Epidemiology, Molecular Characterization and Therapeutic Control of Hard Ticks: Tick-Borne Theileriosis and Anaplasmosis of Ruminants in Pakistan

By

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Epidemiology, Molecular Characterization and Therapeutic Control of Hard Ticks: Tick-Borne Theileriosis and Anaplasmosis of Ruminants in Pakistan

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February 2024

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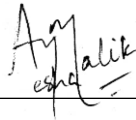
Ayesha Malik:___________________________

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Dedication

I dedicate this effort and work to my loving parents and my teachers.

Thanks to All.

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Comparative Efficacy of Synthetic Acaricides Against Tick Infestations in Goats

LIST OF ABBREVIATIONS

TBPs	Tick-borne Pathogens
KP	Khyber Pakhtunkhwa
ITS-1	Internal Transcribed Spacer Region-1
ITS-2	Internal Transcribed Spacer Region-2
rDNA	Ribosomal DNA
PCR	Polymerase Chain Reaction
R^2	Regression coefficient
CYM	Cypermethrin
IVM	Ivermectin
GOP	Gross Domestic Product
CCHF	Crimean Congo haemorrhagic fever
Cox I	Cytochrome Oxidase I
ECF	East Coast fever
MOT	Malignant Ovine Theileriosis
RLB	Reverse Line Blot
BCCDC	BC Centre of Disease Control
CFIA	Canadian Food Inspection Agency
NCBI	National Center for Biotechnology Information
ITOL	Interactive Tree of Life
C.V	Coefficient of Variation
SD	Standard Deviation
V4	Variable Region
BLAST	Basic Local Alignment Search Tool

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GENERAL ABSTRACT

Ticks are important vectors which cause life-threatening problems in animals worldwide. The first part of the present study aimed to investigate morphometric epidemiology of hard ticks infesting sheep and goats in three agroecological zones of Pakistan. A total of 4488 animals (2184 sheep and 2304 goats) were examined for ticks' infestation. Tick genera were identified based on their morphological features through conventional taxonomic keys. All ticks collected were belonged to four tick genera: *Rhipicephalus, Hyalomma*, *Haemaphysalis,* and *Ixodes.* The results showed slightly higher tick infestation in sheep (23%) compared to goats (20.1%). Male sheep had higher infestation of 23.4%, whereas in goats females had a higher infestation of 23.0%. However, the difference was not significant($P \geq 0.05$). Tick prevalence was similar across the study zones ($P=0.79$), with sheep in Muzaffarabad showing a slightly higher prevalence (47.0%). The prevalence was higher in sheep and goats younger than 1 year (25.7% and 20.8%, respectively) and did not vary significantly ($P=0.99$). Shinwar-White sheep breed 34.4% (132/384), and Taddy goats 29.7% (38/128) were noticeably more infested with ticks when compared to other sheep and goat breeds; however, the difference was not significant ($P=0.79$). The study demonstrates higher infestation of tick suggesting their biological and therapeutic control measures.

In the second part, the molecular characterization of the Ixodid ticks belonging to the genus *Rhipicephalus* was carried out. Prevalence of *Rhipicephalus* ticks was 4.5% in sheep and 3.9% in goats. Molecular characterization based on two mitochondrial and one internal nuclear spacer DNA sequences placed the sequences obtained in this study in paraphyletic clades along with sequences in GenBank. The ITS2 sequence of the *R. sanguineus* (OK642408) and *R. microplus* (OK642409) form a distinct clade with sequences from other countries. The 16S rRNA sequences of *R. sanguineus* (OK560870) clustered with sequences form three lineages, tropical, temperate, and south-eastern lineages. The *Cox I* gene identified *R. turanicus* (OK623472) fall in clad with Pakistan, China, Kazakhstan and Bulgaria, while *R. microplus* (OK623463) form separate clades with sequences from Pakistan, Iran, India and China. The study demonstrates the diversity of *Rhipicephalus* species infesting small ruminants in Pakistan suggesting their possible involvement in the transmission of tick-borne diseases. Further studies on tick-borne diseases are required for control purposes.

In the third part, tick-borne pathogens (TBPs) were investigated in sheep, goats and cattle from Khyber Pakhtunkhwa (KP). This study develops real-time PCR to target Pan-*Theileria*, the primers and probes were designed to target hypervariable V4 region in the *18S* rRNA gene of *Theileria* sp., which allows detection of different *Theileria* sp. We also examined the utility of the *16S* rRNA gene sequence for discriminating *Anaplasma* samples to the species level. Microscopically suspected positive blood samples for *Theileria* and *Anaplasma* were subjected to DNA extraction. A total of fifty-one blood samples for *Theileria* and 14 for *Anaplasma* were subjected to molecular characterization. In *Theileria* qPCR synthetic gBlock™ gene fragments and clinical specimens were used for analytical and clinical validation. PCR positive *Theileria* samples were then confirmed using Sanger sequencing on *18S* rRNA gene. Regression coefficient (R^2) of five reactions was 0.9637 showed a great linearity. Analytical sensitivity ranged from 10 to 100 copies per reaction. The coefficient of variation was less than 5%. Total 51 samples tested for the clinical validation, 47 were positive in Pan *Theileria*, also amplified on the speciation reaction and confirmed by sequencing. Three samples were found positive to *T. orientalis* during sequencing, two were confirmed by qPCR. One sample was found positive by qPCR as *T. ovis*. Overall assay brought us good sensitivity and specificity for our clinical test. *Anaplasma* was observed in the blood of 14 animals, out of these12 *Anaplasma* sequences were of good quality. Across the *Anaplasma* species for which genomes are available, sequence similarity in *16S* rRNA with *Anaplasma marginale* was 99.25% and below this indicating a distinct species. This study adds insight into the epidemiology of TBPs around the KP, province and highlights the need for proactive surveillance of TBPs in livestock.

In the last part, this study investigates the effect of commercial synthetic compounds i.e. Cypermethrine (CYM), Deltamethrin, Trichlorphon + Dimethylester, Ivermectin (IVM) and Fipronil on natural infestations of ticks in goats. The *In-vivo* quantitative assessment of five tick genera i.e. *Hyalomma*, *Rhipicephalus*, *Ixodes*, *Haemaphysalis* and *Boophilus* revealed that both CYM and IVM treated groups resulted in significantly lower ($P<0.05$) tick counts relative to other medicines and controls on all post-treated days. The maximum reduction in mean number of ticks in the CYM and IVM treated group was recorded from days 3 to 4, followed by complete shedding of ticks on day 5. However, Deltamethrin, Trichlorphon+ Dimethylester and Fipronil showed 100% efficacy on sixth day. *In-vitro* efficacy trail fipronil (0.25g/100ml) recorded 100% tick's mortality within 18th hours in post-treated group, while Deltamethrin, Trichlorphon + Dimethylester and CYM were ranked 2nd, 3rd and 5th based of their 100% efficacy within 24-33 hrs, 33-42 hrs and 39-48 hours, respectively. The investigation has shown

that tested acaricides varied in their performance to reduce the tick infestation and further experimentation on different formulations of other members of major acaricidal classes need to be standardized.

GENERAL INTRODUCTION

Ticks are blood feeding ectoparasites considered as vectors infesting all kinds of domestic animals for the transmission of zoonotic infections caused by several pathogens (Fuentem *et al.,* 2017). Ticks belong to suborder of Arachnida, Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitiformes and Suborder Ixodida (Battesti *et al.,* 2006; Urquhart *et al.,* 1996). There are two distinct groups of ticks known as "hard ticks" and "soft ticks," scientifically classified as Ixodidae and Argasidae, respectively. These names are derived from the physical characteristics of these ticks, with hard ticks characterized by a rigid dorsal shield and soft ticks having a flexible, leathery outer layer. According to research by Robert *et al.* (1976) and Latif and Walker (2004), there are a minimum of 702 species of ticks, with approximately 80% (889 species) belonging to the Ixodidae family and the remaining 20% (186 species) belonging to the Argasidae family. These two groups significantly differ from each other in terms of their appearance, behavior, and life cycle, as highlighted by Pegram *et al.* (1987). Ixodid ticks, which can range in size from 2 to 20 mm, have a relatively large physical stature. Their unfed bodies are flattened from top to bottom and consist of two main sections: the anterior gnathosoma, also known as the capitulum, and the posterior idiosoma, which includes the legs (Wall and Shearer, 1997). It has been observed that approximately 10% of all species of hard and soft ticks are capable of transmitting diseases to both domestic animals and humans (Jongejan and Uilenberg, 2004). Ticks that utilize two different host species have larvae that undergo molting to reach the nymphal stage while attached to the first host. Once the nymphs are engorged, they detach from the host, undergo molting in the surrounding environment, and then seek out a new host. Conversely, ticks that rely on only one host remain on the same host for two consecutive molting seasons. Both nymph and larva detached during three-host life cycle from the host to undergo molting, and then find a new host after returning each molting phase. (Zajac et al., 2006).

Pakistan is an agricultural country with a large animal husbandry industry. In Pakistan, livestock presence plays important part in economy as it contributes to the enhancement of socioeconomic conditions within farming communities with limited resources, ultimately helping to alleviate poverty. The livestock industry holds significant importance in Pakistan due to its

provision of various essential products, including milk, meat, protein, and wool. These products serve as the primary source of income for the impoverished population in the country. Pakistan stands as the third-largest country in Asia in terms of the number of sheep and goats in its livestock sector (Devendra, 2005). The Punjab province has the highest proportion of small ruminant population (32.6%), followed by Baluchistan (30.6%), Sindh (20.6%), and Khyber Pakhtunkhwa (KP) (16.1%) (Agricultural Census Organisation, 2020), and small ruminants are the principal source of income for landless farmers (Ghafar *et al.,* 1996). In 2019-2020 milk (1 million), meat (0.75 million), wool (0.47 million), hair (0.29 million), and skins (59.5 million) of small ruminants were added to Pakistan's gross domestic product (GOP, 2008). Globally, hard ticks play a significant role in reducing cattle output potential (Onu *et al.,* 2013). These arthropods sucking blood from various human and animal body areas and transmitting pathogens during feeding, which causes illnesses and reduces milk, meat, and wool production. Animals suffer from immune system depression, blood loss, irritation and overall stress, and skin and hide damage (Ghosh *et al.,* 2007; Garcia, 2003; Biswas, 2003). Tick and Tick-Borne Disease (TBD) epidemiology is expanding with environmental change, resulting in large economic losses of livestock worldwide (Kabir *et al.,* 2011). Tick infestation inflict global financial losses of $14000-18000 million, with US$ 498.7 million spent each year to eradicate ticks and TBDs in India and Pakistan's livestock industries (Minjauw and McLeod, 2003). Australian studies have predicted annual losses of USD 26 million (Sachet and Holmes, 2006). *Rhipicephalus microplus* has caused significant economic losses in the livestock industry (USD 22-30 billion) (Xu *et al.,* 2016).

To evaluate the incidence of ticks, many studies have been documented in different regions of Pakistan. Several tick species namely: *Hyalomma trancatum, H. aegyptium, H. asiatum, Hyalomma anatolicum, Rhipicephalus microplus* and *Haemaphysalis bispinosa* were documented from four agro-climatic zones of Pakistan's Khyber Pakhtunkhwa (Iqbal *et al.,* 2022). Because of the semi-dry climatic condition, *Haemaphysalis bispinosa* and *Hyalomma anatolicum* indicating a widespread occurrence in various districts of KP (Sajid *et al.,* 2009; Irshad, 2010; Singh and Rath, 2013). Ticks can thrive and survive better during the warmer summer months because of the hot, humid weather conditions that prevail in general. Tick genera like as *Hyalomma, Rhipicephalus,*and *Haemaphysalis* have also become acclimated in the Pakistan's northern area, which has dry summers and chilly winters. This demonstrated that these three taxa are widely dispersed across Pakistan's diverse agro-ecological zones. Several researchers (Jongejan and

Uilenberg, 2004; Pegram *et al.,* 1982; Singh and Rath, 2013; Debbarma *et al.,* 2017; Kaur *et al.,* 2015) found that tick prevalence was higher during the wet monsoon than during the dry summer. In the KP province, livestock producers relocate their herds of animals throughout the summer to northern hilly regions where their animals have access to plenty of lush green grazing fields (Iqbal *et al.,* 2022).

In Baluchistan, in many studies different ticks species have been documented for the first time i.e., *Hyalomma (Euhyalomma) schulzei* (Kakarsulemankhel, 2011). *Haemaphysalis flava* infect different sheep breeds *(*Khan, 2000). Several studies have been conducted in Pakistan on ticks distribution (Sultana *et al.,* 2015; Sajid *et al.,* 2017; 2020). Tick species infected goats in Sargodha district includes: *Rhipicephalus* spp. (25.95%), *Hyalomma anatolicum* (31.56%), *Haemaphysalis spp.* (21.07%), *Ixodes* spp. (15.46%), and *Ambylomma spp.* (5.93%) (Manan *et al.*, 2007). Dera Ismail Khan and Lakki Marwat goat population were infected with *Boophilus*, and *Hyalomma* (33.3%) in sheep (Perveen 2011). *Hyalomma* (42.7%) and *Rhipicephalus* (37.6%) were found in Muzaffargarh, Lower Punjab (Sajid *et al.,* 2008), whereas *Hyalomma* (12%), *Boophilus* (8.1%), *Haemaphysalis* (5%) and *Rhipicephalus* (3.1%) were found in Rawalpindi (Durrani *et al.,* 2008). Studying the distribution of ticks is valuable as it helps to estimate how tick-borne diseases are spread and to understand the environmental factors that affect tick populations (Sonenshine *et al.,* 2006).

Ticks and the diseases they transmit, known as tick-borne diseases (TBDs), result in notable economic losses in cattle, particularly in tropical and subtropical regions that are home to 80% of the global cattle population (de Castro, 1997). These diseases also have a significant detrimental impact on the lives of impoverished farming communities, as they lead to reduced productivity in both beef and dairy cattle within these areas (Perry *et al.,* 2002; Jonsson, 2006). In Pakistan, water buffaloes and cattle are significantly affected by infectious diseases such as theileriosis, babesiosis, and anaplasmosis caused by pathogens like *Theileria (T) annulata, Babesia (B) bovis, B. bigemina,* as well as *Anaplasma (A) marginale* and *A. centrale* (Durrani and Kamal, 2008). These diseases are spread by ixodid ticks i.e. *Dermacentor, Haemaphysalis, Hyalomma*, and *Rhipicephalus*, which infest water buffaloes and cattle in Pakistan (Durrani and Kamal, 2008; Durrani *et al.,* 2008; Tasawar *et al.,* 2014). Multiple research studies conducted in Pakistan have indicated that over 80% of bovines, including cattle, are infested with ticks, specifically species of *Hyalomma* and *Rhipicephalus* (Sajid *et al*., 2008; Ghafar *et al.,* 2020). These

ticks serve as vectors for transmitting tick-borne pathogens (TBPs), which can cause diseases such as babesiosis, theileriosis, and anaplasmosis in ruminant animals, as well as Crimean Congo haemorrhagic fever (CCHF) in humans (Jabbar *et al.,* 2015; Ahmed *et al.,* 2006). Tick-borne diseases can spread by human contact through various ways which including contact with infected blood or tissues or tick bites. People who work with livestock, including slaughterhouse employees, veterinarians, laborers, workers in laboratories, and milk delivery personnel, face an increased risk of infection because they encounter ticks that are attached to animals on a regular basis (Carter *et al.,* 2012; Charrel *et al.,* 2004). TBDs, including Lyme disease, rickettsiosis, Crimean Congo haemorrhagic fever (CCHF), and tick-borne encephalitis, are prevalent worldwide, including in Pakistan (Kaisi *et al.,* 2020; Parola *et al.,* 2013).

Controlling ticks poses significant challenges, as conventional methods like biological and chemical interventions have proven ineffective and unsatisfactory. One of the reasons for this is that many types of ticks have become resistant to acaricides, which are chemicals used to kill or control ticks (George *et al.,* 2004; Abbas *et al.,* 2014), unintended toxicity to non-target organisms (Ray *et al.,* 2000; Dahlgren *et al.,* 2012), and the high costs associated with chemical tick control treatments (De Meneghi *et al.,* 2016). In Pakistan, tick control becomes particularly challenging due to the importation of exotic cattle breeds that are more susceptible to tick infestation under the country's climatic conditions (Buczek *et al.,* 2013).

Different strategies have been reported against tick control in domestic animals like buffaloes, cattle, sheep, and goats (Eisen and Dolan, 2016). Burning waste materials near animal sheds has proven effective in reducing tick infestations by eliminating tick stages (Ramzan *et al.,* 2018). Providing separate sheds for each animal, particularly for exotic and native breeds, can effectively minimize tick infestations (Lemcke, 1997). Taking out ticks from animals as soon as possible can greatly decrease the number of ticks on their bodies yearly (Muhammad, 1994). Proper clearance of grasses can also help reduce tick infestations to some extent (Gaff *et al.,* 2015; Mays *et al.,* 2016). Implementing bait boxes to control rodents and ticks on their bodies can be an effective strategy (Dolan *et al.,* 2017). There are different ways to control tick populations, such as using powders, spraying by hand, using mechanical spray races, applying treatments directly with hands, using systemic treatments, and using dipping methods. These methods help reduce the

number of ticks (Konet, 2004). Nevertheless, in developing countries, the most employed method for controlling ticks is still with chemical treatments.

Tick control has been achieved with significant effectiveness using acaricides, including synthetic pyrethroids, organophosphates, macrocyclic lactones, organochlorines, carbamates, and insect growth regulators (Whitnall *et al.,* 1951; Davey and Ahrens, 1984). Specifically, in tropical and subtropical season, tick species populations showed resistance against the acaricides because of extensive utilization in tick management (Abbas *et al.,* 2014; Lenka *et al.,* 2016). Primary methods which are using for control of tick in ruminants which involve the periodic application of acaricides, for instance macrocyclic lactones, trichlorfon, and cypermethrin in Pakistan (Jabbar *et al.,* 2015; Iqbal *et al.,* 2017; Graf *et al.,* 2004). However, studies on assessment of the *In-vivo* efficacy of acaricidal drugs are limited and studies on coumaphos, cypermethrin, diazinon and ivermectin were performed in both sheep and goats in Pakistan (Khan, 1993; Sajid *et al.,* 2011).

Objectives

The objectives of present study were:

1. To find the prevalence and morphometric identification of hard ticks infesting sheep and goats in three agro-ecological zones of Pakistan. We also investigated the epidemiological association of various ticks' species infesting sheep and goats.
2. Molecular characterization to investigate the diversity of *Rhipicephalus* ticks infesting small ruminants in three agro-ecological zones of Pakistan.
3. Molecular identification of tick-borne pathogens *Theileria* and *Anaplasma*.
    a. A multiplex qPCR assay was established for the detection and the quantification of *Theileria* species, compared analytical validation results with clinical samples by designing Pan-*Theileria* and four specific probes to targets *18S* rRNA.
    b. Standard PCR and sequencing of the *18S* rRNA were performed on samples positive in the FRET-qPCR for phylogenetic analysis.
    c. For *Anaplasma 16S* rRNA genetic marker was used for molecular identification and phylogenetic analysis.
4. *In vivo* and *In-vitro* efficacy testing to establish the current level of acaricidal resistance for five products representing every major acaricidal class (cypermethrine and deltamethrin

representing the synthetic pyrethroid; trichlorphon + dimethyl ester representing organophosphates; ivermectin representing the macrocyclic lactones and fipronil representing phenylpyrazole compound) in controlling natural infestations with ticks of goats.

PREVALENCE AND MORPHOMETRY OF HARD TICKS (IXODIDAE) INFESTING SMALL RUMINANTS

ABSTRACT

Ticks are important vectors which cause life-threatening problems in animals worldwide. The present study aimed to investigate morphometric epidemiology of hard ticks infesting sheep and goats in three agroecological zones of Pakistan. A total of 4488 animals (2184 sheep and 2304 goats) were examined for ticks' infestation. Tick genera were identified based on their morphological features through conventional taxonomic keys. All ticks collected were belonged to four tick genera: *Rhipicephalus, Hyalomma*, *Haemaphysalis,* and *Ixodes.* The results showed slightly higher tick infestation in sheep (23%) compared to goats (20.1%). Male sheep had higher infestation of 23.4%, whereas in goats females had a higher infestation of 23.0%. However, the difference was not significant(P≥0.05). Tick prevalence was similar across the study zones (P=0.79), with sheep in Muzaffarabad showing a slightly higher prevalence (47.0%). The prevalence was higher in sheep and goats younger than 1 year (25.7% and 20.8%, respectively) and did not vary significantly (P=0.99). Shinwar-White sheep breed 34.4% (132/384), and Taddy goats 29.7% (38/128) were noticeably more infested with ticks when compared to other sheep and goat breeds; however, the difference was not significant (P=0.79). The study demonstrates higher infestation of tick in small ruminants in three agroecological zones of Pakistan suggesting their biological and therapeutic control measures.

Keywords: Ticks, Taxonomy, Epidemiology, Sheep, goats,

2.1 INTRODUCTION

Ticks are distributed across the world including Asia (Haque *et al.,* 2011; Singh and Rath, 2013), Africa (Reye *et al.,* 2012;), North and South Americas (Lohmeyer *et al.,* 2011), Australia (Kamau, 2011) and Europe (Scharlemann *et al.,* 2008). At present, there are 896 recognized species of ticks across the globe (Guglielmone *et al.,* 2010). These species are classified into three different families: Argasidae (soft ticks), Ixodidae (hard ticks), and Nuttalliellidae, consists of only one species. The family Ixodidae encompasses around 702 species distributed among 14 genera. It is necessary to note that these numbers may change over time as advancements in molecular techniques contribute to resolving taxonomic discrepancies (Guglielmone *et al.,* 2010). Hard ticks are involved in causing economic losses with symptoms of weight loss, paralysis, milk production, skin irritation and transmitting a huge number of pathogens which includes bacteria, rickettsiae, protozoa, spirochetes, and viruses (Mirzaei *et al.,* 2014). The economic losses related to the tick burden have been estimated to be US $498.7 million annually in Asian countries (Minjauw and McLeod, 2003). Livestock plays an important part in Pakistan's economy, making up 55.4% of the total value of the farm sector, respectively, adding 11.9% to the gross domestic product of the country. In Pakistan, 30-50 million people are directly involved in livestock rearing as supplementary work and each farming family owns 5-6 sheep/goats. A total of 31.2 million sheep, 78.2 million goats are present in the country (GOP, 2020).

Numerous studies have been investigated to confirm the presence of different species of ticks and their associated risk factors in different regions of Pakistan. Five ticks' genera were more frequently recorded in Pakistan, namely *Hyalomma, Haemaphysalis, Rhipicephalus, Ornithodoros*, and *Argus* (Karim *et al.,* 2017; Rehman *et al.,* 2017). The frequency of ticks varies in small ruminants with 26.5% *Rh*. *Boophilus annulatus* followed by 6.1 % *Hyalomma anatolicum* and 3.4% *Rhipicephalus turanicus* (Asmaa *et al.,* 2014). From arid and semi-arid zones of country proportion of tick infestation was 11% in sheep (Rehman *et al.,* 2017). In the hilly and plain areas of Khyber Pakhtunkhwa and Gilgit Baltistan higher prevalence of 81.47% was recorded in sheep (Sajid *et al*., 2017).

The morphological characters are frequently used for tick identification (*Nava et al.,* 2009). Hard ticks recognized by a sclerotised scutum and gnathosoma located apically (Hajdušek *et al.*

2013). Ticks are classified based on their morphology and fundamental structural characteristics, particularly the shape of their scutum (dorsal shield) and its ventral structure. Once the tick genus is identified, female adult ticks belonging to each genus can be distinguished by examining specific areas of the scutum on the anterior dorsum (Walker *et al.,* 2003). Morphometrics, which involves measuring distances between anatomical landmarks, is a widely utilized technique for quantifying phenotypic variation (Dujardin, 2011). It can be employed to compare different tick species or to examine variations within a species (Sorensen and Footit, 1992). A very limited detail data on morphometrics of tick genera is available from the study area. Hence, the current study reported the epidemiology, prevalence and taxonomic identification of hard ticks which infests goat and sheep in three ecologies (plain, semi-hilly and highland) of Pakistan. We also investigated the epidemiological association of various ticks' species infesting goats and sheep. The findings of this study will be helpful for further development of control strategies to safeguard the animal as well as human health conditions.

