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

Zia Ul Rehman1*, Sarfraz Ur Rahman1*, Muhammad Zubair Munir2, Abdul Jabar2, Irtaza Hussain3, Muhammad Imran Rashid1

1Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan
2Livestock and Dairy Development Punjab, Lahore, 54000, Pakistan
3Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University Multan, Pakistan

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 56080 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$ 3.24 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...
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 protease 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 (Catalog 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, 56°C for 45 seconds, and 72°C for 1 minute, followed by an extension step at 72°C for 5 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 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 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 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>
<thead>
<tr>
<th>Sr. No.</th>
<th>Gene Targeted</th>
<th>Name</th>
<th>Primer Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18S SSU rRNA</td>
<td>RLB-F</td>
<td>5'-GAG GTAGTGACAAGAAATAACAATA-3'</td>
<td>390-460</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RLB-R</td>
<td>5'-TCTTCGATCCCCCTAATCTTC-3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16SrRNA HER</td>
<td>EHR 16 SD</td>
<td>5'-GGTACCCAGAGGAGAAGCCCG-3</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EHR 16 SR</td>
<td>5'-TACGCCTACTCAGTCCAGC-3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cytochrome b</td>
<td>Tann F</td>
<td>5'-TGGTCAATGAGGCTTCAGGGG-3'</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tann R</td>
<td>5'-TCTCAGATATGAGGTAGGCTTCAC-3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16SrRNA</td>
<td>Babesia bigemina F</td>
<td>5'-AGAGGCACTCTCTGCGGTTCA-3'</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Babesia bigemina R</td>
<td>5'-GGACCAATCGAAAGCAGCCACG-3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16SrRNA</td>
<td>Babesia bovis F</td>
<td>5'-AATAGGTTTGGGCAATGCG-3'</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Babesia bovis R</td>
<td>5'-CCACCCAACAAGAGCAACT-3'</td>
<td></td>
</tr>
</tbody>
</table>

Note. RLB: Reverse line blotting; SSU: Small 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.

**Dissection of Hyalomma Tick**

The dissection of the tick has been done as shown in

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**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

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Saliva</th>
<th>Gut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>-ve</td>
<td>-ve</td>
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<tr>
<td>2</td>
<td>-ve</td>
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<tr>
<td>3</td>
<td>+ve</td>
<td>-ve</td>
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<tr>
<td>4</td>
<td>-ve</td>
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<tr>
<td>5</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>6</td>
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<td>-ve</td>
</tr>
<tr>
<td>7</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Total -ve</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Total +ve</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

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 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


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