identification of ticks and detection of blood protozoa in friesian cattle by polmerase chain reacton test and estimation of blood parameters in district Kasur, Pakistan. Trop Anim Health Prod. 2008;40(6):441-7. doi: 10.1007/s11250-007-9117-y.
13. Shahnawaz S, Ali M, Aslam MA, Fatima R, Chaudhry ZI, Hassan MU, et al. A study on the prevalence of a tick-transmitted pathogen, *Theileria annulata*, and hematological profile of cattle from Southern Punjab (Pakistan). Parasitol Res. 2011;109(4):1155-60. doi: 10.1007/s00436-011-2360-1.
14. Khan MK, He L, Hussain A, Azam S, Zhang WJ, Wang LX, et al. Molecular epidemiology of *Theileria annulata* and identification of 18S rRNA gene and ITS regions sequences variants in apparently healthy buffaloes and cattle in Pakistan. Infect Genet Evol. 2013;13:124-32. doi: 10.1016/j.meegid.2012.09.007.
15. Piesman J, Eisen L. Prevention of tick-borne diseases. Annu Rev Entomol. 2008;53:323-43. doi: 10.1146/annurev.ento.53.103106.093429.
16. Schlatter D, Kinkel L, Thomashow L, Weller D, Paulitz T. Disease suppressive soils: new insights from the soil microbiome. Phytopathology. 2017;107(11):1284-97. doi: 10.1094/phyto-03-17-0111-rvw.
17. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science. 2001;292(5519):1115-8. doi: 10.1126/science.1058709.
18. Narasimhan S, Fikrig E. Tick microbiome: the force within. Trends Parasitol. 2015;31(7):315-23. doi: 10.1016/j.pt.2015.03.010.
19. Gubbels JM, de Vos AP, van der Weide M, Viseras J, Schouls LM, de Vries E, et al. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J Clin Microbiol. 1999;37(6):1782-9. doi: 10.1128/jcm.37.6.1782-1789.1999.
20. Menchaca AC, Visi DK, Strey OF, Teel PD, Kalinowski K, Allen MS, et al. Preliminary assessment of microbiome changes following blood-feeding and survivorship in the *Amblyomma americanum* nymph-to-adult transition using semiconductor sequencing. PLoS One. 2013;8(6):e67129. doi: 10.1371/journal.pone.0067129.
21. Urquhart J. Patient non-compliance with drug regimens: measurement, clinical correlates, economic impact. Eur Heart J. 1996;17 Suppl A:8-15. doi:10.1093/eurheartj/17.suppl_a.8.
22. Jyothimol G, Ravindran R, Juliet S, Ajithkumar KG, Suresh NN, Vimalkumar MB, et al. Low level deltamethrin resistance in ticks from cattle of Kerala, a south Indian state. Vet Parasitol. 2014;204(3-4):433-8. doi: 10.1016/j.vetpar.2014.04.028.
23. Garedaghi Y. Flea infestation in farm animals and its zoonotic importance in East-Azerbaijan province. Am J Anim Vet Sci. 2011;6(4):193-6.
24. Garedaghi Y, Mashayekhi M, Yousefi S. Prevalence of *Hypoderma* infestation and cattle hypodermosis in Tabriz slaughterhouse, Iran. Int J Biosci. 2014;5(1):323-7. doi: 10.12692/ijb/5.1.323-327.
25. Aktas M, Altay K, Dumanli N, Kalkan A. Molecular detection and identification of *Ehrlichia* and *Anaplasma* species in ixodid ticks. Parasitol Res. 2009;104(5):1243-8. doi: 10.1007/s00436-009-1377-1.
26. Berggoetz M, Schmid M, Ston D, et al. Protozoan and bacterial pathogens in tick salivary glands in wild and domestic animal environments in South Africa. Ticks Tick Borne Dis. 2014;5(2):176-185. doi:10.1016/j.ttbdis.2013.10.003.
27. Karim S, Budachetri K, Mukherjee N, et al. A study of ticks and tick-borne livestock pathogens in Pakistan. PLoS Negl Trop Dis. 2017;11(6):e0005681. doi:10.1371/journal.pntd.0005681.