2.2 **MATERIALS AND METHODS**

2.2.1 Study Area

The study area comprised three ecological zones of Pakistan: Punjab (Lahore, Jhelum, Attock, and Rawalpindi), Balochistan (Quetta), and Azad Kashmir (Muzaffarabad) (Fig. 1). These ecological regions have diverse climates: tropical, semi-arid, hot, and subtropical monsoon type in Punjab, continental semiarid in Quetta, and humid subtropical climate in Muzaffarabad. The rainfall pattern varies markedly across the regions.

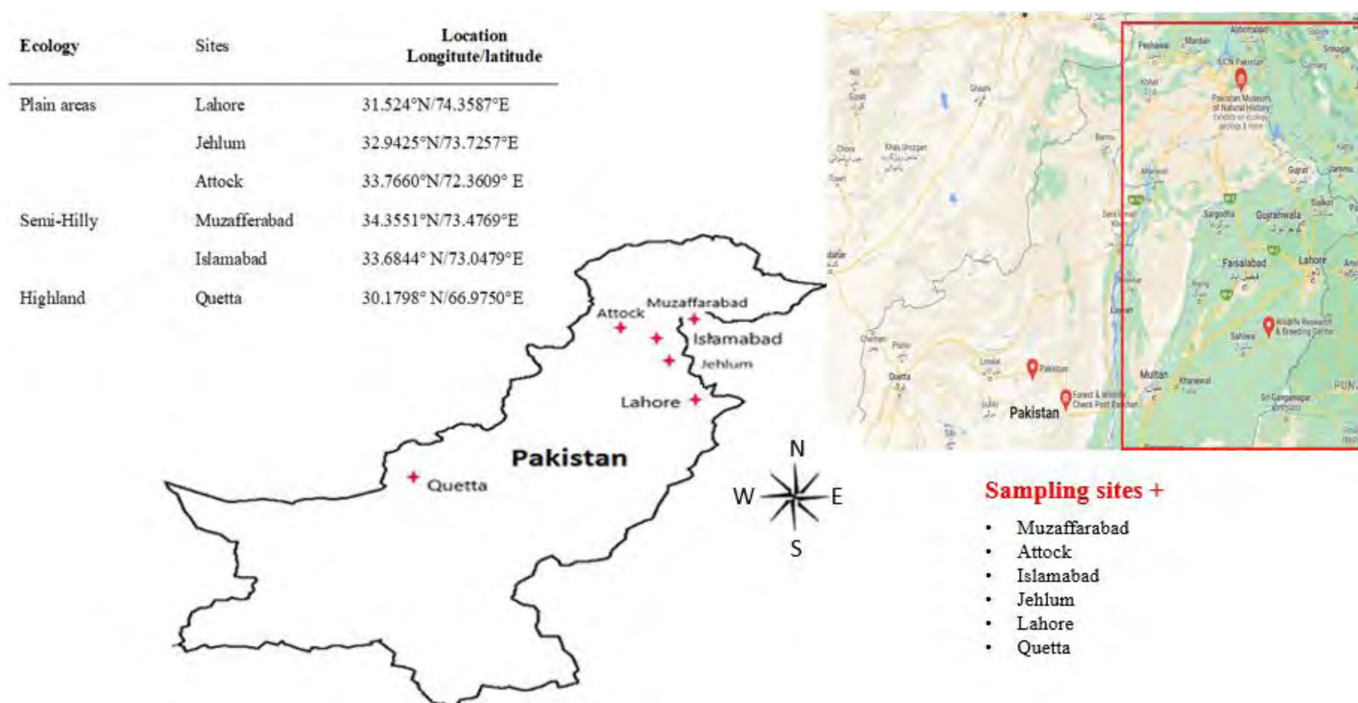


Fig. 2.1. Map of Pakistan indicating six tick sampling areas within three agro-ecological zones.

2.2.2 Tick collection

A total of 4488 animals (2184 sheep, 2304 goats) were examined for tick infestation during April 2018 to August 2019. The animals were managed under natural grazing and stall-feed. Five farms were randomly selected from each sampling site and visited twice a month, all animals (sheep and goats) at the farms were physically examined for the presence of ticks. Relevant information regarding tick infestation, age, breed, sex, and site of infestation was recorded at the time of sampling.

The collection of ticks needs inspection of the head area with proper attention given to the ears, thorough examination of the neck-chest area and legs. Tweezers were used to remove the ticks, ensuring that the mouth parts (Capitulum) remained undamaged and attached to the tick. Ticks were collected from infected sheep and goats directly without application of acaricides. To encourage maximum participation, only up to five ticks per animal were collected for DNA extractions and morphological studies. The ticks were placed in individual tubes, containing 70% ethanol, and labelled with the animal code and collection date.

2.2.3 Ticks Preservation and Slide Preparation

In the laboratory, the ticks were preserved by immersing them in 70% ethyl alcohol, followed by the preparation of permanent mount slides for further examination (Shah et al., 2015). To begin the slide preparation process, the ticks were removed from the ethyl alcohol preservative and kept in water for one hour. Subsequently, the ticks have been transferred to a new tube which contains a 10% KOH solution, where they were kept for a minimum of 24 hours. Afterward, the ticks were thoroughly rinsed with water to eliminate the KOH, followed by a 30-minute immersion in a 5% aqueous solution of glacial acetic acid. To remove any residual acid, the ticks were then transferred to water and allowed to soak for 30 minutes. If needed, specimens that appeared too pale were stained with Acid fuchsin. Then the specimens were dehydrated by keeping for at least 10 minutes in each 80 %, 90 % and absolute alcohol. The ticks were treated with cedar wood oil for at least 24 hours to remove any impurities. After that, they were briefly placed in xylene for 1 minute before being mounted. The ticks were mounted in DPX mountant on a cavity slide. (Gutierrez, 1985)

2.2.4 Morphological Identification

Ticks were morphologically identified (n=450) based on genus level using dichotomous keys (Walker *et al*., 2003; Barker and Walker, 2014). The standard morphological measurements are presented in mean and SD values. All ticks were carefully examined, and characters used for taxonomical differentiation of ticks were measured as previously described (Beati and Keirans, 2001; Walker *et al*., 2000; 2003).

2.2.5 Statistical Analysis

All the ticks prevalence data in sheep and goats were initially entered in MS Excel followed by coding for various factors assessment. Afterward, the coded data were analysed through statistical computer program Statistix version 8.1. To compare the prevalence and association of ticks among breed, age, and sex categories, the Chi-square test was performed by using SPSS 20.0 statistical software. The t-test statistic was applied to find the differences among morphometric measurements of various tick genera. Significance was defined as $P \leq 0.05$.

2.3 RESULTS

2.3.1 Prevalence of Ticks

Of the 2184 sheep and 2304 goats examined, 514 (23.5%) sheep and 462 (20.1%) goats were infested with ticks (Table 2.1). All ticks collected were identified as adults, males and females, or as immature stages belonging to four tick genera: *Rhipicephalus, Hyalomma*, *Haemaphysalis,* and *Ixodes.* Their prevalence in sheep and goats was recorded (Fig. 2.2). Tick prevalence was slightly higher in males 23.4% (266/1092) when compared to females 22.7% (248/1092) in sheep, whereas in goats females had a higher prevalence, 23.0 % (265/1152), than males, 17.1% (197/1152). Tick prevalence was similar across the study zones (P=0.79), with sheep in Muzaffarabad showing a slightly higher prevalence (47.0%; 124/264); and goats in Jhelum showing a somewhat lower prevalence (38.0%; 146/384). The prevalence was higher in sheep and goats younger than 1 year (25.7% and 20.8%, respectively) and did not vary statistically (P=0.99) from sheep and goats older than 1 year. Finally, the Shinwar-White sheep breed 34.4% (132/384), and Taddy goats 29.7% (38/128) were noticeably more infested with ticks when compared to other sheep and goat breeds; however, the difference was not significant (P=0.79).

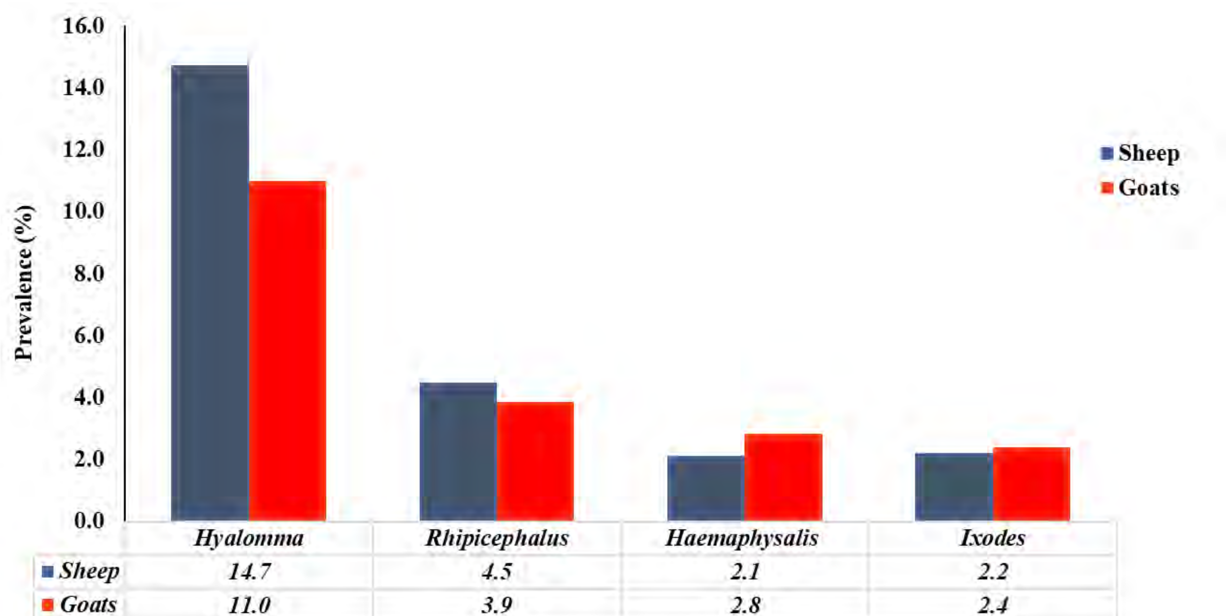


Fig. 2.2 Prevalence of four tick genera identified in sheep and goats from Pakistan.

Table 2.1: Prevalence and tick infestation rate in sheep and goats with respect to geographical location, host, sex, age, and breed from Pakistan.

Characteristics	Number of Animals			Ticks' infestation				
	Examined	Infested	Prevalence (%)	*Hyalomma*	*Rhipicephalus*	*Haemaphysalis*	*Ixodes*	Chi-square
Sheep Sites								
Lahore	384	60	15.6	33	13	4	6	χ^2=7.22, p=0.99^{NS}
Jehlum	384	154	40.1	106	29	9	12	
Attock	384	110	28.7	71	20	13	9	
Muzafferabad	264	124	46.97	77	23	11	13	
Rawalpindi	384	27	7.0	13	5	3	4	
Quetta	384	39	10.2	22	8	6	4	
Goats Sites								
Lahore	384	49	12.8	27	13	6	5	χ^2=14.6, p=0.79^{NS}
Jhelum	384	146	38.0	65	43	25	12	
Attock	384	112	29.2	55	9	15	18	
Muzaffarabad	384	103	26.8	67	16	15	15	
Rawalpindi	384	20	5.2	14	4	3	2	
Quetta	384	32	8.3	25	4	1	3	
Host (Type)								
Sheep	2184	514	23.5	322	98	46	48	χ^2=0.82; p=0.93^{NS}
Goats	2304	462	20.1	253	89	65	55	
Age (years)								
Sheep								
One year and below	1092	281	25.7	181	50	20	26	χ^2=3.35, p=0.99^{NS}
Above one year	1092	233	21.3	141	48	26	22	
Goats								
One year and below	1152	240	20.8	135	45	40	21	χ^2=3.35, p=0.99^{NS}
Above one year	1152	222	19.3	118	44	25	34	
Sex								
Sheep								
Male	1092	266	24.4	160	46	27	23	χ^2=4.02, p=0.94^{NS}
Female	1092	248	22.7	162	52	19	25	
Goats								
Male	1152	197	17.1	121	22	34	20	χ^2=4.02, p=0.94^{NS}
Female	1152	265	23.0	132	67	31	35	
Breeds								
Sheep								
Balochi	192	34	17.7	32	4	9	6	χ^2=21.6, p=0.79^{NS}
Balkhi	708	192	27.1	127	40	13	19	
Shinwar-white	384	132	34.4	71	20	9	9	

Non-Discreptive	900	156	17.3	92	34	15	14	
Goats								
Beetal	896	217	24.2	129	41	38	29	χ^2=21.6, p=0.79^{NS}
Lehri	192	14	7.3	11	2	1	2	
Taddy	128	38	29.7	20	5	6	5	
Non-Discreptive	1088	193	17.7	93	41	20	19	

*^{NS} not significant (P>0.05)

2.3.2 Morphological Identification of Tick Species

The prevalence of *Hyalomma* spp. was the highest recorded at 14.7% and 11.0%, followed by *Rhipicephalus* 4.5% and 3.9%, *Haemaphysalis* 2.1% and 2.8%, and *Ixodes* 2.2% and 2.4% in sheep and goats, respectively (Fig. 2.2). Unengorged tick specimens from each species were morphologically measured (Fig. 2.3 and Table 2.2). Differences in physical characteristics were observed among *Rhipicephalus* tick species, particularly in terms of size, pattern on their hard outer covering (scutal pattern), female genital opening, length of spiracular plate and male adanal plate. Among the various measured characteristics, only the length-to-width ratio of the front part of the tick's body (basis capituli) was significantly different between *R. sanguineus* and both *R. turanicus* and *R. microplus* ticks. The length/width ratio of the male *R. sanguineus* was significantly (P=0.001) different from male *R. microplus*. Members of the genus *Hyalomma* are all large ticks with a relatively long hypostome, beady eyes, banded legs, and three pairs of anal plates in male ticks. Small ticks belonging to the genus *Haemaphysalis* differ from those belonging to other genera by lacking eyes, having festoons present at all life stages, having rectangular-shaped capituli, and not having adanal plates on the males. *Ixodes* ticks are characterized by their medium size and golden-brown coloration. They possess a notably enlarged first article in their palps, densely arranged teeth on the hypostome, strong pointed spurs on all coxae, and an open anal groove at the posterior end.

Table 2.2: Morphological measurements of hard ticks (Ixodidae) presented in values of Mean±SD, all measurement were taken in millimetres (mm).

Characters	*R. sanguineus*		*R. turanicus*	*R. microplus*		*Hyalomma*	*Haemaphysalis*		*Ixodes*
	Male (n=50)	**Female (n=50)**	**Male (n=50)**	**Male (n=50)**	**Female (n=50)**	**Male (n=50)**	**Female (n=50)**	**Male (n=50)**	**Female (n=50)**
Body L	3.66±0.07	3.85±0.09	3.85±0.19	5.2±0.0	4.42±0.15	5.25±0.25	1.98±0.08	2.21±0.01	4.25±0.25
Body W	1.82±0.03	1.90±0.13	1.81±0.20	3.52±0.0	2.82±0.11	2.55±0.15	0.85±0.07	0.98±0.12	2.46±0.05
Ratio BL/BW	2.01	2.03	2.13	1.48	1.57	2.06	2.33	2.26	1.73
Adanal plates L	0.81±0.03	0.82±0.06	0.93±0.06	1.1±0.0	0.81±0.04	0.96±0.02	0.47±0.02		
Adanal plates W	0.30±0.01	0.33±0.04	0.57±0.20	0.97±0.01	0.39.9±0.04	0.35±0.005	0.23±0.01		
Basis capituli L	0.36±0.05	0.39±0.16	0.26±0.04	0.33±0.02	0.28±0.02	0.33±0.0.3	0.36±0.004	0.11±0.01	0.14±0.005
Basis capituli W	0.59±0.07	0.51±0.10	0.75±0.07	0.99±0.01	0.59±0.05	0.47±0.22	0.17±0.004	0.25±0.08	0.38±0.03
Ratio BCL/BCW	0.61	0.76	0.35	0.33	0.47	0.70	2.12	0.44	0.37
Distance b/w Porose areas	0.26±0.01	0.35±0.05	0.23±0.02	0.29±0.0	0.18±0.011	0.39±0.01	0.12±0.009	0.10±0.00	0.19±0.03
Idiosoma L	2.99±0.09	3.09±0.06	3.23±0.17	4.9±0.0	3.48±0.16	3.85±0.05	1.39±0.09	1.91±0.08	3.43±0.22
Idiosoma W	1.80±0.04	1.90±0.13	1.81±0.20	3.52±0.02	2.82±0.11	2.55±0.15	0.85±0.07	0.98±0.12	2.46±0.05
Palp L	0.31±0.007	0.35±0.02	0.29±0.03	0.46±0.01	0.32±0.01	0.72±0.07	0.38±0.01	0.19±0.01	0.38±0.03
Palp W	0.15±0.01	0.13±0.006	0.18±0.002	0.16±0.00	0.15±0.008	0.19±0.01	0.16±0.01	0.11±0.01	0.15±0.01
Spiracular area L	0.31±0.04	0.40±0.10	0.38±0.08	0.35±0.0	0.28±0.01	0.84±0.13	0.29±0.01	0.14±0.03	0.28±0.03
Spiracular area W	0.20±0.02	0.20±0.009	0.14±0.01	0.26±0.00	0.18±0.005	0.31±0.01	0.20±0.004	0.10±0.01	0.17±0.01
Posterior grooves L	0.54±0.03	0.47±0.04	0.68±0.02	0.86±0.0	0.57±0.03	1.01±0.01	0.53±0.03		0.44±0.01
Posterior grooves W	0.23±0.02	0.37±0.10	0.37±0.03	0.46±0.0	0.32±0.02	0.97±0.01	0.30±0.23		0.28±0.03
Accessory Adanal Shield L	0.35±0.04	0.32±0.02	0.35±0.03	0.45±0.0	0.25±0.008	0.30±0.05	0.235±0.012		0.29±0.005
Accessory Adanal Shield W	0.17±0.02	0.15±0.02	0.19±0.02	0.21±1.0	0.16±0.005	0.18±0.00	0.22±0.004		0.20±0.005
Genital aperture L	0.19±0.01	0.16±0.02	0.22±0.01	0.25±0.0	0.18±0.005	0.30±0.005	0.11±0.006	0.07±0.01	0.21±0.005
Genital aperture W	0.17±0.01	0.15±0.02	0.17±0.01	0.26±0.0	0.15±0.006	0.27±0.07	0.10±0.006	0.10±0.005	0.19±0.005
Palp article I L	0.09±0.009	0.13±0.03	0.07±0.008	0.13±0.01	0.09±0.004	0.11±0.00	0.10±0.009	0.05±0.01	0.05±0.005
Palp article I W	0.10±0.007	0.11±0.008	0.08±0.004	0.10±0.01	0.06±0.005	0.08±0.03	0.09±0.004	0.06±0.02	0.05±0.01
Palp article II L	0.09±0.004	0.10±0.003	0.09±0.005	0.16±0.00	0.11±0.004	0.22±0.005	0.125±0.006	0.09±0.01	0.10±0.005
Palp article II W	0.11±0.008	0.13±0.004	0.014±0.02	0.13±0.01	0.09±0.005	0.18±0.005	0.10±0.005	0.10±0.00	0.09±0.005
Palp article III L	0.11±0.01	0.10±0.01	0.14±0.02	0.19.0±0.0	0.11±0.006	0.27±0.04	0.12±0.004	0.07±0.01	0.14±0.005
Palp article III W	0.10±0.007	0.09±0.01	0.11±0.02	0.11±0.00	0.07±0.003	0.14±0.005	0.082±0.005	0.08±0.00	0.09±0.005
Scutum L	2.73±0.20	2.25±0.41	3.17±0.17	2.15±0.05	0.72±0.024	3.77±0.07	1.38±0.10	1.90±0.10	3.40±0.20
Scutum W	1.76±0.06	1.60±0.03	1.81±0.20	2.10±0.0	0.80±0.012	2.50±0.10	0.84±0.07	0.98±0.12	2.46±0.05

Ratio SL/SW	1.55	1.41	1.75	1.02	0.90	1.51	1.64	1.94	1.38
Hypostome L	0.74±0.04	0.85±0.03	0.94±0.05	0.94±0.00	0.52±0.043	1.60±0.10	0.55±0.01	0.35±0.06	0.55±0.04
Hypostome W	0.18±0.01	0.19±0.003	0.22±0.02	0.42±0.00	0.18±0.014	0.21±0.10	0.19±0.01	0.09±0.01	0.28±0.03
Coxa I L	0.44±0.02	0.38±0.08	0.50±0.01	0.98±0.02	0.43±0.015	0.70±0.00	0.42±0.04	0.22±0.02	0.51±0.07
Coxa I W	0.36±0.03	0.44±0.03	0.30±0.03	0.75±0.0	0.26±0.014	0.45±0.15	0.21±0.006	0.17±0.01	0.33±0.01
Coxa II L	0.50±0.02	0.44±0.05	0.63±0.02	1.15±0.05	0.47±0.023	0.75±0.05	0.43±0.03	0.19±0.01	0.47±0.02
Coxa II W	0.42±0.02	0.51±0.06	0.35±0.02	1.35±1.5	0.39±0.023	0.71±0.11	0.28±0.02	0.18±0.005	0.44±0.21
Coxa III L	0.43±0.02	0.41±0.04	0.48±0.02	0.91±0.0	0.41±0.027	0.57±0.07	0.50±0.03	0.23±0.04	0.39±0.04
Coxa III W	0.48±0.03	0.58±0.02	0.36±0.03	0.85±0.0	0.37±0.021	0.75±0.05	0.36±0.02	0.18±0.04	0.60±0.20
Coxa IV L	0.41±0.01	0.41±0.007	0.51±0.02	0.96±0.0	0.40±0.020	0.74±0.01	0.44±0.011	0.25±0.005	0.46±0.14
Coxa IV W	0.50±0.03	0.60±0.03	0.33±0.05	0.65±0.0	0.35±0.015	0.56±0.14	0.41±0.15	0.27±0.01	0.47±0.25
Cornua L	0.28±0.08	0.47±0.21	0.20±0.007	0.35±0.00	0.15±0.009	0.21±0.01	0.18±0.004	0.06±0.00	0.19±0.01
Cornua W	0.13±0.009	0.30±0.16	0.20±0.006	0.22±0.0	0.21.5±0.03	0.22±0.005	0.14±0.012	0.15±0.03	0.25±0.03
Tibia L	0.66±0.03	0.66.±0.09	0.73±0.02	0.69±0.0	0.35±0.018	1.17±0.07	0.66.±0.03	0.24±0.04	0.60±0.24
Tibia W	0.26±0.01	0.26±0.02	0.25±0.02	0.22±0.01	0.14.6±0.009	0.40±0.00	0.355±0.01	0.14±0.00	0.22±0.06
Tarsus L	0.58±0.04	0.69±0.09	0.63±0.03	0.87±0.02	0.38±0.016	0.85±0.05	0.66±0.01	0.29±0.005	0.60±0.19
Tarsus W	0.22±0.03	0.15±0.01	0.20±0.01	0.18±0.0	0.139±0.007	0.25±0.00	0.15±0.01	0.09±0.01	0.18±0.02
Anal groove L	1.00±0.03	0.82±0.21	0.91±0.05	0.98±0.02	0.675±0.06.4	1.36±0.14	0.37±0.02		0.85±0.40
Anal groove W	0.47±0.04	0.70±0.17	0.38±0.03	0.69±0.0	0.469±0.032	1.20±0.00	0.45±0.00		0.67±0.42
Anus L	0.18±0.01	0.26±0.06	0.22±0.01	0.22±0.00	0.174±0.004	0.33±0.02	0.14±0.02	0.09±0.00	0.25±0.04
Anus W	0.19±0.01	0.20±0.02	0.19±0.01	0.20±0.00	0.16.±0.007	0.32±0.02	0.11±0.00	0.09±0.01	0.24±0.07
Sub Anal Plates L						0.14±0.00	0.10±0.01		
Sub Anal Plates W						0.14±0.00	0.08±0.007		
central festoon L						0.15±0.05	0.087±0.003		
central festoon W						0.21±0.01	0.07±0.003		

***L: length; W: width**

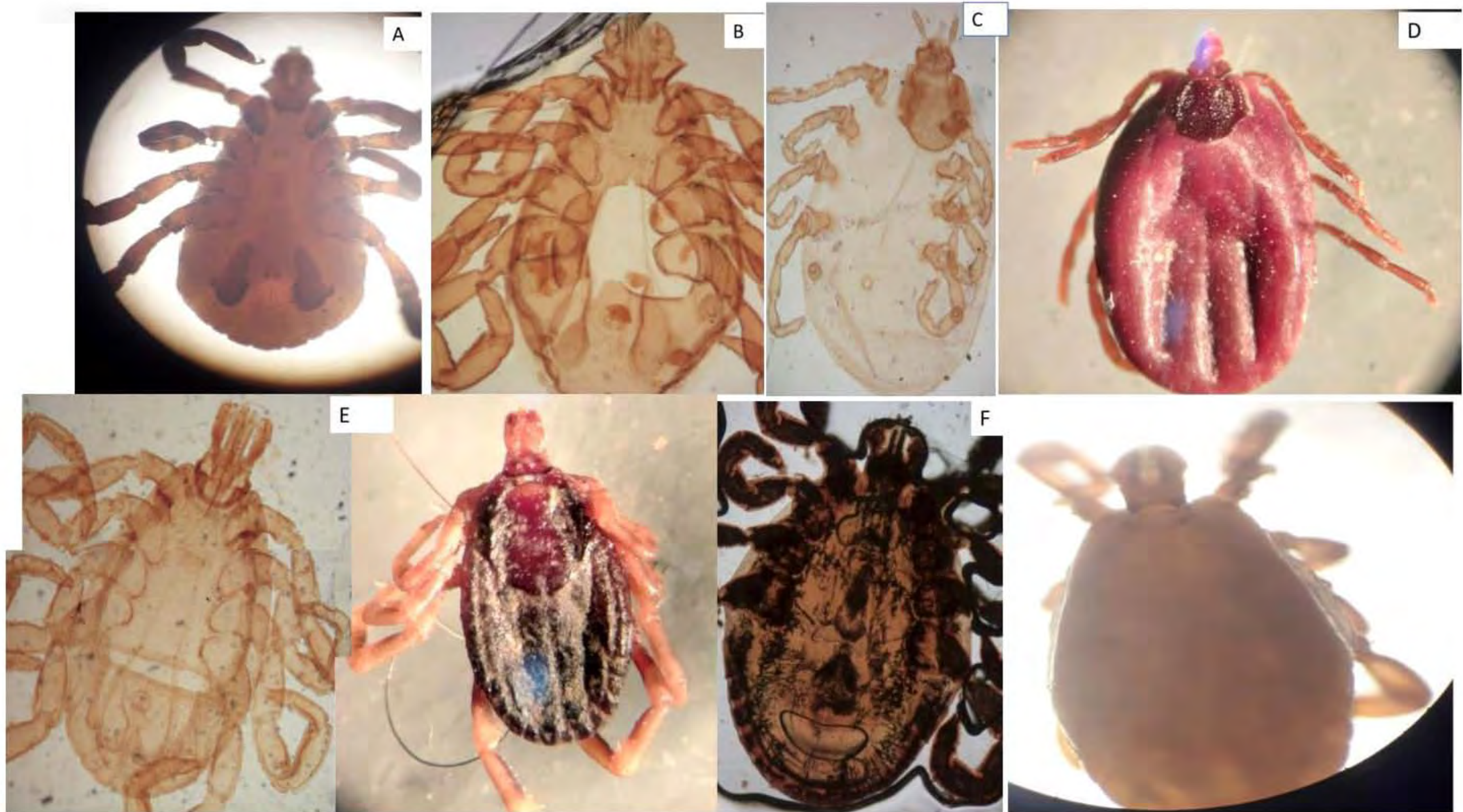


Fig. 2.3: Ventral view of A) *R. sanguineus* B) *R. turanicus* and C) *R. microplus* D) Dorsal view of *Ixodes* E) *Hyalomma* vental and dorsal view F) *Haemaphysalis* vental and dorsal view.

2.4 DISCUSSION

The present investigation provides insight into the distribution and morphological identification of hard ticks, mainly *Rhipicephalus, Hyalomma, Haemaphysalis and Ixodes* sp, from small ruminants in Punjab, Balochistan, Islamabad and Azad Kashmir, Pakistan. Numerous researchers have identified, in several areas of Pakistan, ticks that carry numerous pathogens of veterinary and medical significance, such as *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Hepatozoon*, *Rickettsia* and *Theileria* (Ghafar *et al.,* 2020a, b).

Previously, studies conducted on tick infestation in small ruminants of tribal areas of Pakistan identified the occurrence of *Hyalomma*, *Haemaphysalis,* and *Rhipicephalus* species (Ghafar *et al.,* 2020a, b), consistent with the current findings. However, the current work provides the first detailed morphological evidence for *Rhipicephalus, Hyalomma, Haemaphisalis and Ixodes* in small ruminants of Pakistan.

We recorded the prevalence of *Hyalomma* was highest, followed by *Rhipicephalus* in sheep and goats, consistent with previous studies (Ali *et al.,* 2013; Sultana *et al.,* 2015; Rehman *et al.,* 2017; Ganjali *et al.,* 2014; Chhillar *et al.,* 2014). *Hyalomma* ticks are a potential vector for the transmission of *Theileria* in Pakistan (Ali *et al.,* 2013). The results of the current research showed the presence of three *Rhipicephalus* species, i.e., *R. sanguineus*, *R. turanicus,* and *R. microplus* based on morphological evidence in all examined small ruminants from the three agro-ecological zones of Pakistan. Among *Rhipicephalus* species, *R. microplus* was the most prevalent species, consistent with previous study in northern Punjab (Rehman *et al.,* 2017). The variations in temperature and climatic conditions may be the cause of the variability in tick infestation between research areas (Iqbal *et al.,* 2014; Batool *et al.,* 2019). Various researchers have identified a range of environmental elements that contribute to the survival of ticks within a particular area. These factors encompass temperature, humidity, rainfall (Greenfield *et al.,* 2011), vegetation (Gray, 2002), the presence of suitable hosts, seasonal fluctuations (Teel *et al.,* 1996), animal age, Lactation stage, nutritional status and breed (Alonso *et al.,* 2007; Yacob *et al.,* 2008) altitude (Cadenas *et al.,* 2007), physical condition (Rony *et al.,* 2010), acaricide application method (Bianchi *et al.,* 2003), methods and techniques that associated with animal husbandry (Sajid *et al.,* 2011), and the patterns of animal movement (Hassan and Osman, 2003).

The data regarding the prevalence of ticks in different hosts and breeds of animals revealed the highest prevalence in Shinwar-White sheep (34.4%) compared to all goat breeds studied. The low tick infestation in goats could be explained by their feeding and grooming behaviour which helps to prevent the attachment of ticks and detaches attached ticks from the body (Malla *et al.,* 2021). The tick prevalence might be linked to habitats, diverse vegetation patterns and exposure to the same community grazing space (Sajid *et al.,* 2009; Estrada-Peña, 2009). The results showed that animals aged one year and below were more heavily infested with ticks (24.7% in sheep and 21.8% in goats) compared to older animals, consistent with reports from a previous study (Negi and Arunachalam 2019). This might be due to the pliant and soft skin of young animals which enables easier attachment of the ticks (Kabir *et al*., 2011). Another study recorded nearly similar findings with our results that young animals were discovered to have a greater number of parasites comparatively adult animals (Sultana *et al.,* 2015), this could be justified might be due to reduce behavioural activities (Sajid *et al.,* 2011). In addition, the development of acquired immunity and the thicker skin of older animals can contribute to their resistance against tick infestations (Obi *et al.,* 2014). Our findings differ from those of Riaz *et al.* (2017), who reported a higher incidence of tick infestation (52.3%) in animals older than three years, followed by animals aged 1-2 years (45.23%), and a lower incidence in animals younger than one year (44.0%). Asmaa *et al*. (2014) also disagreed with our results that the highest infestation rates recorded in older ages, above three years (78.8%) followed by at age of two months (57.8%) in sheep (22.9%), respectively.

Among various breeds, the Shinwar-White sheep showed higher prevalence (34.4 and 24.2%) followed by Balkhi sheep (27%), non-Descriptive sheep (17.3%) and Balochi sheep (12.5%). There is limited reference available regarding the specific breeds targeted in present study. Shahid (2017) reported lower prevalence than present study, in Shinwar sheep breed (10%) and Balochi sheep (10%).

Four ticks' genera were identified in present study, among these ticks similar were also reported by Ramzan *et al*. (2020) i.e., *Haemaphysalis punctata, Rhipicephalus singuanieus, Dermacentor 9ijkkkmarginatus, Hyalomma dromedarii, H. excavatum, H. anatolicum*, and *Rhipicephalus microplus* in Multan, Pakistan. In a study conducted by Irshad *et al.* (2010), the tick species observed on goats consisted of *Rhipicephalus* spp (73.36%), *Haemaphysalis* spp (23.36%),

Ixodes spp (2.17%), and *Ambylomma* spp (1.08%). For sheep, the two identified genera were *Rhipicephalus* spp (73.68%) and *Haemaphysalis* spp (26.31%). Similarly, Dimanopoulou *et al.* (2017) identified different tick species in Greece, including *Haemaphysalis punctata* (13%), *Hyalomma anatolicum* (4.47%), *Rhipicephalus sanguineus* (4.47%), and *Ixodes ricinus* (3.58%). In contrast Fatemian, *et al*. (2018) recorded some other tick species like *Hyalomma scupense*, *Dermacentor marginatus* in sheep and goats. Rubel *et al.* (2018) identified different species in Russia. Tadesse *et al.* (2012) identified *Ambylyomma cohaerens, Amblyomma variegatum* and *Amblyomma gemma* in Ethopia.

In this study, the *Rhipicephalus* genus was identified, and differences were observed in terms of body length/width and the ratio of basis capituli length/width, indicating potential geographic variations within the species (Li *et al.,* 2017). However, distinguishing between *R. sanguineus* and *R. turanicus* based on morphology alone is challenging due to similarities in spiracle plates, scutum shape, punctuations, and anal accessory shields (Estrada Pena *et al.,* 2004). To accurately differentiate between tick species from various genera, both morphological and molecular-based methods have proven valuable (Balinandi *et al.,* 2020; Ernieenor *et al.,* 2017; Estrada-Peña *et al.,* 2017).

Conclusion

The study concluded tick's infestations were prevalent in sheep as compared to goats. Morphologically four hard ticks genera, mainly *Rhipicephalus, Hyalomma, Haemaphysalis and Ixodes* spp were identified. However, the morphological discrimination among *Rhipicephalus* spp. is challenging and molecular identification of these ticks are required for the accurate identification. Identification of these ticks is necessary as these further resulted in many other diseases in animals and causes higher economic losses. Many predisposing factors like animal shed management practices, improper animal health inputs resulting long term existing ticks infestation in animals. Therefore, it is recommended that all the three main approaches like physical recovery, biological and therapeutic measures may be properly adopted for ticks control at each farms level subject to the appearance of ticks attack in different seasons particularly during summer.

MOLECULAR CHARACTERIZATION OF THE GENUS *Rhipicephalus* (IXODIDAE) INFESTING SMALL RUMINANTS IN THREE AGRO-ECOLOGICAL ZONES OF PAKISTAN

ABSTRACT

In this study, the molecular characterization of the Ixodid ticks belonging to the genus *Rhipicephalus* was carried out. In total, 4488 infested small ruminants are collected from three agro-ecological zones of Pakistan. Prevalence of *Rhipicephalus* ticks was 4.5% in sheep and 3.9% in goats. Molecular characterization based on two mitochondrial and one internal nuclear spacer DNA sequences placed the sequences obtained in this study in paraphyletic clades along with sequences in GenBank. The ITS2 sequence of the *R. sanguineus* (OK642408) and *R. microplus* (OK642409) form a distinct clade with sequences from other countries. The 16S rRNA sequences of *R. sanguineus* (OK560870) clustered with sequences form three lineages, tropical, temperate, and south-eastern lineages. The *Cox I* gene identified *R. turanicus* (OK623472) fall in clad with Pakistan, China, Kazakhstan and Bulgaria, while *R. microplus* (OK623463) form separate clades with sequences from Pakistan, Iran, India and China. The study demonstrates the diversity of *Rhipicephalus* species infesting small ruminants in Pakistan suggesting their possible involvement in the transmission of tick-borne diseases. Further studies on tick-borne diseases are required for control purposes.

Keywords: *Rhipicephalus,* sheep, goats, molecular characterization, Pakistan.

3.1 INTRODUCTION

Worldwide, ticks are associated with significant medical and veterinary challenges (Brites-Neto *et al.,* 2015). which cause tissue injury, body paralysis, and anaemia in heavy infestation (Betancur-Hurtado and Geraldo-Ríos, 2018), and ticks can serve as vectors for a wide range of disease-causing microorganisms, which encompass bacteria, fungi, protozoa, rickettsiae, spirochetes, and viruses. (Anderson and Magnarelli, 2015). The financial impact of the tick burden in Asian countries has been approximated to be around US $498.7 million per year (Minjauw and McLeod, 2003).

Several studies have been found in small ruminants regarding prevalence of ticks ruminants but mainly focused on the morphological characterization (Khan *et al.,* 2019, Ahmed *et al.,* 2012; Ali *et al.,* 2013, Sajid *et al.,* 2011). In a recent study conducted in the federally administered tribal area of Pakistan, six types of ixodid ticks *(Rhipicephalus haemaphysaloides, R. microplus, R. turanicus, Haemphysalis punctata, Hae. sulcata,* and *Hyalomma anatolicum*) were identified in small ruminants using molecular analysis (Ghafar *et al.,* 2020a). Hence, it is preferable to employ a molecular method as well to assess the range of tick species affecting small ruminants in the various agro-ecological regions of Pakistan.

The morphological characters are frequently used for tick identification (Nava *et al.,* 2009). However, the morpho taxonomical differentiation has been challenged in the past few years on the identification of *R. microplus* because of the existence of its four closely related taxa namely *R. australis*, *R. annulatus*, *R. microplus* clade A *sensu.* Burger *et al.* (2014) and *R. microplus* clade B *sensu* (Burguer *et al.* 2014; Barker and Walker, 2014). The challenging task of distinguishing *Rhipicephalus* ticks arises from their morphological variations, and up until now, multiple valid *Rhipicephalus (Boophilus*) species have been identified (Estrada-Peña *et al.,* 2012; Guglielmone *et al.,* 2014; Ali *et al.,* 2016; CoimbraDores *et al.,* 2018; Roy *et al.,* 2018). Immature engorged or damaged tick specimens are unable to identify through morphological methods (Caporale *et al.,* 1995; Walker *et al.,* 2003). Therefore, molecular approaches are the most preferred methods to study species identity and diversity in *Rhipicephalus* ticks (Burger *et al.,* 2014), offers a reliable

and alternative method for accurately identifying ticks (Cuickshank, 2002). The examination of *R. microplus* ticks from America, Asia, Australia, and Africa using mitochondrial markers (12S rRNA and 16S rRNA) and microsatellite markers has provided evidence that *R. microplus* comprises at least two distinct species (Labruna *et al.,* 2009; Low *et al.,* 2015; Ali *et al.,* 2016; Baron *et al.,* 2018). The molecular studies confirmed that 16S rRNA and mitochondrial cytochrome oxidase I (*Cox I*) and the second internal transcribed spacer (ITS2) of the nuclear ribosomal gene are powerful genetic markers to define species identification, interspecies variations, and phylogenetic relationship (Low *et al.,* 2015; Cruckshank 2002).

Therefore, in the present study, we employed molecular tools to investigate the diversity of *Rhipicephalus* ticks infesting small ruminants in the Punjab, Balochistan, and Azad Kashmir the agro-ecological zones of Pakistan.

3.2 MATERIALS AND METHODS

The detail of study area, animal examined, ticks' collection and storage from each animal is given in methodology section of chapter 2.

3.2.1 DNA Extraction

For operational reasons, only up to four adult ticks, after morphologically identification as belonging to the *Rhipicephalus* genus, were selected from infected sheep (n=25 ticks) and goats (n=24 ticks) from each of the agro-ecological study zones and processed for molecular identification. Genomic DNA was extracted from a total of 49 ticks (male=22, female=27) representative of each ecological zone and animal host using a commercial DNA extraction kit (Bio-Rad Lab, Inc. USA) according to the manufacturer's instructions.

3.2.2 PCR amplification and sequence analysis of ribosomal and mitochondrial DNA

PCR amplification was performed to target the second internal transcribed spacer (ITS2) of rDNA and two mitochondrial loci (*Cox I* and 16S). Previously reported primers (listed in Table 3.1) were used for the amplification process. The PCR reactions were prepared using a total

volume of 25µl comprised of 2µl of PCR buffer (1×), 2µl of $MgCl_2$ (25 mM), 2µl of 2.5 mM dNTPs, 0.7µl of each primer (10 pmol/µl), 2µl of gDNA, and 0.3µl of Taq DNA polymerase (5 U/µl), and 16µl of ddH_2O was added. The thermocycler conditions were set at an initial denaturation 96 °C for 8 min followed by 39 cycles of denaturation at 96°C for 1 min, annealing at 59 °C (for ITS2-F/ITS2-R), 61 °C (for 16S-F/16S-R), and 56 °C (for Cox1-F/Cox1-R) for 1 min, and extension at 72 °C for 1 minute. A final extension step was performed at 72 °C for 8 minutes. Subsequently, 5 µl aliquots of each individual amplicon were loaded onto 1.5% (w/v) agarose gels and visualized by capturing images using a GelDoc system.

Table 3.1: Primer sequences for the amplification of *Rhipicephalus* species ITS2, 16S and Cox1 regions.

Primer name	Sequences (5′ -3′)	Annealing temperature	References
ITS2-F	CGGATCACATATCAAGAGAG	59°C	Csordas *et al.,* 2016
ITS2-R	CCCAACTGGAGTGGCCCAGTTT		
16S-F	CTGCTCAATGAATATTTAAATTGC	61°C	Black and Piesman,
16S-R	CGGTCTAAACTCAGATCATGTAGG		1964
Cox1-F	CTTCAGCCATTTTACCGCGA	56°C	Csordas *et al.,* 2016
Cox1-R	CTCCGCCTGAAGGGTCAAA		

The amplified DNA fragments were purified using a WizPrepTM Gel/PCR Purification Mini kit from Seongnam, South Korea (Catalog number 13,209). The purified amplicons were then sent for sequencing on an Applied Biosystems 3730Xl genetic analyzer by Eurofins Genomics LLC. The sequencing was performed using the same primers used for amplification. The resulting sequences from both strands of ITS2, *16S,* and *Cox* I for each tick specimen were assembled, aligned, and edited. The newly identified sequences were submitted to GenBank and assigned

accession numbers. They were subsequently compared and aligned with existing rDNA, 16S, and Cox1 sequences of *Rhipicephalus* from the NCBI GenBank database.

3.2.3 Molecular phylogeny of the rDNA (*ITS2*) and mitochondrial loci (*16S* and *Cox I*)

The molecular phylogeny of *ITS2* and *Cox I* sequence data were inferred by using the maximum likelihood method and the Kimura 2-parameter model (Kimura 1980**),** while the Hasegawa-Kishino-Yano model (Hasegawa *et al.,* 1985) was used for *16S* sequence data. The trees with the highest log likelihood (*ITS2* (-1011.33), *Cox I* (-1547.30), *16S* (-1251.19)) are shown. To account for evolutionary rate variations among sites, a discrete Gamma distribution was employed to model the differences in ITS2 (4 categories (+G, parameter = 13.9487), *16S* (4 categories (+G, parameter = 2.0585) and *Cox 1* (4 categories (+G, parameter = 0.2590) sequence data. Complete deletion options were used to remove gaps and missing data. The sequences used in comparison and out groups (*Haemaphysalis longicornis* (EU604103); *Argas persicus* (MK555333; FN394341)) to construct trees were downloaded from the NCBI Genbank database. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

3.3 RESULTS

3.3.1 *ITS2* rDNA

The ITS2 rDNA sequences of each of the 49 ticks (male=22, female=27) were aligned for intraspecific variations. The analysis of the evolutionary relationships was conducted using ITS2 sequences obtained from this study, in addition to 20 sequences sourced from GenBank (Fig. 3.1). Ticks identified herein as *R. sanguineus* Pakistan (OK642408) and *R. microplus* Pakistan (OK642409) formed a distinct sub-clade. The identified species blast results showed 88.19% to 89.76% identity with reference *R. sanguineus* and 97.3% - 97.8% similarity with reference *R. microplus* sequences from different countries.

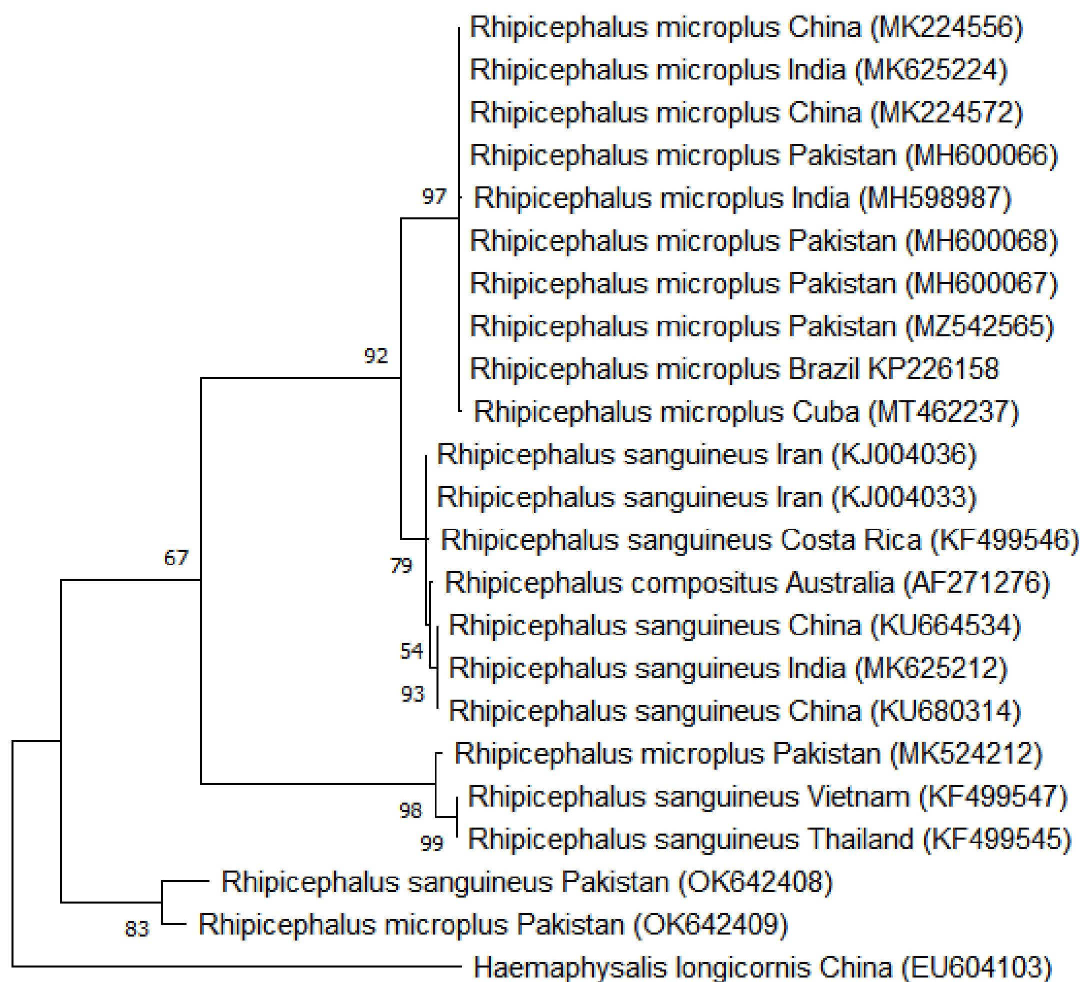


Fig. 3. 1. Phylogenetic relationships of partial sequences of the ITS2 of the nuclear ribosomal DNA of *R. microplus* and *R. sanguineus* ticks collected from small ruminants in the three agro-ecological zone of Pakistan.

3.3.2 16S *rRNA*

Phylogenetic analyses of the 16S sequences data set involved 31 reference nucleotide sequences from the NCBI GenBank database (Fig. 3.2). The 16S *R. turanicus* (OK560873) sequences acquired in this study were clustered into clade II, which included three sequences from Afghanistan, Krygyzstan, and Israel. It revealed 98.5% to 99.3% similarity with sequences from Pakistan and Afghanistan. The identified *R. sanguineus* (OK560870) was in clade II along with closely related species from China, France, Israel, Greece, and Romania. Blast results showed a 94.4%–98.2% similarity to *R. sanguineus* from other countries.

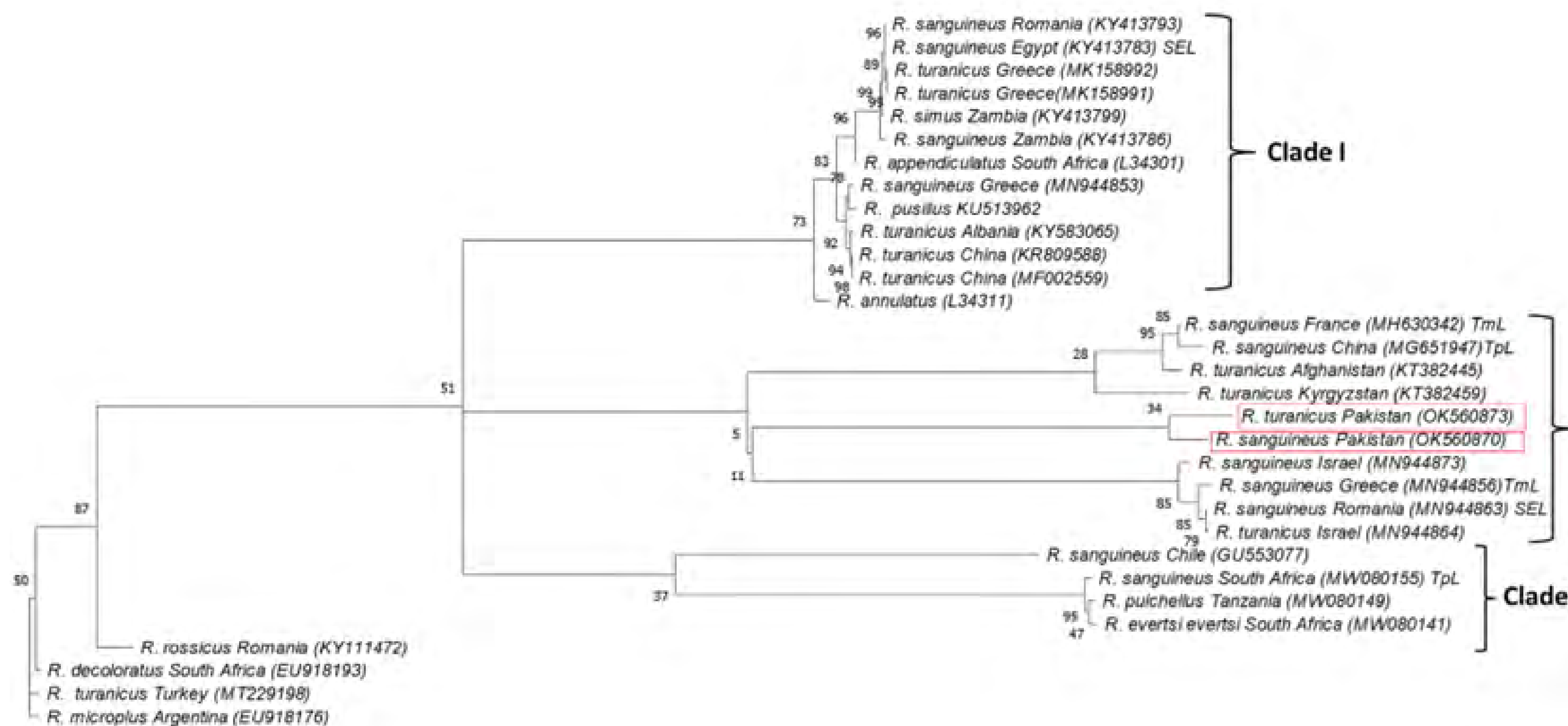


Fig. 3.2 Phylogenetic relationships of partial 16S rRNA sequences of *R. turanicus* and *R. sanguineus* ticks collected from small ruminants from the three agro-ecological zones of Pakistan.

3.3.3 *Cox I* Sequences

The *Cox I* sequence of sufficient quality was generated for 34 tick samples. These were aligned with 12 nucleotide sequences available on the public NCBI Genbank database. The resulting phylogram based on the *Cox I* gene identified the *R. turanicus* (OK623472) and clustered it in clade with sequence from Pakistan, China and Kazakhstan (Fig. 3.3). The *R. microplus* (OK623463) clustered with sequences from China, Bangladesh, Pakistan and India.

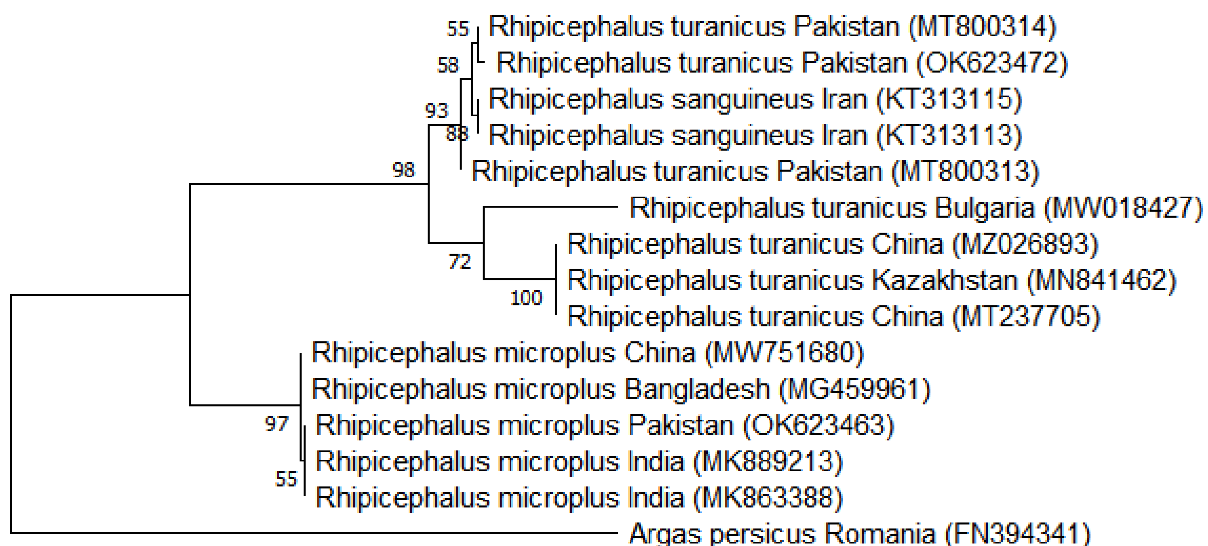


Fig. 3.3: Phylogenetic relationships of partial *Cox I* sequence of *R. microplus* and *R. turanicus* ticks collected from small ruminants from the three agro-ecological zones of Pakistan.

3.4 DISCUSSION

The results of the current research showed the presence of three *Rhipicephalus* species, i.e., *R. sanguineus*, *R. turanicus,* and *R. microplus* based on morphological and molecular evidence from all the examined small ruminants from the three agro-ecological zones of Pakistan. Previous studies have shown the value of morphological and molecular-based methods for the accurate species differentiation between various tick's genera (Balinandi *et al.,* 2020; Ernieenor *et al.,* 2017; Estrada-Peña *et al.,* 2017). The specimens collected from six study sites were identified as *Rhipicephalus* genus, but overlapping morphological traits prevented their accurate species designation. The observed differences between body length: width and ratio of basis capituli length: width, might be partialy interpreted as geographically related intraspecific morphological variations (Li *et al.,* 2017). However, the morphological discrimination of *R. sanguineus s.s.* and *R. turanicus* is difficult because of the similarity in the form of spiracle plates, scutum shape, punctuations, and anal accessory shields (EstradaPena *et al.,* 2004).

Our analyses of ITS2, 16S, and Cox I loci sequences of *R. sanguineus*, *R. turanicus* and *R. microplus* with sequences from the public database showed consistent interspecific variations. The ITS2 molecular marker, which also displayed inter-specific changes, corroborated the differences between *R. microplus* (OK642409) and *R. sanguineus* (OK642408). The relatively low percentage similarity values observed in the identified species compared to the corresponding accession sequences of *R. microplus* and *R. sanguineus* species could potentially be attributed to factors such as cryptic hybridization or geographical isolation (Taberlet *et al.,* 1992) which results in nucleotide substitutions (Rees *et al.,* 2003). Accurate species description relies on the intra-specific and inter-specific variations in the selected markers. For species discrimination of ticks, ITS2 should be the most efficient marker, but ITS2 has no advantage over Cox I, 16S rDNA, and 12S rDNA in its resolution power of species identification (Lv *et al.,* 2014).

The 16S sequence analysis of the identified *R. sanguineus* ticks revealed the formation of a clade consisting of three lineages: tropical, temperate, and south-eastern lineages. This finding aligns with previous studies conducted by Chitimia-Dobler *et al. (*2017) and Nava *et al.* (2012). The significant differences observed within these lineages could be attributed to various factors,

including ecological variations, vector competence, interbreeding, and other biological characteristics, as discussed in studies by Eremeeva *et al.* (2011), Labruna *et al.* (2017), Levin *et al.* (2012), and Zemtsova *et al.* (2016). Our study reinforces the notion that certain ticks belonging to the *R. sanguineus* complex in Pakistan comprise multiple distinct entities and are composed of closely related yet different species.

Likewise, the *Cox I* sequence of *R. turanicus* (OK623472) and *R. microplus* (OK623463) forms a separate clade and showed similarity with previously reported species from Pakistan and China. Recently, the monophyletic identity of *R. turanicus* was contradicted, a report on a new species, i.e., *R. afranicus* with the existence of two lineages from southern Europe and the Middle East/Asia was recorded (Bakkes *et al.,* 2020). Another study found a similar pattern, where *R. turanicus* clustered into two distinct clades (Ghafar *et al.,* 2020a). Like the present work, *R. microplus* was genetically confirmed in the northern part of Punjab and FATA, Pakistan (Rehman *et al.,* 2017; Ghafar *et al.,* 2020a).

Conclusion

In conclusion, our findings present the identity of three *Rhipicephalus* species, i.e., *R. sanguineus*, *R. turanicus,* and *R. microplus* collected from small ruminants. The other three tick genera were not characterized due to operational reasons, problems with DNA extraction and primers optimization. Further, research on genetic identification of *Hyalomma*, *Haemaphysalis,* and *Ixodes* to confirm the species identity, as well as on tick-borne pathogens belonging to *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Hepatozoon*, *Rickecttsia* and *Theileria* genera, are required.

MOLECULAR CHARACTERIZATION OF TICK BORNE *Theileria* AND *Anaplasma* SPECIES IN RUMINANTS

ABSTRACT

Tick-borne diseases (TBPs) pose significant challenges to raising livestock and also pose a risk to public health in Pakistan. The present study investigated TBPs in sheep, goats and cattle from Khyber Pakhtunkhwa (KP) region of Pakistan. This study develops real-time PCR to target Pan-*Theileria*, the primers and probes were designed to target hypervariable V4 region in the 18S rRNA gene of *Theileria* sp., which allows detection of different *Theileria* sp. Furthermore, we assessed the effectiveness of utilizing the 16S rRNA gene sequence to differentiate *Anaplasma* samples at the species level. Microscopically suspected positive blood samples for *Theileria* and *Anaplasma* were subjected to DNA extraction. A total of fifty-one blood samples for *Theileria* and 14 for *Anaplasma* were subjected to molecular characterization. For analytical validation, gBlock findings were serially diluted to assess linearity and sensitivity. The regression coefficient (R2) for the five reactions was determined to be 0.9637, demonstrating a strong linear relationship. The sensitivity of the analysis covered from 10 to 100 copies per reaction. The coefficient of variation was below 5%, indicating high precision. Out of the 51 samples examined during the clinical validation, 47 samples tested positive for Pan *Theileria* in the speciation reaction and were further confirmed through sequencing. Three samples tested positive for *T. orientalis* during sequencing, two of which were validated by qPCR. One sample was positive for *T. ovis* by qPCR. Overall, the assay provided us with high sensitivity and specificity for our clinical test. *Anaplasma* was observed in the blood of 14 animals, out of these12 *Anaplasma* sequences were of good quality. The sequence similarity between *Anaplasma marginale* and the reference genome were 99.25%, while below this value indicating a distinct species. This study contributes to the epidemiology of TBPs in the KP province and highlights the importance of proactive TBP surveillance in livestock.

4.1 INTRODUCTION

Tick-borne diseases causes financial burdens on the livestock sector in tropical and subtropical regions worldwide (Berggoetz *et al.,* 2014; Karim *et al.,* 2017). Tropical theileriosis is transmitted by *Hyalomma* ticks and distributed worldwide including Asia (Nene *et al.,* 2016; Bilgic *et al.,* 2010). *T. annulata* is recognized as the most pathogenic and economically significant species of *Theileria*, responsible for causing severe infections in large ruminant animals. Another notable species is *T. parva,* which leads to East Coast fever (ECF), and *T. mutans,* causing benign theileriosis, as well as *T. orientalis,* which is associated with Oriental theileriosis (OT) or *Theileria*-associated bovine anemia (TABA). Moreover, *T. lestoquardi, T. uilenbergi* and *T. luwenshuni* are also considered in one of the pathogenic diseases which causes malignant ovine theileriosis (MOT), *T. taurotragi*, and *T. ovis,* mostly cause asymptomatic infections in livestock (Nene *et al.,* 2016; OIE, 2021). Theileriosis is regarded as a significant tick-borne disease in the livestock industry, posing a major challenge worldwide (Jenkins, 2018). In the absence of acaricide usage and limited access to veterinary healthcare facilities, untreated animals experience a high mortality rate, resulting in a significant impact on farming communities with limited resources. (Gul 2015; Khan *et al.,* 2017) and meat and milk production (Jabbar *et al.,* 2015; Rashid *et al.,* 2018). Main clinical signs associated include pyrexia (40–41.5°C), ocular and enlargement of superficial dyspnea, lymph nodes, nasal discharge, leucopenia, anemia, and jaundice (Zeb *et al.,* 2020; Brown, 2008).

Bovine anaplasmosis is a bacterial disease transmitted by ticks, primarily caused by *Anaplasma marginale.* This gram-negative bacterium infects red blood cells and belongs to the order Rickettsiales (Ashraf *et al.,* 2013; Tana-Hernández *et al.,* 2017). The primary mode of transmission of this bacterial agent belonging to the Rickettsiales order to animal hosts is through ticks. Extensive research has identified 20 tick species as efficient vectors of *A. marginale* (Kocan *et al.,* 2004). Among the commonly reported tick species involved in *A. marginale* transmission are Ixodes spp., *Dermacentor* spp., *Rhipicephalus* spp., and *Amblyomma* spp. (Hairgrove *et al.,* 2015). Clinical symptoms commonly observed in cattle suffering from anaplasmosis include fever, progressive anemia, jaundice, loss of appetite, depression, decreased milk production, abortion in pregnant animals, and a higher risk of mortality, particularly in exotic breeds (Camus *et al*., 2010).

Although anaplasmosis is considered a zoonotic concern, with the potential to infect humans, *A. marginale* has not been found to infect humans (Kumar *et al.,* 2015).

To control tick born disease several diagnostic techniques were applied. The clinical diagnosis of acute theileriosis is typically conducted through the examination of Giemsa-stained peripheral blood smears or lymph node biopsy smears to detect piroplasms (Hayati *et al.,* 2020). To detect and identify multiple *Babesia* and *Theileria* species simultaneously, various standard hybridization methods with high multiplexing capacity have been developed, such as Reverse Line Blot (RLB) and LuminexW suspension arrays (Santos *et al.,* 2013; Bilgic *et al.,* 2013). However, these diagnostic procedures are not enough sensitive to allow the reliable estimation of tropical theileriosis in carrier animals (Pienaar *et al.,* 2020). To overcome these limitations, novel advanced techniques such as next-generation genomic resources have been developed. These techniques utilize variations in the hyper-variable region of the 18S rDNA sequence, enabling differentiation of different piroplasm species (Gubbels *et al.,* 1999; Lee *et al.,* 2015). In several studies, various PCR methods such as reverse line blot (RLB)-PCR, quantitative PCR (qPCR), and multiplex PCR have been described as rapid means of amplifying the 18S region (Bilgiç *et al.,* 2013; Kundave *et al.,* 2018). However, none of these methods allow for reliable quantification of the parasite. The effective utilization of the 18S rRNA gene has been demonstrated in the detection and identification of different species of *Theileria* (Mans *et al.,* 2015; Sivakumar *et al.,* 2014).

The main methods for detecting *A. marginale* involve serological and molecular diagnoses, which have high sensitivity and specificity, enabling accurate detection of the pathogen (Rafael *et al.,* 2021). Polymerase Chain Reaction (PCR) method provides a reliable and sensitive approach to identify the presence of the *A. marginale* and efficacy of the method extends to the testing of intricate blood samples containing hemolyzed and coagulated bovine blood. (Ben *et al.,* 2018).

Here, we described a multiplex qPCR assay using g-Block for analytical validation that can be used for both the detection and the quantification of *Theileria* species, compared analytical validation results with clinical samples by designing Pan-*Theileria* and four specific probes to targets *18S* rRNA. Samples that tested positive in the FRET-qPCR were further analyzed using standard PCR and sequencing of the 18S rRNA gene to conduct phylogenetic analysis. For *Anaplasma 16S* rRNA genetic marker was used for molecular characterization. The molecular assays showed high sensitivity and specificity and provide good diagnostic results.

4. 2. MATERIALS AND METHODS

4. 2.1. Study Area

Pakistan is categorized into five different agro-ecological zones based on climate variations: Drought/hyper-arid, Arid, Humid, Wetland, and Cold drought (Zomer *et al.,* 2008; Javid *et al.,* 2019). Samples were collected from Charsadda (34.1495°N, 71.7428°E), Mardan (34.2062°N, 72.0298°E), and Peshawar (34.0000°N, 71.7500°E) located in the Khyber Pakhtunkhwa (KP) province of Pakistan (Fig. 4.1). These areas have a transitional climatic profile, and due to extensive irrigation systems, certain study areas exhibit variable climatic conditions with a semi-arid and humid agro-ecological pattern (Javid *et al.,* 2019). The maximum temperature of summers is hot and dry with little rainfall in these regions and sub-tropical and temperate dry mixed deciduous scrub forest. The winter temperature is mild with 4 °C, but summer is quite hot with 38-44 °C, with 65% humidity.

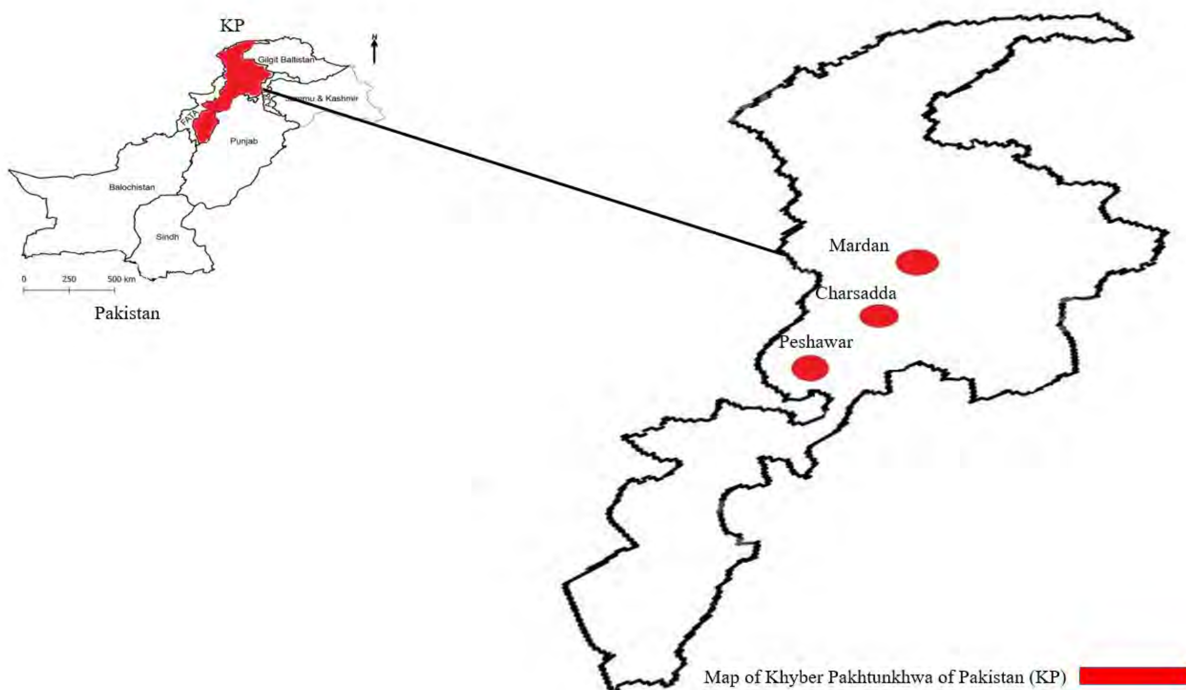


Fig. 4.1. Geographical map of Pakistan showing three regions Mardan, Peshawar and Charsadda of Khyber Pakhtunkhwa province where blood samples were collected.

4.2.2 Sample Collection, Screening and DNA Extraction

A total number of 514 sheep, 462 goats and 50 cattle infested with ticks were examined. The majority of these animals appeared to be in good health and had adult and nymph tick infestations from four different tick genera: *Rhipicephalus*, *Hyalomma, Haemaphysalis,* and *Ixodes*. Blood sample taken from jugular veins only if they were symptomatic. Approximately 4-5 mL of blood was collected of each animal in EDTA tube, proper labelled and kept in ice until reaching laboratory of Parasitology at Quaid-i-Azam University, Islamabad Pakistan. For each blood sample, a thin blood smear was created and allowed to air dry. For 5 minutes, the smear was then fixed in methanol 96%. Subsequently, the smear was stained using Giemsa stain (5%) for a period of 30 minutes, following the methodology described by Benjamin (1986). Microscopically suspected positive blood samples for *Theileria* and *Anaplasma* were subjected to DNA extraction. A total of fifty-one blood samples for *Theileria* and 14 for *Anaplasma* were subjected to molecular characterization. DNA was extracted by using the standard Phenol-Chloroform protocol (Sambrook, 1989). Molecular tests were performed in BC Centre of Disease Control (BCCDC, Affiliated with University of British Columbia) in Canada.

4.2.3 Ethics Approval

Permission to import samples were issued by Canadian Food Inspection Agency (CFIA) department in accordance with the Import Permit Regulations. Prior shipping DNA samples were treated with RNAase, DNAase-free kit (Millipore Sigma; Product number, 11119915001). Protocol has been reviewed and approved by CFIA and Biohazard Containment and Safety (OBCS).

4.2.4 *Theileria* Molecular Characterization

4.2.4.1 Development of the Pan-*Theileria* real-time PCR and speciation

Available sequences in GenBank of *Theileria* species were aligned. We used PrimerQuest™ Tool software to design the primer pairs and probes and specificity was evaluated nucleotide BLAST online, which showed 100% homology with the target sequences. Two panels were designed with two pairs of forward and one reverse primer; F1: forward [5’-

GCGGTAATTCCAGCTCCAA-3’], F2: forward [5’-TTAAAAAGCTCGTAGTTGAATTTCTGCTGCA-3’], reverse [5’-GCCTGCTTTGAGCACTCT -3’] to amplify 178 and 135 base pairs fragment to target most conserved and hypervariable V4 region 18S rRNA gene of *Theileria* spp., enables us to distinguish the most common *Theileria* spp., *Theileria annulata, Theileria parva, Theileria lestoquardi,* and *Theileria ovis*. In this assay, in total five probes were designed in two panels to avoid cross reactivity between species: in panel one, Pan-*Theileria*, which has ability to detect more *Theileria* species and *T-parva.* Panel two included three species-specific probes for *T. annulata, T. lestoquardi* and *T. ovis* (Sigma Aldrich, St. Louis, USA) (see Figure 4.2).

Table 1. Sequences of real-time PCR primers and probes.

PCR	Panel	P/P	Name	Nucleotide Sequence (5'-3')	Length	Reference
qPCR	I	Primers	Th18S_F1	GCGGTAATTCCAGCTCCAA	19	This study
			Th18S_R	GCCTGCTTTGAGCACTCT	18	This study
		Probes	*Tparv*_P	56-FAM/ATTTCGGAC/ZEN/GGAGTTCGCTTTGTCTGG/3IABkFQ	27	This study
			PanTh_P	NED-TTAAAAAGCTCGTAGTTGAATTTCTGCTGCA-MGBNFQ	31	This study
	II	Primers	Th18S_F2	TTAAAAAGCTCGTAGTTGAATTTCTGCTGCA	31	This study
			Th18S_R	GCCTGCTTTGAGCACTCT	18	This study
		Probes	*T. ovis*_P	NED-CTCCTTTACGAG TCTTTGCATTGTGGCT-MGBNFQ	28	This study
			*T. annu*_P	56-FAM/TCCCTCTGG/ZEN/GGTCTGTGCATG/3IABkFQ/	21	This study
			*T. lest*_P	VIC-TGTCCCTCCGGGGTCTGTGCAT-QSY	22	This study
cPCR		Primers	F-Bab-GF2	GYYTTGTAATTGGAATGATGG	21	Bonnet et al., 2007
			R-Bab-GR2	GYYTTGTAATTGGAATGATGG	21	Bonnet et al., 2007

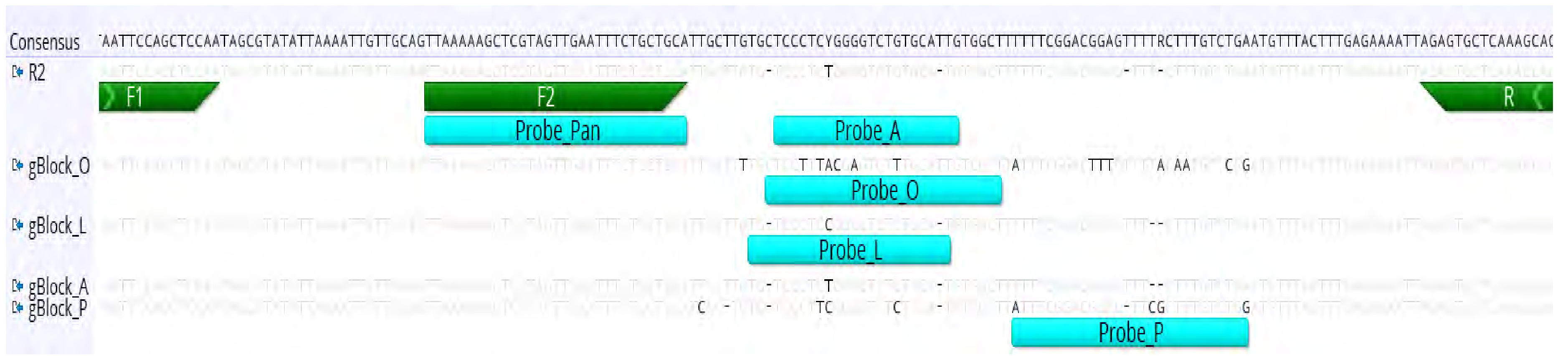


Fig. 4.2: *18S rRNA* gene variable region (V4) nucleotide sequence alignment. Sequence analyses include *T. annulata, T. parva, T. lestoquardi and T. ovis.*

4.2.4.2 Development of the Multiplex TaqMan assay

On an Applied Biosystems 7500 Fast Real-Time PCR System, the Real-Time Multiplex TaqMan^{TM} test was carried out using the TaqMan^{TM} Fast Advanced Master Mix kit. (Catalogue No. 01259720) using Fast Advanced Master Mix (1x) of 20 µl reaction total 0.03 µl (20x) of each Probe 4 µl ddH_2O, both reverse and forward primer 0.1 (20x) and 5 µl of template DNA with the cycling profile was as follows: 1 min at 95 °C, 40 cycles of 15 s each at 95 °C for amplification, and then an annealing or extension step set up at 60 °C for 30 s.

4.2.4.3 Analytical and clinical validation

To determine the analytical validation, synthetic gBlocks ^{TM} gene fragments were used. Ten-fold serial dilutions (10^9-1 copy) were made to validate the assay with three replicate reactions per dilution step to evaluate the specificity, sensitivity, PCR efficiency, precision, and accuracy. To challenge the analytical validation results, clinical DNA samples were used.

4.2.4.4 Sanger sequencing-based confirmation method

To confirm qPCR positive specimen, a Sanger sequencing confirmation was done. Forward and reverse primers targeting *18S* rRNA for pan-*Theilieria spp* (Bonnet *et al.,* 2007) are detailed in Table 4.1. PCR was amplified using a GeneAmp® PCR System 9700, utilizing the Qiagen HotStart PCR Master Mix. The following program was employed: Following a 15-minute initial denaturation stage at 95°C, there will be 40 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds at either 50°C or 55°C to identify Theileria spp., and elongation for 1 minute at 72°C. A final elongation step was performed at 72°C for 10 minutes. The Qiagen PCR Purification Kit was then used to purify the PCR products and amplicons were quantified using a Lunatic spectrophotometer. The BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit was used for cycle sequencing and purification. On a genetic analyzer by ABI 3130xl, sequencing was done.

4.2.4.5 Phylogenetic Analysis

The assembled sequences were imported into the Geneious 9.8.1 programme (Biomatters, New Zealand) and trimmed to remove sequence troubleshoot and primer sections. Sequences that are

homologous were found in the NCBI database and aligned with the acquired sequences, along with an outgroup, using Geneious 9.8.1 software. The maximum-likelihood model was used to construct the phylogenetic tree, and 500 bootstrap replicates were performed to assess the reliability of the tree. The Kimura two-parameter approach was used to calculate the evolutionary distances (Kimura 1980).

4.2.4.6 Data analysis

All qPCR results and sequence results summarized in Excel. qPCR calling speciation was based on lowest value of Ct value. The Gold standard contingency table (2x2) was based on clinical samples used to determine sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated (Alonzo and Pepe 1999) with the following formula:

$$\text{Accuracy} = \frac{TP + TN}{TP+TN+FP+FN} \times 100$$

4.2.5 *Anaplasma* Molecular Characterisation

4.2.5.1 Standard PCR for the *16S* rRNA

To characterize the *Anaplasma* species detected in blood samples of 14 animals (Table 4.2), we carried out standard PCRs for the *16S* rRNA of *Anaplasma* spp with Pan-primers designed for the study (Forward: 5′ GCCACTAGAAATGGTGGGTAATA and Reverse: 3′ AAAGCAGCTCCAGGGTTAAG).

The PCR procedure was performed using the HotStarTaq Master Mix kit (Catalogue No. 203446, QIAGEN Inc., Mississauga, Ontario, Canada) on an ABI GeneAmp 9700 Thermocycler. The PCR reaction mixture was prepared (see Table 4.3) and thermocycler conditions are mentioned in table 4.4. The PCR products obtained were confirmed through 2% agarose gel electrophoresis. Subsequently, the PCR products were purified using the Qiagen PCR Purification Kit, and the resulting amplicons were quantified using a Lunatic spectrophotometer.

Table 4.2: Animal information detected positive for microscopy and used in PCR analysis.

Code	Host animal	Location	Blood Type
A1	Goat	Abbotabad, KP	Whole Blood
A2	Cattel	Serdheri, KP	Whole Blood
A3	Cattel	Charsadda, KP	Whole Blood
A4	Sheep	Mardan, KP	Whole Blood
A5	Goat	Mardan, KP	Whole Blood
A6	Goat	Mardan, KP	Whole Blood
A7	Goat	Mardan, KP	Whole Blood
A8	Goat	Mardan, KP	Whole Blood
A9	Goat	Serdheri, KP	Whole Blood
A10	Goat	Serdheri, KP	Whole Blood
A11	Goat	Serdheri, KP	Whole Blood
A12	Cattel	Charsadda, KP	Whole Blood
A13	Cattel	Abbotabad, KP	Whole Blood
A14	Cattel	Abbotabad, KP	Whole Blood

Table 4.3 : PCR reaction mixture recipe for *Anaplasma* 16S rRNA.

	Master Mix Recipe	
Component	**1 rxn (uL)**	**Total**
HotStart Taq Mix	12.5	62.5
Primer R (10 uM)	2	10
Primer F (10 uM)	2	10
H2O	6	30
Total volume	**22.5**	112.5
DNA	**2.5**	
T. Vol.	25	

Table 4.4: Thermocycler conditions for *Anaplasma* 16S rRNA.

	PCR Program	
Temp ©	**Stage**	**Duration**
95	Initial heat activation	15 min
94	Denaturation	30 sec
55	Annealing	30 sec
72	Extension	1 min
72	Final Extension	10 min
	Cycle no. 45	

4.2.5.2 Data Analysis

To align the sequences, CLUSTALW was employed as a tool (accessible at https://www.genome.jp/tools-bin/clustalw). The evolutionary tree was inferred using the Maximum Likelihood method and the Kimura 2-parameter model (Kimura 1980). 1000 replicates were used to create a bootstrap consensus tree, which shows the evolutionary relationships between the taxa under consideration (Felsenstein 1985). A discrete Gamma distribution with 4 categories (+G, parameter = 2.4272) was used to account for changes in evolutionary rates between sites. 11 nucleotide sequences made up the dataset, and any gaps or incomplete data were fully removed (full deletion option). The final dataset included 177 places in it. Using MEGA X, evolutionary analysis was carried out (Kumar *et al*., 2018). The phylogenetic tree was generated utilizing the interactive tree of life (ITOL) tool, available at https://itol.embl.de/upload.cgi. The 28 reference sequences used for comparison were acquired from NCBI, with information provided in table 4.5.

Table 4.5 Reference Sequences downloaded from NCBI GenBank for comparison.

Organism	Host	Country	Accession No.
Anaplasma bovis	Tick	China	KP314249
	Tick	Mauritania	MZ476211
Anaplasma camelii	Camel	Pakistan	MZ411410
Anaplasma capra	Sheep	Pakistan	ON238131
Anaplasma centrale	Cattle	Kyrgyzstan	MW672119
Anaplasma marginale	Cattle	China	KX279690
	Sheep	Pakistan	OL307000
	Goat	Pakistan	OL305725
Anaplasma odocoilei	Deer	China	JX876644
Anaplasma ovis	Tick	Russia	MW600413
	Tick	China	KJ410245
	Goat	China	KX279688
	Goat	Russia	MW600407
Anaplasma phagocytophilum	Tick	Ireland	OL519484
	Tick	Ireland	OL519483
	Cat	Pakistan	ON796007
Anaplasma platys	Dog	Pakistan	MW019671
	Dog	Brazil	KC989957
	Dog	Egypt	LC632659
	Dog	Egypt	MZ068099
	isolate WH	China	MN630836
	Dog	Egypt	MT053461
Anaplasma sp.	Dog, South	South Africa	AY570538
	Sheep	Senegal	MN317253
	Procapra gutturosa	China	KM227008
	Goat	China	FJ389575
	Cattle	Mongolia	MK575506
Ehrlichia ruminantium (out group)		South Africa	KF786045

4.3 RESULTS

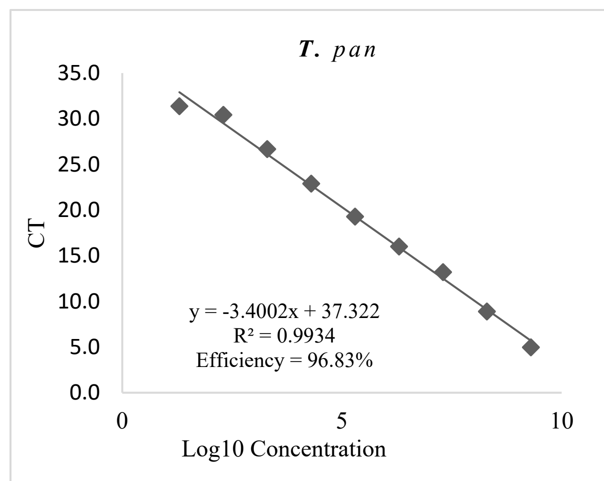
4.3.1 *Theileria*

4.3.1.1 *Theileria* Real-Time PCR Analytical Validation

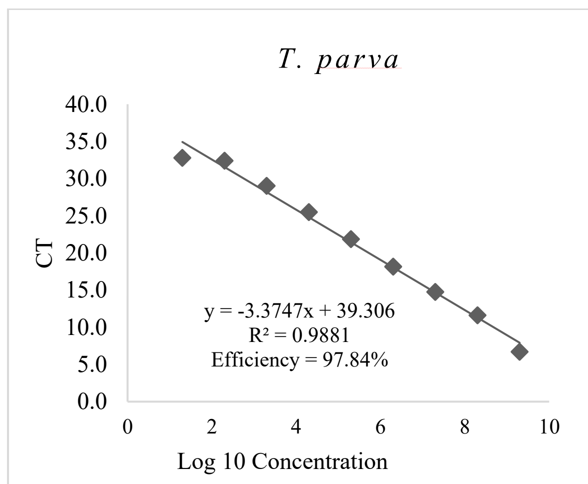
4.3.1.1.1 Linearity and PCR efficiency

Linearity was measured using the Coefficient of Variation (R^2) of a standard curve and PCR efficiency was calculated using the slope of the linear regression using serial dilution (10^0 to 10^7) of g-Block results. R^2 values were above 0.99, suggesting a highly linear assay (Fig. 4.3). Acceptable PCR efficiency, defined as PCR efficiency 90-110% and all reactions showed > 96% including Pan-*Theileria*.

A



B



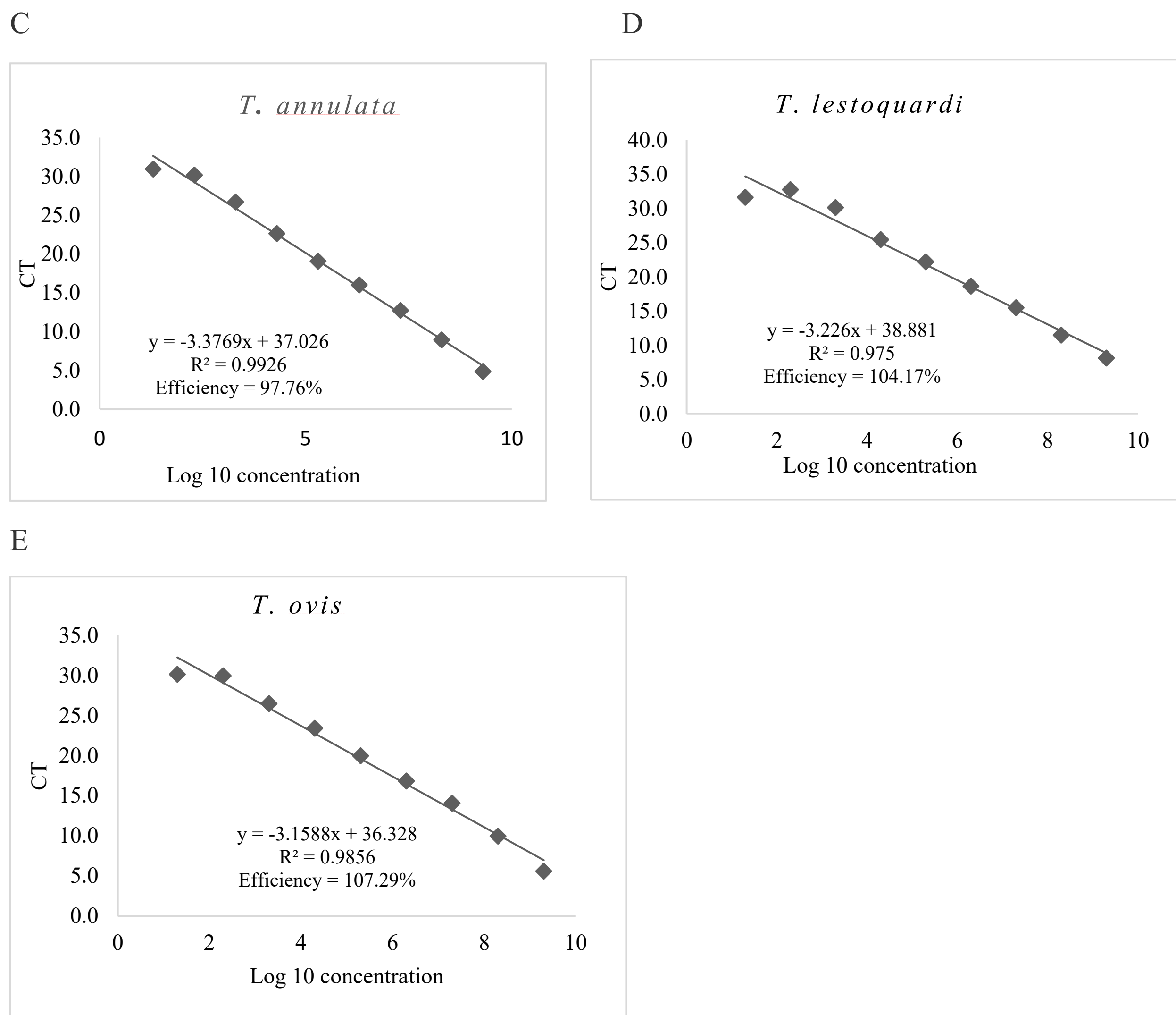


Figure 4.3. Acceptable linearity ($R^2 > 0.99$) and PCR efficiency (96.83% Pan-*Theileria*; 97.8% *T. parva*; 97.7% *T. annulata*; 104.1% *T. lestoquardi* and 107.2% *T. ovis*) for 18S rRNA targets. Triplicate measurements of serially diluted gBlocks™ were measured in three runs to generate a standard curve. Efficiency was calculated using the slope of the standard curve.

4.3.1.1.2 Sensitivity and limit of detection

To determine the limit of detection for all reactions, low concentrations of synthetic gene fragments were analyzed in triplicate over three runs. Limit of detection was determined as the lowest dilution producing consistent positive (Ct < 40) results. Sensitivity determined for all reactions were 10 and 100 DNA copies/reaction, respectively (Table. 4.6).

Table 4.6. Serial dilutions of Gene fragments to quantify gene copies of 18S rRNA in triplicate measurements.

Dilution	*T.* pan			*T. parva*			*T. annulata*			*T. lestoquardi*			*T. ovis*		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
1.00+07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+06	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+05	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+04	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+03	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+02	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.00+00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.3.1.1.3 Precision

To calculate precision, a low, moderate, and high concentration of target synthetic gene fragments were tested in triplicate over three runs. The total coefficient of variation (C.V. %) was calculated for each concentration. An acceptable precision was defined as C.V.% < 15. Maximum total C.V. % were less than 5% and thus, indicate high precision as shown in Table 4.7.

Table 4.7. **Precision (Mean, SD, COV) calculated of each target within acceptable range.**

Dilution	*T.* pan			*T. parva*			*T. annulata*			*T. lestoquardi*			*T. ovis*		
	M	S.D	CV %	M	S.D	CV %	M	S.D	CV %	M	S.D	CV %	M	S.D	CV %
1.00+07	14.3	0.7	5.2	15.4	0.1	0.7	15.2	0.6	3.8	16.9	0.2	1	14	0.6	4.4
1.00+06	16	1.1	6.8	18.6	0.2	0.8	18.7	0.4	1.9	19.8	0	0.2	16.8	0.7	4
1.00+05	19.4	0.9	4.9	21.8	0.2	1	22.3	0.7	3.2	22.9	0.1	0.3	20	0.7	3.4
1.00+04	23.7	1	4.2	24.8	0.2	0.6	26	0.7	2.7	25.9	0.1	0.4	23.4	0.8	3.4
1.00+03	27.3	0.9	3.2	28.7	1.3	4.6	29.9	1.1	3.8	30.3	0.5	1.6	26.5	0.4	1.6
1.00+02	30.2	1.5	4.9	31.8	0.2	0.5	33.2	0.6	1.9	32.7	0.2	0.5	29.9	0.8	2.7
1.00+01	32.6	1.2	3.5	31.1	0.9	2.8	33.7	0.7	1.9	32	0.9	2.9	30.1	1.3	4.4

M: Mean CV%: Co-efficient of variation; S. D: Standard Deviation

4.3.1.1.4 Specification

In panel I, Pan-*Theileria* successfully amplified the DNA fragment of the *Theileria* species with high affinity. The multiplex PCR assay was tested for specificity using a G-Blocks serial dilutions, firstly detected by Pan-*Theileria* with strong Ct values and showed high affinity overall. No cross reactivity observed in panel I. However, *T. annulata* and *T. lestoquardi* showed cross reactions, in panel II, *T. annulata* carried low Ct values which indicated that it is highly specific for the detection of *T. annulata* as shown in Table 4.8.

Table 4.8: Serial dilution of gene fragments validated analytical results.

	Panel I					Panel II						
G-Blocks	**qPCR CT**	**Result**	**qPCR CT**	**Result**	**Species-specific**	**qPCR CT**			**Result**	**Species-specific**		
	Pan- *T*	***T. spp.***	***T. par***	***T. spp.***	***T. par***	***T. annu***	***T. lesto***	***T. ovis***	***T. spp***	***T. annu***	***T. lesto***	***T. ovis***
T-parva_E7	13.7	POS	11.2	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E6	17.5	POS	14.8	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E5	20.7	POS	18.1	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E4	24.5	POS	21.9	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E3	28.2	POS	25.6	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E2	31.9	POS	29.4	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E1	36	POS	33.6	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-parva_E0	36.7	POS	36.1	POS	POS	U. D	U. D	U. D	POS	NEG	NEG	NEG
T-annu_E7	10.4	POS	11.7	POS	NEG	9.7	13.9	U. D	POS	POS	NEG	NEG
T-annu_E6	14	POS	15.3	POS	NEG	13.3	17.3	U. D	POS	POS	NEG	NEG
T-annu_E5	17.7	POS	19.1	POS	NEG	16.8	20.9	U. D	POS	POS	NEG	NEG
T-annu_E4	21.3	POS	22.6	POS	NEG	20.4	24.3	U. D	POS	POS	NEG	NEG
T-annu_E3	24.7	POS	25.8	POS	NEG	23.8	27.4	U. D	POS	POS	NEG	NEG
T-annu_E2	28.3	POS	29	POS	NEG	27.5	30.9	U. D	POS	POS	NEG	NEG
T-annu_E1	31.7	POS	32.6	POS	NEG	31.3	34.6	U. D	POS	POS	NEG	NEG
T-annu_E0	34.6	POS	35.7	POS	NEG	33.5	37	U. D	POS	POS	NEG	NEG
T-ovis_E7	12.9	POS	U. D	POS	NEG	U. D	U. D	13	POS	NEG	NEG	POS
T-ovis_E6	16.7	POS	U. D	POS	NEG	U. D	U. D	16.6	POS	NEG	NEG	POS
T-ovis_E5	20.4	POS	U. D	POS	NEG	U. D	U. D	20	POS	NEG	NEG	POS
T-ovis_E4	24	POS	U. D	POS	NEG	U. D	U. D	23.7	POS	NEG	NEG	POS
T-ovis_E3	27.7	POS	U. D	POS	NEG	U. D	U. D	27.5	POS	NEG	NEG	POS
T-ovis_E2	34	POS	U. D	POS	NEG	U. D	U. D	33.8	POS	NEG	NEG	POS
T-ovis_E1	37	POS	U. D	POS	NEG	U. D	U. D	37.2	POS	NEG	NEG	POS
T-ovis_E0	36.9	POS	U. D	POS	NEG	U. D	U. D	U. D	POS	NEG	NEG	POS
T-lesto_E7	11.4	POS	12.5	POS	NEG	12.9	9.7	U. D	POS	NEG	POS	NEG
T-lesto_E6	15.1	POS	16.2	POS	NEG	16.5	12.9	U. D	POS	NEG	POS	NEG
T-lesto_E5	18.6	POS	20	POS	NEG	20.1	16.6	U. D	POS	NEG	POS	NEG
T-lesto_E4	22.3	POS	23.5	POS	NEG	23.6	20.3	U. D	POS	NEG	POS	NEG
T-lesto_E3	26	POS	26.9	POS	NEG	27.2	23.9	U. D	POS	NEG	POS	NEG
T-lesto_E2	29.5	POS	30	POS	NEG	30.8	27.6	U. D	POS	NEG	POS	NEG
T-lesto_E1	33.3	POS	33.9	POS	NEG	34.1	31.2	U. D	POS	NEG	POS	NEG
T-lesto_E0	36.3	POS	36.9	POS	NEG	36.9	34.2	U. D	POS	NEG	POS	NEG

Pos: Positive; U.D: Undetermined; T-lesto: *T. lestoquardi* T. annu: *T. annulata*

4.3.1.1.5 Speciation calling

In total fifty-one (51) clinical samples, 47 samples Pan-*Theileria* firstly detected positive with high affinity and yielded good fluorescence intensity, which indicated that the multiplex qPCR assay was highly specific for the detection of *Theileria* species, while four (4) samples confirmed negative by Pan-*Theileria* and sequencing agreed with Pan-*Theileria.* G-Blocks were used as positive control to compare results with clinical samples. No amplification signals were detected for *T. parva, T. lestoquard* in panel II. One sample detected *T. ovis* but cleared negative by sequencing primer. Three samples detected as *T. orientalis* by sequencing, the results were same of two samples by qPCR, but one sample was not clearly confirmed by qPCR. Thirty-three (33) samples were confirmed positive *T. annulata* by qPCR, sequencing from both PCR were 100 % identical. However, few samples showed poor peaks, which were unable to sequence (Table 4.9).

Table 4.9. Clinical performance

Sample	Pan *Theileria*	*Theileria* Speciation	Sequencing Result	Gold Standard*
T10	NEG	NEG	NEG	NEG
T21	NEG	NEG	NEG	NEG
T31	NEG	NEG	NEG	NEG
T40	NEG	NEG	NEG	NEG
R10	POS	NEG	*T. orientalis*	*Theileria* spp
R12	POS	NEG	*T. orientalis*	*Theileria* spp
R20	POS	*T. annulata*	*T. orientalis*	*Theileria* spp
T1	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T2	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T3	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T4	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T5	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T6	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T7	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T8	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T9	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T11	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T12	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T13	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T15	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T16	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T17	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T32	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T33	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T34	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T36	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T37	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T38	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T39	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R2	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R3	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R6	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R8	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R13	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R14	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R15	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R16	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R17	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R18	POS	*T. annulata*	*T. annulata*	*Theileria* spp
R19	POS	*T. annulata*	*T. annulata*	*Theileria* spp
T14	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
T18	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
T19	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
T20	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
T35	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
R1	POS	*T. ovis*	Unable to sequence **	*Theileria* spp
R4	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
R5	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
R7	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
R9	POS	*T. annulata*	Unable to sequence **	*Theileria* spp
R11	POS	*T. annulata*	Unable to sequence **	*Theileria* spp

4.3.1.1.6 Accuracy

To determine 18S rRNA target accuracy, clinical *Theileria* positive samples were tested. All specimens were *Theileria* positive with a calculated accuracy of 100% (Table. 4.10). However, all 51 clinical samples were tested and 47 were confirmed positive while 4 were detected negative, with calculated accuracy of 100%.

Table 4.10. Assessment of *Theileria* clinical samples for determination of *18S* rRNA target accuracy. Reference methods used for determining positive specimen include qPCR and PCR.

		Positive	Negative	Total
Reference methods	Positive	47	0	47
	Negative	0	4	4
	Total	47	4	51

Sensitivity and specificity	
Positive predictive value	100.00%
Negative predictive value	100.00%
Sensitivity	100.00%
Specificity	100.00%
False positive rate	0.00%
False negative rate	0.00%
Power	100.00%
Likelihood ratio positive	0.00%
Likelihood ratio negative	0.00

4.3.1.2 Sanger Sequencing

The current results identified 33 isolates of *T. annulata* and 3 isolates of *T. orientalis* infection discriminated based on results of blood smear microscopy and conventional PCR in studied animals (see Annexure). All blood samples were analysed for the presence of a sting-ring or comma-shaped parasite that indicated positive *Theileria* spp. However, 18S rDNA marker confirmed 36 DNA samples were positive for *Theileria* species. The amplicons with size of ~359 base pairs were observed in all positive examined samples as shown in (Fig. 4.4). The 18S rRNA gene marker was successfully used by PCR to amplify a total of 36 DNA samples. The negative control sample showed no amplification (Fig. 4.4).

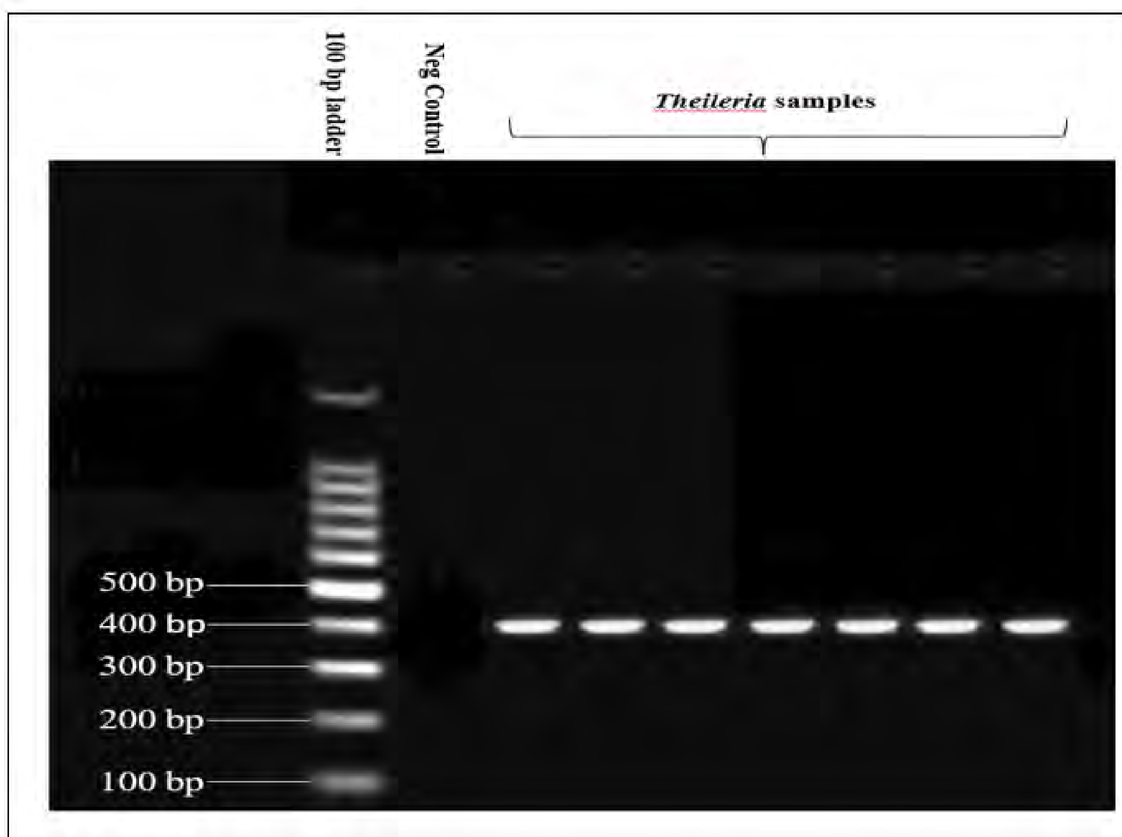


Fig.4.4: Agarose gel electrophoresis (2%) of PCR product band of 359bp for *Theileria* isolates targeting 18SrDNA.

In this study, the sequence diversity of *Theileria* species' 18S rRNA genes will be examined, all published sequences were retrieved from GenBank and multiple aligned with query sequences, reading each nucleotide that demonstrated that the bootstrap values and topologies had not changed significantly. The phylogenetic tree was created utilising multiple *Theileria* strains that exhibited similarities with query sequence searches (Query coverage: 99-100%) with the same isolates reported from Asia, Africa, and Europe. In total, thirty-three sequences acquired in this investigation fell into the clade of *T. annulata,* whereas the remaining three sequences exhibited similarities with *T. orientalis* (Fig. 4.5 and 4.6). The results showed that all new sequences in this study have close relationship appeared as past descendants to *T. annulata* and *T. orientalis.*

Phylogenetic analysis of the current 18S rDNA sequences of *T. annulate* revealed similarities to isolates from Pakistan (Accession Nos. MG599098, MG599090), India (Accession Nos. MK849886.1, MF287929), Bangladesh (Accession No. MF576178), and Russia (Accession *No. MW774588).* The *T. orientalis* isolates acquired in the current investigation, however, formed a cluster with related isolates from Bangladesh, India, Australia, Thailand, and Russia, according to the application of the neighbor-joining algorithm (Fig. 4.6). To calculate the evolutionary distances, the Tamura-Nei technique with 1000 replications was used.

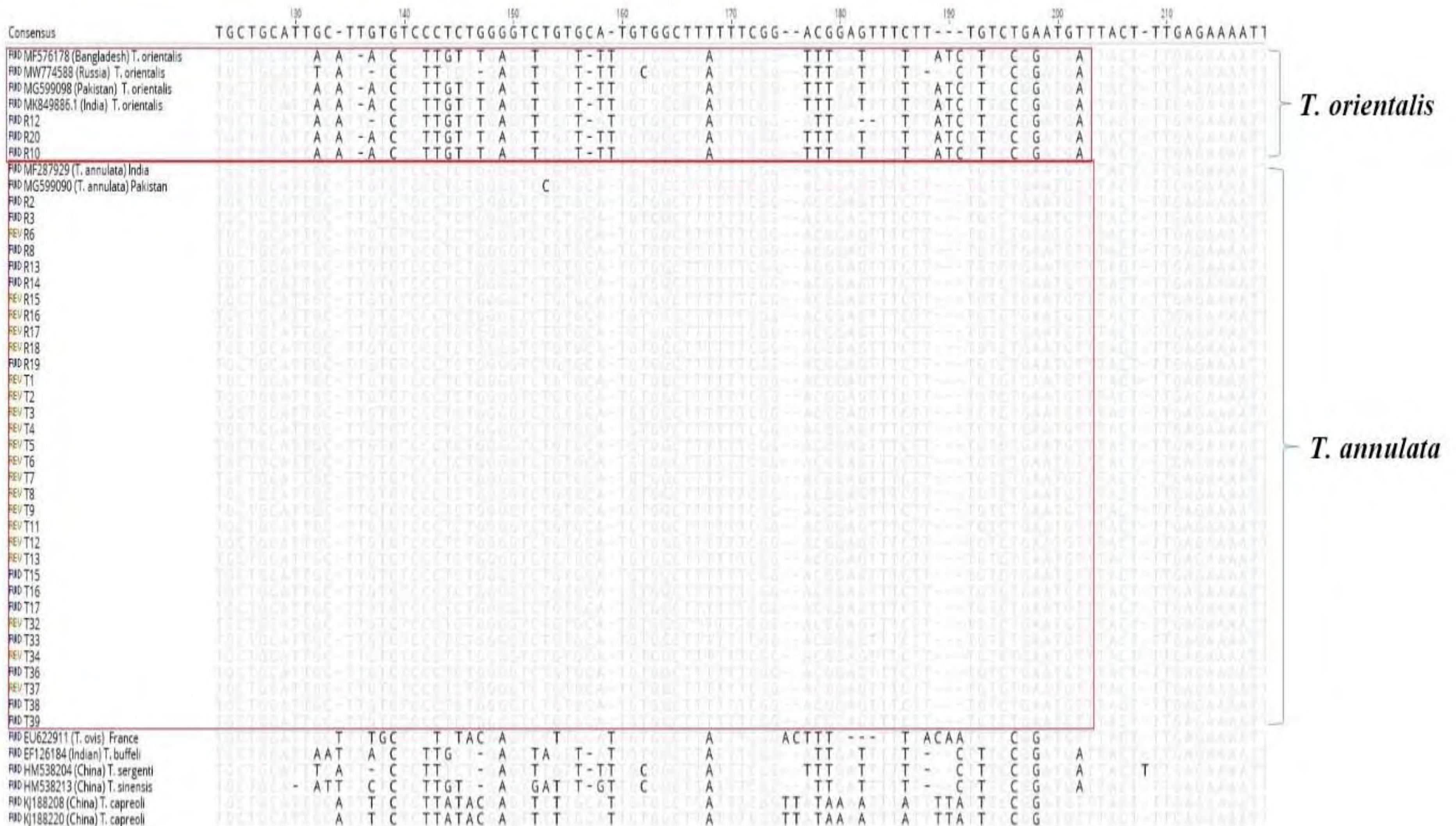


Fig. 4.5: 18S rRNA gene variable region (V4) nucleotide sequence alignment of *Theileria* samples from the current study and previously published isolates from neighbouring countries.

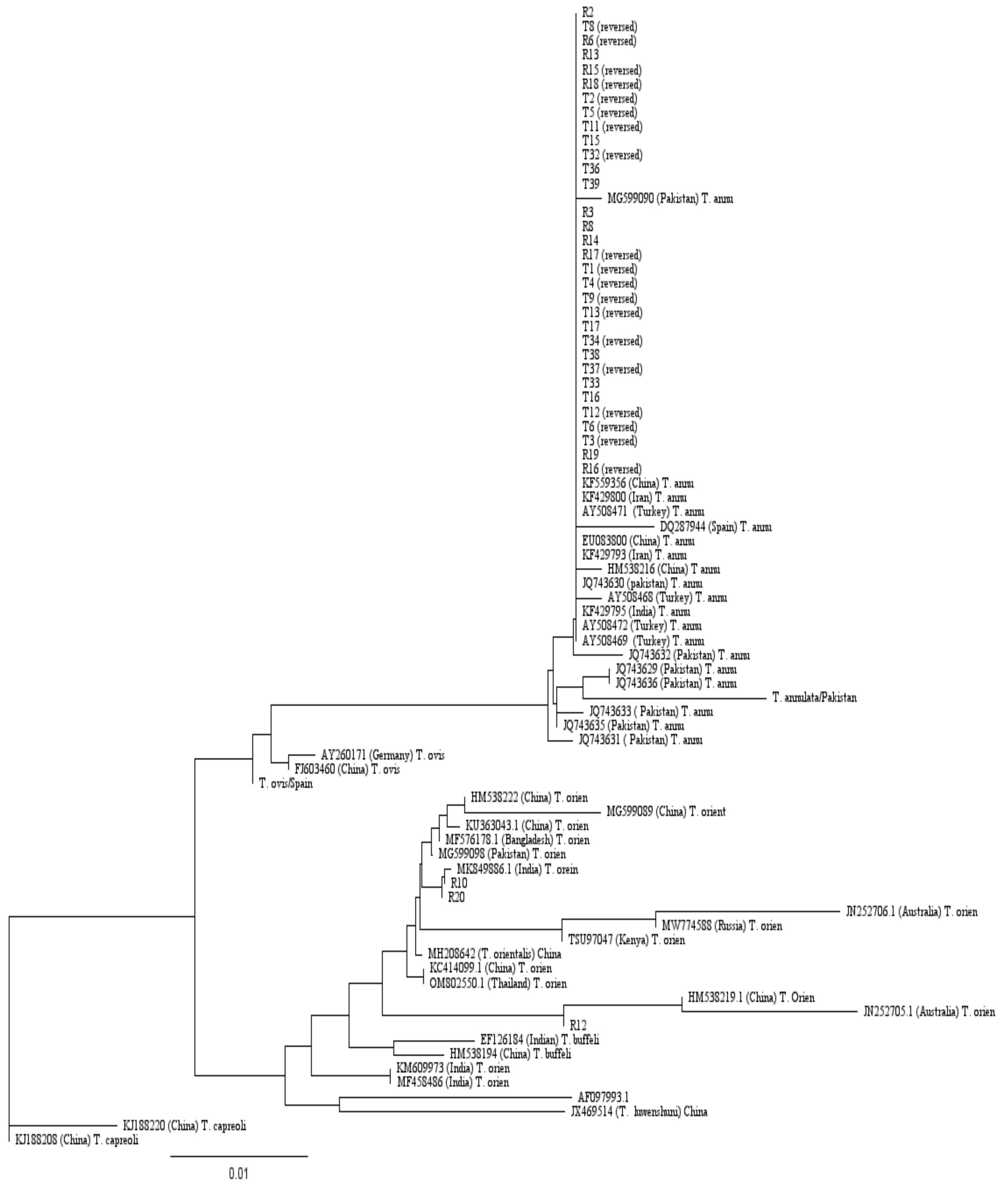


Fig. 4.6. Phylogenetic tree for the 18S rDNA sequences of *T. annulate* and *T. orientalis. T. capreoli* (KJI88220, KJI88208) was used as an outgroup.

4.3.2 *Anaplasma* Molecular Characterisation

Anaplasma marginale was observed in the blood of 14 samples amplified with PCR showing positive detection of *Anaplasm*a with ~ 400 base pairs of 16S rRNA fragments on 2% gel (Fig. 4.7). Out of these 14 sequences isolated from sheep, cattel and goats 12 *Anaplasma* sequences were of good quality and aligned with 28 NCBI GeneBank sequences of different *Anaplasma* species (Fig. 4.8).

The genetic distance search of *Anaplasma* 16S rRNA sequences (present study) shows 99.5% to 99.25% similarity to a previously identified uncultured *Anaplasma sp.* (OP297688, OP297687, OP297686, OP297680) and *Anaplasma marginale* (MK310487, MK310488, MH020201) (Fig. 4.9). Further comparison showed similarities between *Anaplasma ovis* (MK855077). However, phylogenetic tree of *Anaplasma* from Pakistan (present study) revealed a close link with *Anaplasma marginale* and *Anaplasma sp.* (Fig. 4.10). Similarly, the phylogenetic comparison showed that *A, bovis, A. ovis, A. platys, A. phagocytophilum* and *A. capra* form distinct subclades (Fig. 4.8).

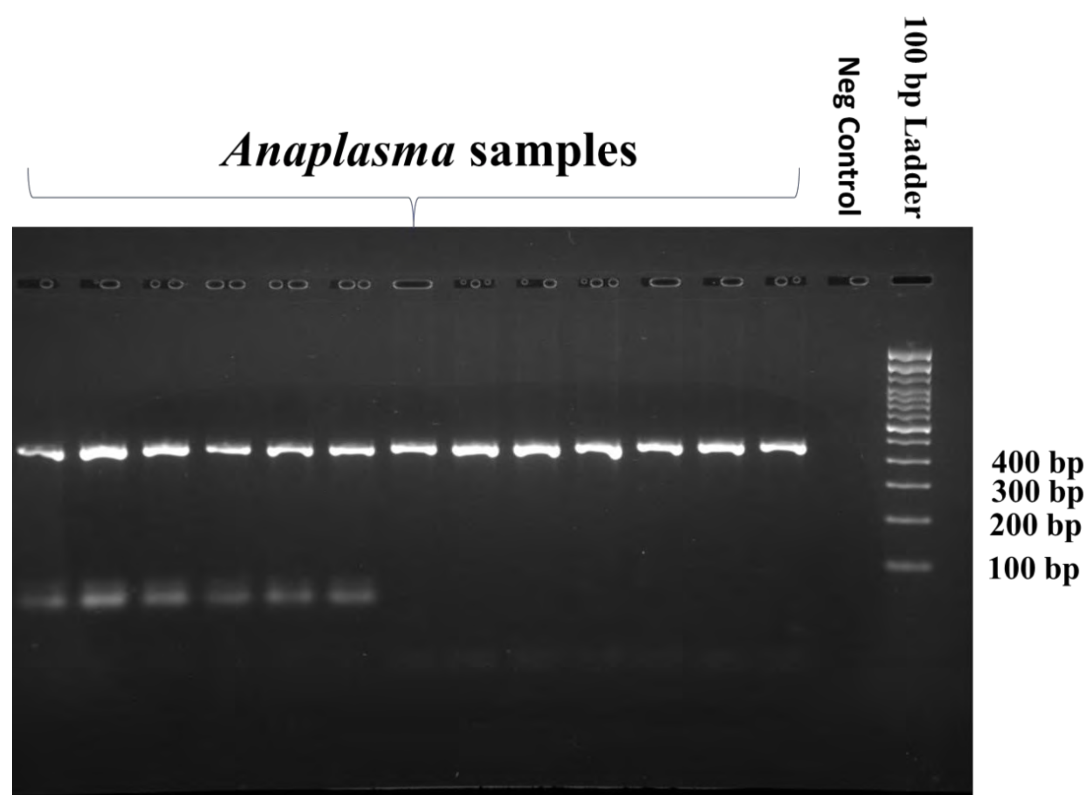


Fig. 4.7: Agarose gel electrophoresis (2%) of PCR product band of 400 bp for *Anaplasma* isolates targeting 16S rRNA.

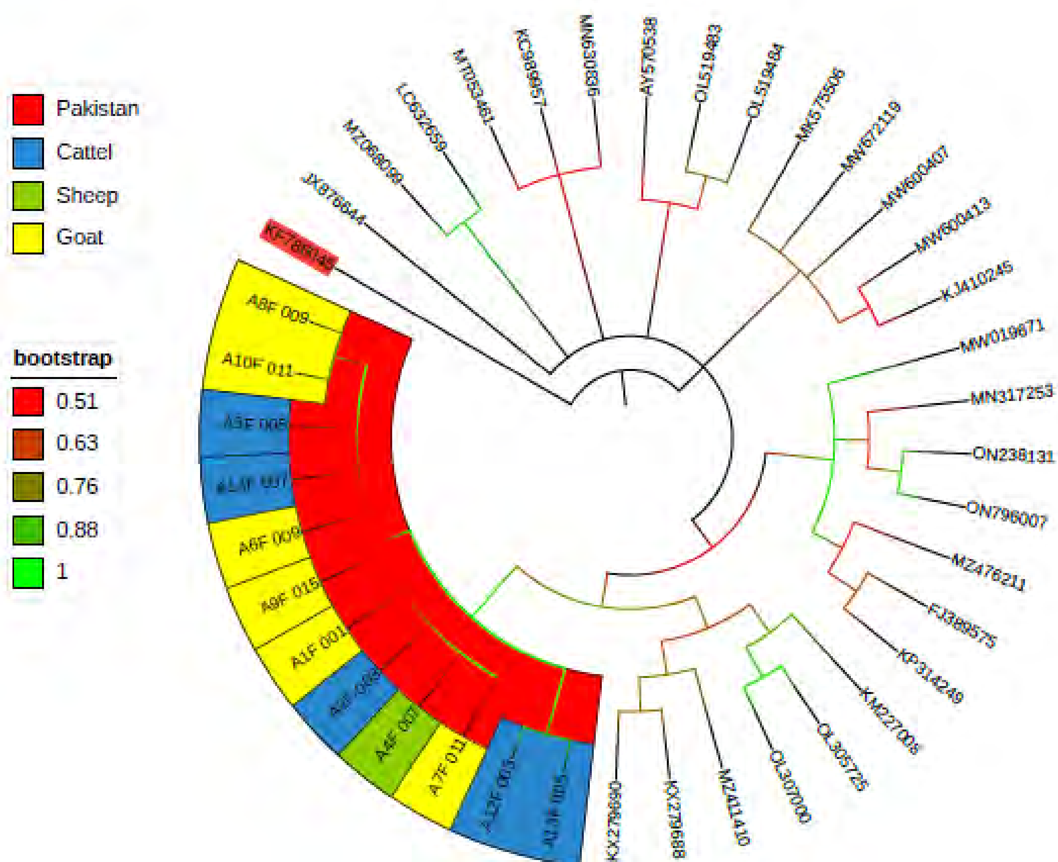
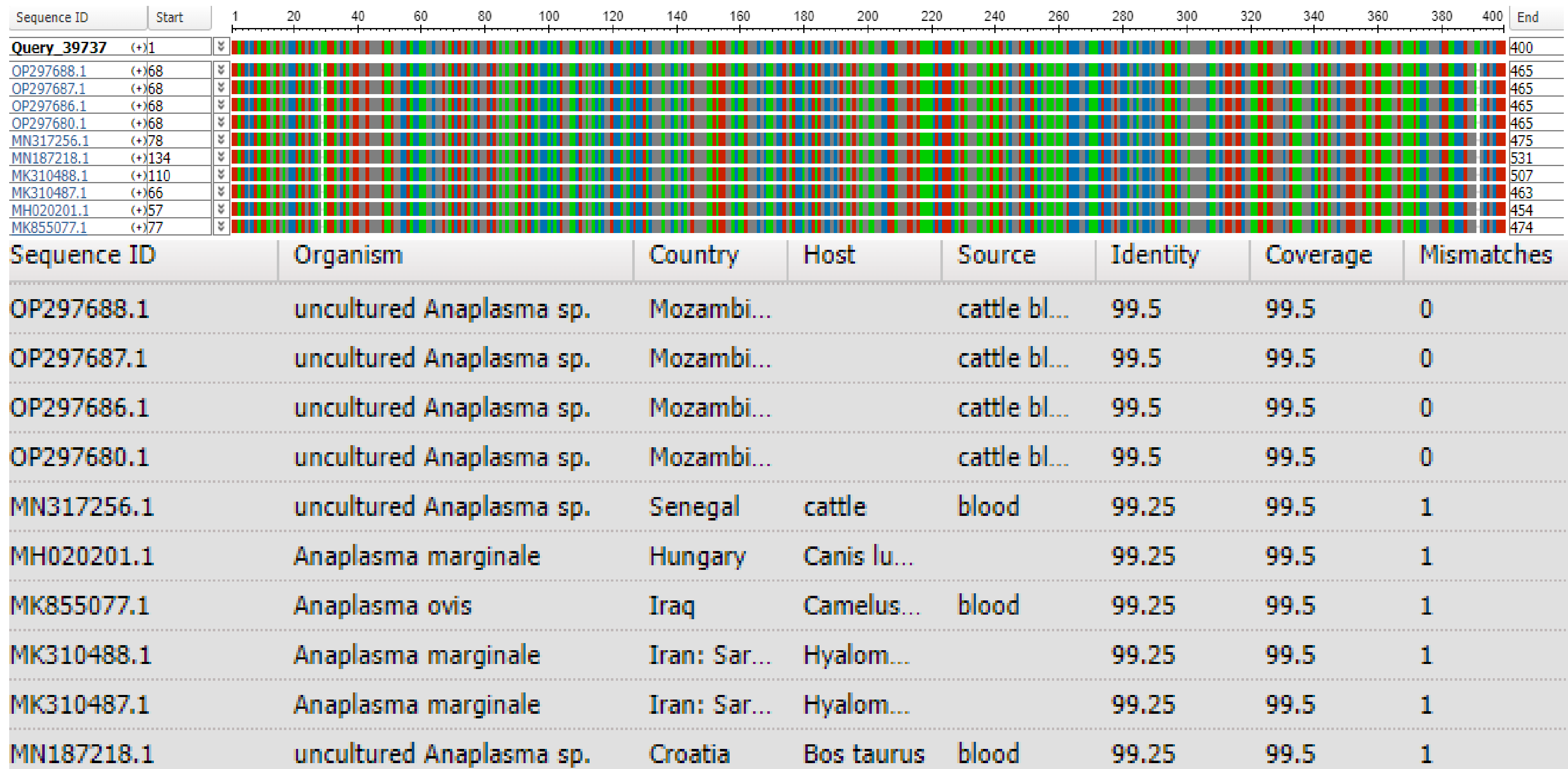


Fig 4.8: Phylogenetic tree for the 16S rRNA sequences of *Anaplasma* isolates from sheep, goats and cattel.



Sequence ID	Organism	Country	Host	Source	Identity	Coverage	Mismatches
OP297688.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297687.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297686.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297680.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
MN317256.1	uncultured Anaplasma sp.	Senegal	cattle	blood	99.25	99.5	1
MH020201.1	Anaplasma marginale	Hungary	Canis lu...		99.25	99.5	1
MK855077.1	Anaplasma ovis	Iraq	Camelus...	blood	99.25	99.5	1
MK310488.1	Anaplasma marginale	Iran: Sar...	Hyalom...		99.25	99.5	1
MK310487.1	Anaplasma marginale	Iran: Sar...	Hyalom...		99.25	99.5	1
MN187218.1	uncultured Anaplasma sp.	Croatia	Bos taurus	blood	99.25	99.5	1

Fig. 4.9: The Blast results showed Query sequence identity with *Anaplasma marginale and Anaplasma* sp. color lines shows different nucleotides position.

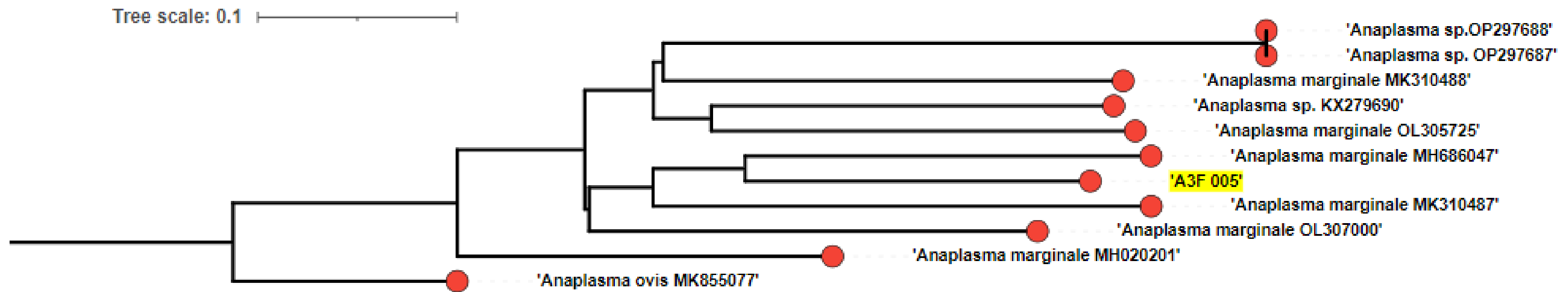


Fig. 4.10: The evolutionary tree based on 16S rRNA sequence by using the Maximum Likelihood method and Kimura 2-parameter model.

4.4 DISCUSSION

This investigation offers information about the diversity of TBPs in sheep, goats and cattle from KP, province of Pakistan. The result shows presence of *T. annulata*, *T. orientalis* and *A. marginale* in the blood samples. The current study created a multiplex real-time PCR test for the rapid detection and speciation of *Theileria* sp. his assay revealed 100% specificity and sensitivity for all Pan-*Theileria* sp. (including: *T. annulata, T. parva, T. lestoquardi, and T. ovis*).

In current study, newly designed Pan-*Theileria* probe to target *18S* rRNA, enabled us to detect more than fifteen *Theileria* species. Four probes specifically target *18S* rRNA gene were designed to detect *T. annulata, T. parva, T. lestoquardi* and *T. ovis*. Similarly, a study reported by Criado-Fornelio *et al.* (2008) developed a duplex TaqMan assay, which improved the detection on species level of bovine piroplasmids. Lobanov *et al.* (2018) and Kunying *et al.* (2022) employed a duplex real-time qPCR approach based on two distinct genes (EMA1 and 18S rRNA) for piroplasm identification. These researchers created a pair of unique primers and fluorescent probes based on the 18S rRNA sequences of *T. equi* and *B. caballi,* respectively. In contrast, Dandasena *et al.* (2018) established a real-time PCR technique based on the absolute quantification, but assay could not differentiate between infected and vaccinated animals. In present study to assess the sensitivity, detection limit and precision, we used serial dilution (10^0 to 10^7) of gBlocks™ gene fragments to validate the assay analytically. Lobanov *et al.,* (2018) study showed high diagnostic sensitivity for *T. equi,* which is consistent with the high diagnostic sensitivity estimated for molecular assays developed for other *Theileria* spp. Kunying *et al.,* (2022) determined nested PCR sensitivity by serially diluting the standard positive plasmid 10-fold. In current study including Pan-*Theileria,* all *Theileria* species showed efficiency in range of 96% to 100% which indicated an acceptable efficiency by using gBlocks™ gene fragment. With an above regression coefficient (R2) of 0.99, this test successfully assessed good linearity for all reactions spanning at least seven log units. Ros-Garca *et al.* (2012) demonstrated homology linear detection with an efficiency of 94% and a regression coefficient (R2) of 0.999. Serial dilutions of gBlocks™ gene fragments were tested in two panels by developed multiplex qPCR, to determine sensitivity and specificity with high affinity. Cross reactivity observed in panel II between *T. lestoquardi* and *T. annulata* due to single base pair nucleotide but based on Ct values speciation was confirmed.

Unfortunately, no data were found to compare the Ct values of our study with published studies. Yang *et al.* (2014) devised a pan-Theileria FRET-qPCR that demonstrated similarities, allowing us to amplify our reference with a minimum of two copies of the *Theileria* 18S rRNA gene per PCR. Although real-time PCR is helpful for analysing clinical specimens and may be able to reach comparable levels of specificity and sensitivity. The qPCR assay's key benefits are its capacity for quantifying infection levels and its lower potential for false-positive detection due to cross-contamination (Mackay 2007). Bhoora *et al.,* (2010) demonstrated disagreement by creating primers specific to the *caballi 18S* rRNA gene and a hydrolysis probe for piroplasmosis, which were not attributable and produced poor results. In terms of detection and quantification of *T. annulata*, Gotia *et al.,* (2016) and Sibeko *et al.,* (2008) used targeted *18S* rRNA qPCR, which showed poor amplification on clinical samples.

According to our data, Theileria infection in ruminants is common in Pakistan. During testing, we detected positive animals in one province, and the prevalence of T. annulata was recorded in 33 samples. Interestingly, 3 samples were declared *T. orientalis,* compared and confirmed results using *in-silico* analysis via NCBI BLAST. One sample showed *T. ovis* according to qPCR results, detected positive by Pan-*Theileria* and *T. ovis* probe but unable to sequence and remained unclear with noisy and poor peaks.

Th results showed that 36 DNA samples were sequenced for *18S* rDNA marker of *Theileria* species by using species-specific primer (Bonnet *et al.,* 2007). The expected amplicons with size of ~359 base pairs were seen in positive samples compared to negative controls. In Pakistan, limited data found based on the genetic diversity of *Theileria* species. Phylogenetic analysis providing helpful information based on genetic variations and evolutionary relationships between species (Alanazi *et al.,* 2021). The phylogenetic tree was constructed using different *Theileria* type strains which showed similarities with isolates reported from Asia, Africa, and Europe (George *et al.,* 2015; Zeb *et al.,* 2019; Khan *et al.,* 2013; Hassan *et al.,* 2018; Ros-García *et al.,* 2012). Similar study found from the Moscow region of Russia targeting *18S* rRNA genetic marker to detect *Theileria* species in cattle (Bursakov and Kovalchuk 2019; Kovalchuk 2022).

One of the most significant tick-borne infections worldwide is *Anaplasma marginale* (Palmer *et al.,* 2000). In present study fourteen blood samples were suspected positive in microscopy for anaplasmosis and further investigated for molecular speciation. Twelve sequences were produced with good quality and blast results showed 99.25% similarity with *A. marginale* and 99.5% similarity with uncultured *Anaplasm*aa spp. from Mozambique. In current work animals were mainly infected with *Rhipicephalus* and *Hyalomma* ticks. According to several investigations, the tick species *Hyalomma excavatum* and *Rhipicephalus annulatus* were both implicated as vectors for *A. marginale* (Samish *et al., 1993;* Shkap *et al.,* 2009).

The phylogenetic analysis of present study *Anaplsama* spp. based on *16S* rRNA gene revealed close similarity with *A. marginale* isolates from Iran, Vietnam, and Pakistan. And form distinct clad from other *Anaplasma* spp. circulating in other countries. The presence of comparable isolates at different locations could be due to introduction exotic species in the country, cross border or unrestricted transportation of live animals for selling. We show that the genus *Anaplasma's* available sequencing samples can be classified to the species level using 16S rRNA sequences, and *A. marginale* can be distinguished from *A. phagocytophilum, A. centrale,* and *A. ovis.* In another study, the 16S rRNA sequence for species categorization of *Anaplasma* samples revealed identities below the 98.7% threshold, implying that two bacteria are distinct species (Caudill *et al.,* 2022). Moreover, it was observed that utilizing sequences that only encompass a few hypervariable regions of the 16S rRNA gene should be avoided as it may lead to misclassification of species. The intraspecies population heterogeneity for each *Anaplasma* 16S rRNA sequence remains impossible to determine rigorously, but these preliminary findings warrant further investigation.

Conclusion

The present study introduced a multiplex real-time PCR assay that targeted the 18S rRNA gene, enabling sensitive detection and quantification of various species of *Theileria.* The serial dilution plots demonstrated good linearity and regression coefficients ($R^2 > 0.975$). For the real-time PCR performance, all reactions showed good efficiency (all >96.83%) with detection

limits of 10 to100 copies per reaction. Out of 51 samples, 47 were positive by Pan *Theileria* PCR. When pan-Theileria positive samples were compared, 33 yielded *Theileria annulata* and 3 samples clearly confirmed *T. orientalis* results, which were supported by their sequencing confirmation results. Both species showed homology with isolates from India, China, Turkey, Iran and Pakistan. Further molecular characterization of *A. marginale* based on *18S rDNA* genetic marker was investigated and phylogenetic analysis revealed close similarity with *A. marginale* isolates from Iran, Vietnam and Pakistan. The study concluded the real-time PCR assay to be most reliable method for screening *Theileria* infection in bovine populations up to species level without sequence confirmation. Understanding the phylogenetic and epidemiological connections between the isolates from any geographic locations more specimens are needed. Additionally, synthetic gBlocks may not be accurate representations of target specimen, and thus, genomic DNA or clinical specimen should be used for clinical validation. This research could contribute to showed that *T. annulata* and *T. orientalis* causes a potential economic loss and severe diseases in bovine populations which needs attention at local and global levels. Additionally, the intraspecies population heterogeneity for each *Anaplasma* 16S rRNA sequence remains impossible to determine rigorously, but these preliminary findings warrant further investigation.

COMPARATIVE EFFICACY OF SYNTHETIC ACARICIDES AGAINST TICK INFESTATIONS IN GOATS

ABSTRACT

Four commercial synthetic compounds, pyrethroid, organophosphates, macrocyclic lactones and phenylpyrazole have been used for tick control worldwide. However, periodic monitoring of effectiveness of acaricides has not been fully explored, although such information could contribute to a more effective application, economic analysis and harmful impact on other organisms and environmental contamination. This study investigates the effect of Cypermethrine (CYM), Deltamethrin, Trichlorphon + Dimethylester, Ivermectin (IVM) and Fipronil on natural infestations of ticks in goats. The *In-vivo* quantitative assessment of five tick genera i.e. *Hyalomma*, *Rhipicephalus*, *Ixodes*, *Haemaphysalis* and *Boophilus* revealed that both CYM and IVM treated groups resulted in significantly lower ($P<0.05$) tick counts relative to other medicines and controls on all post-treated days. The maximum reduction in mean number of ticks in the CYM and IVM treated group was recorded from days 3 to 4, followed by complete shedding of ticks on day 5. However, Deltamethrin, Trichlorphon+ Dimethylester and Fipronil showed 100% efficacy on sixth day. *In-vitro* efficacy trail fipronil (0.25g/100ml) recorded 100% tick's mortality within 18th hours in post-treated group, while Deltamethrin, Trichlorphon + Dimethylester and CYM were ranked 2nd, 3rd and 5th based of their 100% efficacy within 24-33 hrs, 33-42 hrs and 39-48 hours, respectively. The investigation have shown that tested acaricides varied in their performance to reduce the tick infestation and further experimentation on different formulations of other members of major acaricidal classes need to be standardized.

Keyword: Efficacy trials, goats, acaricides, Ticks

5.1 INTRODUCTION

Ticks are one of the leading vectors of diseases of economic importance to the livestock industry in tropical and sub-tropical countries of the world. In tropical country like Pakistan, the warm, humid climate favors perpetuation and propagation of ticks. Tick fauna of Pakistan is rich in number of genera and species (Jabbar *et al.*, 2015). In Pakistan, the overall rate of tick infestation has been detected about 50%. Economic losses creating food insecurity (Habeeb 2010), reduced growth and milk production and causes estimated global cost of control and productivity losses of 7 billion USD per year (Nchu *et al.*, 2012). The adverse effects include paralysis/toxicosis and tick-transmitted haemoparasites that reduce production or cause mortality (Walker *et al.*, 2003).

In Pakistan tick fauna comprises of at least 40 species belonging to mainly three genera i.e. *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* (Ghafar *et al.,* 2020a). The prevalence of tick infestation in small ruminants was estimated as 27.85%. Tick infestation was apparently found higher in goats (30.67%) than sheep (23%). A significant variation in the prevalence (22.2%-70.5%) of bovine ticks i.e. *Hy. anatolicum*, *Hy. hussaini*, *Hy. scupense*, *Rh. annulatus* and *Rh. microplus* was recorded across five agroecological zones of Pakistan (Ghafar *et al.,* 2020b).

Ticks are the major constraints to small ruminant production, and worldwide its control is based mainly on the repeated use of acaricides (Pirali-Kheirabadi and da Silva 2011; Shyma *et al.*, 2013). Number of methods exists to suppress tick's population i.e., dusting, hand spraying, mechanical spray race, hand dressing, systemic and dipping (Koney 2004). However chemotherapeutic control remains the most extensively applied method in the developing world. Acaricides such as synthetic pyrethroids and organophosphates, macrocyclic lactones, organochlorines, carbamates, and insect growth regulators have been found with significant efficacy for tick control (Whitnall *et al.*, 1951; Davey and Ahrens 1984; Ware 2000). However, populations of several tick species mainly in tropical and subtropical countries have developed resistance to all major classes of these compounds due to the high intensity of their use in tick management (Abbas *et al*., 2014; Lenka *et al.*, 2016).

In Pakistan the main tick control methods in small and large ruminants are periodic application of acaricides i.e. macrocyclic lactones, trichlorfon and cypermethrin (Jabbar *et al.,* 2015; Iqbal *et al.,* 2017; Khan 1993). However, studies on assessment of the in vivo efficacy of acaricidal drugs are limited and to date few in vivo efficacy testing studies on coumaphos,

cypermethrin, diazinon and ivermectin were performed in both sheep and goats in Pakistan (Khan 1993; Sajid *et al.,* 2011). Therefore, it is necessary to undertake periodic monitoring of effectiveness of acaricides to provide updated information on the efficacy of commercial acaricides for effective control against tick infestations on animals. Here, current study presented in vivo and in vitro efficacy testing to establish the current level of acaricidal resistance for five products representing every major acaricidal class (cypermethrine and deltamethrin representing the synthetic pyrethroid; trichlorphon + dimethyl ester representing organophosphates; ivermectin representing the macrocyclic lactones and fipronil representing phenylpyrazole compound) in controlling natural infestations with ticks of goats.

5.2. MATERIAL AND METHODS

5.2.1 Site/Experimental Animal's Selection

The present research was carried out at Livestock Research Station (LRS), National Agricultural Research Centre (NARC), Islamabad (33.6844° N, 73.0479° E) involving natural heavy ticks infected doses (female-goats) between 2-5 years aged. Ticks were collected from infected goats with the help of forceps avoid damage to mouth parts. Identification of ticks was performed through observation of morphological characteristics under stereoscope with the help of taxonomic keys (Walker *et al.*, 2003).

5.2.2 In-Vivo Acaricidal Efficacy Trials

The *In-vivo* acaricidal efficacy trials were conducted per guideline of WAAVP (Holdsworth *et al.,* 2006). Briefly, 60 adult female goats, age between 2-5 years, with semi intensive management, no history of acaricidal treatment and tick infestation rate of 100-120 ticks peranimal. Five compounds were subjected to acaricidal treatment viz., Cypermethrine, Deltamethrin, Trichlorphon + Dimethylester, Ivermectinand Fipronil. These compounds were selected based on their extensive usage in livestock farms for tick control.

The animals were divided into six equal groups A through F (Table 5.1). Groups A to E were treated with acaricidal compounds as per manufactory instructions, while groups F served as control. After treatment with either of the above mentioned acaricides, the animals were examined quantitatively through "finger counting" (Rugg and Hair 2007), the number of ticks shed after the first 24 hour and the duration for which the treatment remained effective was

calculated from the data. The data are expressedas post-treatment tick burden on days 0, 1, 2, 3, 4, 5, and 6.

***5.2.3 In-Vitro* Acaricidal Efficacy Trials**

The fully engorged ticks (4-5 mm in size) were collected from natural infectedgoats managed at livestock research stations. Two different dilutions of each acaricidal compounds were prepared (Table 5.2), and 30 adult ticks were used in each *In vitro* test dilutions, while one group served as control treated with distilled water. The Petri dishes were kept at 25 ± 2°C and 80 ± 5% relative humidity in an incubator for 24 hours. The mortality of ticks in all groups was observed after different time intervals.

5.2.4 Statistical Analysis

Descriptive analyses were performed according to the scale of infestation as recommended[26]. The raw data of the ticks count were transformed in a natural logarithm of 10 (count +1). The data were analyzed using the analysis of variance test (ANOVA) followed by least significant difference (LSD) test for means comparisons. The level of significance used was $p \leq 0.05$. The threshold of 90% reduction in the counts of ticks in treated dogs compared to untreated ones was considered as of acceptable efficacy for tick control agents, as recommended (Marchiondo *et al*., 2013).

The data of five acaricides efficacy in *In-vivo* trail were initially analysed by descriptive statistics (mean, standard error) using Statistix 8.1 program. The efficacy was determined as follows:

$$Efficacy\ (\%) = C\text{-}T/C \times 100.$$

Where: C= present mean number of ticks per animal in the control group and T=mean number of ticks per animal in the treatment group.

The data of acaricides efficacy in *In-vitro* trail were as follows:

$$Efficacy\ (\%) = N_0 - N/N_0 * 100$$

Where N_0 is the number of ticks prior to acaricidal treatment and N is the number of ticks recorded post-treatment (Holdsworth *et al.,* 2005).

Table 5.1: *In-vivo* therapeutic trial against tick's infestation in goats

Experimental groups	Composition and packing	Dilution of medicines	Dose rate/Mode of application
A (10 goats)	Cypermethrine 25%	5 ml /liter	Spray on animals 20 ml/animal
B (10 goats)	Deltamethrin 2.5% W/V (100 ml)	4 ml/liter	Spray on animals 20 ml/animal
C (10 goats)	Trichlorphon 98% W/W, Dimethylester of (2,2,2- trichloro-1 hydroxy-ethyl phosphoric acid) (100gm)	2 g/liter	Spray on animals 20 ml/animal
D (10 goats)	Ivermectin-1gm Vit-A-2500,000U Vit-D-375000U Vit-E-2.5gm	As such	Sub-cut administration 1ml/50 kg live-body weight
E (10 goats)	Fipronil 0.25g in each 100ml	As such	Spray on animals 20 ml/animal
F (10 goats)	Control (Water)	-	-

Table 5.2. ***In-vitro* acaricide efficacy trial against ticks collected from goats.**

Medicine	Petri Dish #	Concentration
Cypermethrine 25%	A1	5ml/liter (0.125g/ml)
	A2	4ml/liter (1g/m)
Deltamethrin 2.5% W/V (100 ml)	B1	4 ml/liter (0.1mg/ml)
	B2	3 ml/liter (0.075mg/ml)
Trichlorphon 98% W/W, Dimethylester of (2,2,2-trichloro-1 hydroxy-ethyl phosphoric acid) (100gm)	C1	2mg/ml
	C2	1.5mg/ml
Fibronil 0.25g in each 100ml	D 1	0.0025g/100ml
	D2	0.002g/100ml
Control	E	Water

5.3 RESULTS

5.3.1 In-Vivo Experiment

The experimental goats were infested with five tick genera i.e. *Hyalomma*, *Rhipicephalus*, *Ixodes*, *Haemaphysalis* and *Boophilus*. The *In-vivo* post-treatment quantitative assessment of tick burden revealed that both cypermethrine and ivermectin treated groups resulted in significantly lower (P<0.05) tick counts relative to other medicines and controls on all post-treated days. The finger counts were significantly higher (P=0.00) in group A (cypermethrine -treated group) than in group D (ivermectin treated), as shown in Table 5.3. From day 0 (pre-treatment) day 1st (post-treatment), the reduction in the mean number of ticks was not significant (P>0.05) in all treatments. The maximum reduction in mean number of ticks in the CYM and IVM treated group was recorded from days 3 to 4, followed by complete shedding of ticks on day 5. However, deltamethrin, trichlorphon-dimethylester and fipronil showed 100% efficacy on sixth day. The ticks in control group were almost remained the same with no significant (P>0.05) changes during the experimental period.

The fipronil treated group showed an average of 117 ticks, 99.9, 36, 15.6, 1.3, and 0.5 on days 1, 2, 3, 4 and 5, respectively. The formulation presented acaricidal efficacy of 90.8% and 100% on day 4 and 6, respectively.

5.3.2 *In-Vitro* Experiment

The results of in-vitro efficacy trail showed that fipronil recorded 100% tick's mortality within 18th hours post-treatment with higher concentration (0.25g/100ml) and 24 hours with lower concentration (0.2g/100ml). Deltamethrin, trichlorphon + dimethylester and cypermethrine were on 2nd, 3rd and 5th ranked based of their 100% efficacy within 24-33 hrs, 33-42 hrs and 39-48 hours, respectively (Fig. 5.1). However, all the four acaricides i.e., trichlorphon+ dimethylester, deltamethrin and cypermethrine showed 100% tick mortality within 48 hours of post application. The cypermethrine and trichlorphon+ dimethylester treated group (A2 and C2) showed lowest efficacy, as 83.3% after 36th hours.

Table 5.3: Mean (±SD) and percentage reduction (%) in tick burden against different acaricidal treatments in goats.

Acaricide	Pre-treatment	Post-treatment days					
		1st	2nd	3rd	4th	5th	6th
Cypermethrine	117.7±4.2 (0.4)	117.3±4.1^{a} (33.6)	75.9±1^{c} (73.3)	18.9±3.4^{c} (81.91)	4.4±1.4^{c} (87.12)	0.9±0.3^{b} (100)	
Deltamethrin	118.9±6.12 (0.7)	118.1±6.16^{a} (21)	92.1±3.2^{b} (67.4)	29.8±4.8^{bc} (66.7)	8.2±0.9^{c} (72.1)	2.1±1.1^{b} (95.8)	0.1±0.0^{b} (100)
Trichlorphon Dimethylester	116.7±1.81 (0.3)	116.3±1.94^{a} (22.1)	91±9.2^{bc} (65.6)	29.4±2.3^{bc} (69.2)	8.5±0.83^{c} (78.6)	1.9±0.6^{b} (95.5)	0.2±0.0^{b} (100)
Ivermectin	113.9±2.67 (0.3)	113.5±2.3^{a} (22.3)	87.9±6.6^{b} (70.8)	25.5±4.2^{bc} (73.3)	6±1^{c} (96.2)	0.2±0.6^{b} (100)	
Fibronil	118±3.46 (0.8)	117±3.17^{a} (14.6)	99.9±4^{b} (63.6)	36±5.5^{b} (59.4)	15.6±8.5^{b} (90.8)	1.3±0.4^{b} (56.7)	0.5±0.4^{b} (100)
Control	118.1±0.63 (0.9)	117.2±1.13^{a} (0.1)	116.3±1.42^{a} (-0.6)	116.9±1.4^{a} (0.2)	117.6±0.8^{a} (0.2)	117.5±0.8^{a} (-1.4)	118.8±0.4^{a} (0.2)
P- Value		0.90	0.00	0.00	0.00	0.00	0.00

Parenthesis indicates the tick's mortality percentage; Mean with different letters are significantly different ($P<0.05$).

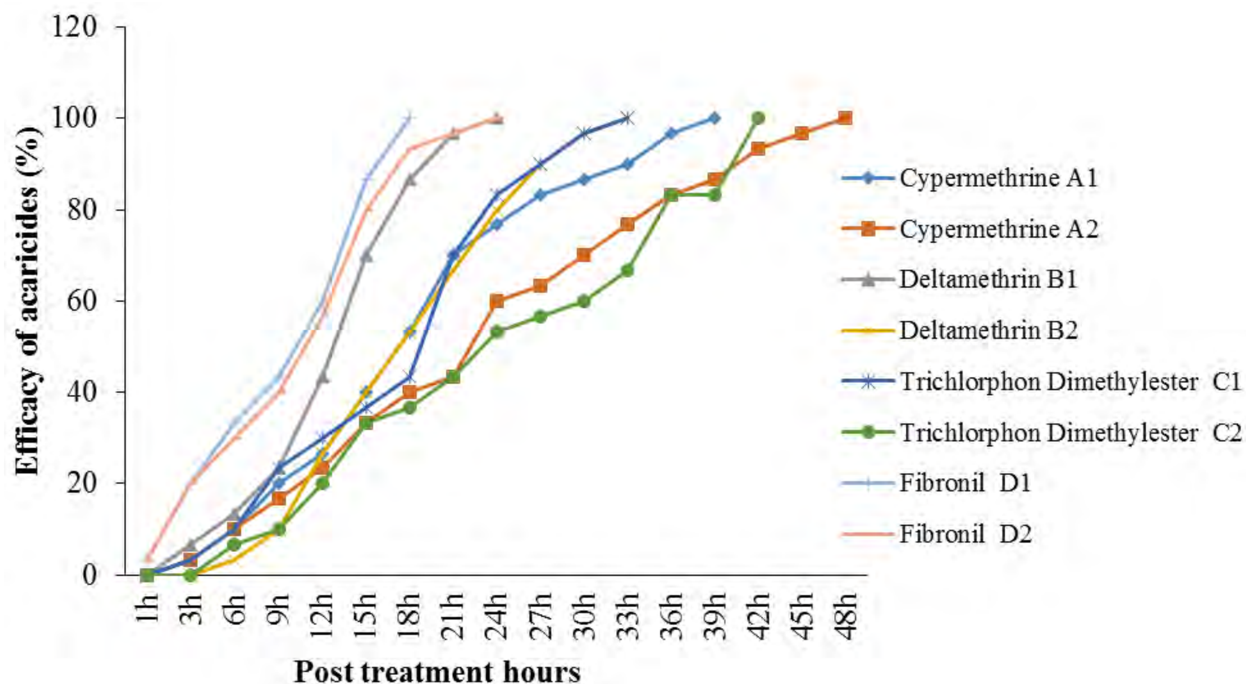


Figure 5.1: *In-vitro* percentage reduction of ticks on goats against two different concentrations of five commercial acaricides.

5.4 DISCUSSION

The application of acaricides may significantly reduce the abundance of the tick species (Benelli *et al.,* 2017), and help to mitigate the risk of tick-borne diseases (Otranto *et al.,* 2010). However, application of acaricides may lead to development of tick resistance to several chemical compounds (Abbas *et al.,* 2014), which needs regular monitoring of acaricides. The present investigation was designed to measure the comparative efficacy of five different formulations of acaricides. The current *in vivo* trials showed 100% tick mortality with cypermethrin and ivermectin o n the 5th day of post-treatment. Similar findings were recorded on larval stages of different species of ticks *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* with cypermethrin (Rani *et al.,* 2018). *Ixodes ricinus* showed 100% mortality at the 9th day of ivermectin post-treatment (Kröber and Guerin 2007), while another study reported even longer period of 21 days against *R. microplus* (Muhammad *et al.,* 2019). The resistance of ivermectin against *I. ricinus* was also reported (Martins and Furlong 2001; Lopez-Arias *et al.,* 2014).

Comparative to the present study, a higher efficacy of cypermethrine as 50% tick's mortality was recorded within 10 min and 100% in 30 min with the dose rate of 1.0 mg/mL or 10.0 mg/m (Myung-Jo 2014). In contrast to these results, lower mortality (92% and 96.7%) was recorded with cypermethrin application on unfed female of *R. sanguineus* and engorged females, respectively (Bicalho *et al.,* 2001). The differences among the mortality rates may dependent on the dose formulation, mode of application and the type of tick species.

In current research, trichlorphon showed complete reduction of ticks on the 6th day of post treatment. Several studies recorded lower efficacy, resistance, and reinfection to tick populations after trichlorphon treatment (Muhammad *et al.,* 2019; Vudriko *et al.,* 2016; Katuri *et al.,* 2017). In vitro trichlorphon concentrations 2 mg/mL and 1.5 mg/mL resulted 50% ticks ' mortality within 9th and 24th h and 100% at 18th and 24th h, respectively (Rodriguez-Vivas *et al.,* 2017).

The post treatment efficacy of deltamethrin was 100% at the 6th day in the present investigation, which is not consistent with the previous findings (Katuri *et al.,* 2017; Brito *et al.,* 2011). Lower efficacy of 13.2%, 12.3% and 16.2% was observed at 3, 7, and 14 days of post-treatment for immature ticks, respectively (Barre *et al.,* 2008). Deltamethrin produced about 52.8% reduction of semi engorged females at 3 days post-treatment and lower percentages were observed at 7 and 14-days post-treatment. The present deltamethrin trials with two concentrations i.e., 0.1 mg/mL and 0.075 mg/mL caused 50% tick's mortality in 12th and 18th h and 100% in 24th and 33rd h, respectively. A previous study on *R. microplus* and *H. anatolicum* ticks showed both susceptibility and resistance to deltamethrin (Gaur *et al.,* 2017). The deltamethrin (0.0025) tested for *R. sanguineus* engorged female showed low sensitivity (Shyma *et al.,* 2015). However, resistance with deltamethrin concentration of 0.1 mg/mL was 86.7% (26/30) (Vudriko *et al.,* 2016) and for commercial preparation of 1.25% against *R. microplus* was 63% (Cid *et al.,* 2016). The possible reasons for differences in results are inconsistent experimental conditions, route of administration, formulation, sampling and analytical methods.

The fipronil *in vivo* formulation presented acaricidal efficacy of 90.8% and 100% on day 4 and 6, respectively in the current study, which agrees with the study recorded maximum efficacy (99.39%) against *R. microplus* female after nine days post-treatment (Cid *et al.,* 2016).

The mean efficacy of fipronil at a dose of 1 mg/kg in cattle on adults, nymphs and larvae of *R. microplus* female was 74.96%, 92.24% and 80.13%, respectively (Cid *et al.,* 2016). Longer period of 17 days of 100% effectiveness was also recorded for fipronil against *R. sanguineus* (Tiawsirisup *et al.,* 2013). However, a study on tick's counts of dogs calculated efficacy of fipronil on weekly basis (2nd, 4th, 8th, 12th) and were 97.6%, 93.8%, 100% respectively (Rohdich *et al.,* 2014). These difference in the effectiveness of fipronil may be dose dependent, as higher dosage caused mortality of both adults and larva of *R. microplus* (Tiawsirisup *et al.,* 2013).

Conclusion

The study concluded that application of the tested compounds can reduce the abundance of successive generations of four tick genera namely: *Hyalomma, Rhipicephalus, Ixodes and Haemaphysalis*, which may contribute to reduction of population of tick species. Furthermore, experimentation on acaricidal efficacy testing with different formulations of other members of major acaricidal classes needs to be standardized.

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Appendix

Sample ID	PCR Result			Meta Data				
	qPCR	Phylogenetic analyses	Speciation	Host	Sample Type	City	Province	Coordinate of city
T10	NEG	NEG	NEG	Sheep	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T21	NEG	NEG	NEG	Cattle	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T31	NEG	NEG	NEG	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T40	NEG	NEG	NEG	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
R10	POS	*T. orientalis*	NEG	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R12	POS	*T. orientalis*	NEG	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R20	POS	*T. orientalis*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
T1	POS	*T. annulata*	*T. annulata*	Cow	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T2	POS	*T. annulata*	*T. annulata*	Sheep	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T3	POS	*T. annulata*	*T. annulata*	Sheep	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T4	POS	*T. annulata*	*T. annulata*	Sheep	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T5	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T6	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T7	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T8	POS	*T. annulata*	*T. annulata*	Cow	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T9	POS	*T. annulata*	*T. annulata*	Sheep	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T11	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T12	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T13	POS	*T. annulata*	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T15	POS	*T. annulata*	*T. annulata*	Buffalo	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T16	POS	*T. annulata*	*T. annulata*	Buffalo	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T17	POS	*T. annulata*	*T. annulata*	Buffalo	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T32	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T33	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T34	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T36	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N

T37	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T38	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
T39	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
R2	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R3	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R6	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R8	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R13	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R14	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R15	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R16	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R17	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R18	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R19	POS	*T. annulata*	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
T14	POS	Unable to sequence **	*T. annulata*	Goat	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T18	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T19	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T20	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Charsadda	KP	34.1682° N, 71.7504° E
T35	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Peshawar	KP	71.5249° E, 34.0151° N
R1	POS	Unable to sequence **	*T. ovis*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R4	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R5	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R7	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R9	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E
R11	POS	Unable to sequence **	*T. annulata*	Cattle	Whole Blood	Mardan	KP	34.1986° N, 72.0404° E

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RESEARCH ARTICLE

Comparative Efficacy of Synthetic Acaricides Against Tick Infestations in Goats

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Abstract

Four commercial synthetic compounds, pyrethroid, organophosphates, macrocyclic lactones and phenylpyrazole have been used for tick control worldwide. However, periodic monitoring of the effectiveness of acaricides has not been fully explored, although such information could contribute to a more effective application, economic analysis and harmful impact on other organisms and environmental contamination. This study investigates the effect of cypermethrine (CYM), deltamethrin, trichlorphon + dimethylester, ivermectin (IVM) and fipronil on natural infestations of ticks in goats. The *in vivo* quantitative assessment of four tick genera i.e. *Hyalomma, Rhipicephalus, Ixodes* and *Haemaphysalis* revealed that both CYM and IVM treated groups resulted in significantly lower (P<0.05) tick counts relative to other compounds and controls on all post-treated days. The maximum reduction in the mean number of ticks in the CYM and IVM treated group was recorded from days 3 to 4, followed by complete shedding of ticks on day 5. However, deltamethrin, trichlorphon + dimethylester and fipronil showed 100% efficacy on the sixth day. *In-vitro* efficacy trials showed a 100% tick's mortality based upon the use of fipronil (0.25 g/100 mL) within the 18th h in the post-treated group, while deltamethrin, trichlorphon + dimethylester and CYM were ranked 2nd, 3rd and 5th based on their 100% efficacy within 24-33 h, 33-42 h and 39-48 h, respectively. The investigation has shown that tested acaricides varied in their efficacy to reduce the tick infestation and further experiments on different formulations of the other members of the major acaricidal classes need to be standardized.

Keywords: *Acaricides, Efficacy trials, Goats, Tick*

Keçilerde Kene Enfestasyonuna Karşı Sentetik Akarisitlerin Karşılaştırmalı Etkinliği

Öz

Dünya genelinde kene kontrolü için piretroid, organofosfatlar, makrosiklik laktonlar ve fenilpirazol olmak üzere dört ticari sentetik bileşik kullanılmıştır. Fakat, akarisitlerin etkinliği periyodik olarak tam olarak izlenmemiştir ki bu tür bilgiler daha etkili bir uygulamaya, ekonomik analize ve diğer organizmalar ve çevresel kontaminasyon üzerindeki zararlı etkilere katkıda bulunabilir. Bu çalışmada, sipermetrin (CYM), deltametrin, triklorfon + dimetilester, ivermektin (IVM) ve fipronil'in keçilerde doğal kene enfestasyonu üzerine etkisi araştırılmıştır. *Hyalomma, Rhipicephalus, Ixodes* ve *Haemaphysalis* gibi dört kene cinsi üzerinde yapılan *in vivo* kantitatif değerlendirmede hem CYM hem de IVM ile sağaltılan grupların, tedavi sonrası tüm günlerde diğer bileşiklere ve kontrollere oranla önemli ölçüde daha düşük kene popülasyonuna sahip olduğu saptanmıştır (P<0.05). CYM ve IVM ile tedavi edilen gruplardaki ortalama kene sayısındaki maksimum azalma, 3 ile 4. günler arasında kaydedilmiş, takiben 5. günde kenelerin tamamen döküldüğü izlenmiştir. Bununla birlikte, deltametrin, triklorfon + dimetilester ve fipronil, uygulamanın 6. gününde %100 etkinlik göstermiştir. *In vitro* etkinlik denemelerinde, tedavi sonrası grupta fipronil'in (0.25 /100 mL) oranında kullanımına bağlı olarak 18. saatte %100 kene ölüm oranı saptanırken, deltametrin, triklorfon + dimetilester ve sipermetrin'in %100 etkinlikleri sırasıyla 24-33. saat, 33-42. saat ve 39-48. saatler içerisinde saptanmış ve buna göre etkinlik açısından 2., 3. ve 5. sıralarda yer almışlardır. Bu çalışma, test edilen akarisitlerin kene enfestasyonunu azaltmada etkinliklerinde farklılıklar olduğunu ve temel akarisit sınıflarının diğer üyelerinin farklı formülasyonları üzerinde daha fazla deneylerin standartlaştırılması gerektiğini göstermiştir.

Anahtar sözcükler: *Akarisit, Etkinlik denemeleri, Keçi, Kene*

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INTRODUCTION

Ticks are one of the leading vectors of diseases of economic importance to the livestock industry in tropical and sub-tropical countries of the world. In tropical country like Pakistan, the warm-humid climate favors perpetuation and propagation of ticks. Tick fauna of Pakistan is rich in number of genera and species [1]. In Pakistan, the overall rate of tick infestation has been detected about 50%. Economic losses creating food insecurity [2], reduced growth and milk production and causes estimated global cost of control and productivity losses of 7 billion USD per year [3]. The adverse effects include paralysis/toxicosis and tick-transmitted haemoparasites that reduce production or cause mortality [4].

In Pakistan tick fauna comprises of at least 40 species belonging to mainly three genera i.e. *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* [5]. The prevalence of tick infestation in small ruminants was estimated as 27.85%. Tick infestation was apparently found higher in goats (30.67%) than sheep (23%). A significant variation in the prevalence (22.2%-70.5%) of bovine ticks i.e. *Hy. anatolicum*, *Hy. hussaini*, *Hy. scupense*, *Rh. annulatus* and *Rh. microplus* was recorded across five agroecological zones of Pakistan [6].

Ticks are the major constraints to small ruminant production, and worldwide its control is based mainly on the repeated use of acaricides [7,8]. Number of methods exists to suppress tick's population i.e. dusting, hand spraying, mechanical spray race, hand dressing, systemic and dipping [9]; However, chemotherapeutic control remains the most extensively applied method in the developing world. Acaricides such as synthetic pyrethroids and organophosphates, macro-cyclic lactones, organochlorines, carbamates, and insect growth regulators have been found with significant efficacy for tick control [10-12]. However, populations of several tick species mainly in tropical and subtropical countries have developed resistance to all major classes of these compounds due to the high intensity of their use in tick management [13,14].

In Pakistan the main tick control methods in small and large ruminants are periodic application of acaricides i.e. macrocyclic lactones, trichlorfon and cypermethrin [1,15,16]. However, studies on assessment of the in vivo efficacy of acaricidal drugs are limited and to date few in vivo efficacy testing studies on coumaphos, cypermethrin, diazinon and ivermectin were performed in both sheep and goats in Pakistan [17,18]. Therefore, it is necessary to undertake periodic monitoring of effectiveness of acaricides to provide updated information on the efficacy of commercial acaricides for effective control against tick infestations on animals. Here, current study presented *in vivo* and *in vitro* efficacy testing to establish the current level of acaricidal resistance for five products representing every major acaricidal class (cypermethrine and deltamethrin representing the synthetic pyrethroid; trichlorphon + dimethyl ester representing organophosphates; ivermectin representing the macrocyclic lactones and fipronil representing phenylpyrazole compound) in controlling natural infestations with ticks of goats.

MATERIAL AND METHODS

Site/Experimental Animal's Selection

The present research was carried out at Livestock Research Station (LRS), National Agricultural Research Centre (NARC), Islamabad (33.6844° N, 73.0479° E) involving female goats between 2-5 years aged naturally and heavily infested with ticks. Ticks were collected from infested goats with the help of forceps avoid damage to mouth parts. Identification of ticks was performed through observation of morphological characteristic under stereomicroscope following the taxonomic keys [19,20].

In vivo Acaricidal Efficacy Trials

The *in vivo* acaricidal efficacy trials were conducted per guideline of WAAVP [21]. Briefly, 60 adult female goats, age between 2-5 years, with semi-intensive management, no history of acaricidal treatment and tick infestation rate of 100-120 ticks per animal, are used. Five compounds were subjected to acaricidal treatment viz., cypermethrine, deltamethrin, trichlorphon + dimethylester, ivermectin and fipronil. These compounds were selected based on their extensive usage in livestock farms for tick control.

The animals were divided into six equal groups named A through F *(Table 1)*. Groups A to E were treated with acaricidal compounds as per manufactory instructions, while group F served as control. After treatment with either of the above mentioned acaricides, the animals were examined quantitatively through "finger counting" [22], the number of ticks shed after the first 24 h and the duration for which the treatment remained effective that calculated from the data. The data were expressed as post-treatment tick burden on days 0, 1, 2, 3, 4, 5, and 6.

In vitro Acaricidal Efficacy Trials

The fully engorged ticks (4-5 mm in size) were collected from naturally infected goats managed at livestock research stations. Two different dilutions of each acaricidal compounds were prepared *(Table 2)*, and 30 adult ticks were used in each *in vitro* test dilutions, while one group served as control treated with distilled water. The petri dishes were kept at 25±2ºC and 80±5% relative humidity in an incubator for 24 h. The mortality of ticks in all groups was evaluated after different time intervals.

Statistical Analysis

Descriptive analyses were performed according to the

Table 1. *In vivo therapeutic trial against tick's infestation in goats*			
Experimental Groups	**Composition and Packing**	**Dilution of Medicines**	**Dose Rate/Mode of Application**
A (10 goats)	Cypermethrine 25%	5 mL /liter	Spray on animals 20 mL/animal
B (10 goats)	Deltamethrin 2.5% W/V (100 mL)	4 mL/liter	Spray on animals 20 mL/animal
C (10 goats)	Trichlorphon 98% W/W, Dimethylester of (2,2,2- trichloro-1 hydroxy-ethyl phosphoric acid) (100gm)	2 g/liter	Spray on animals 20 mL/animal
D (10 goats)	Ivermectin-1gm Vit-A-2500,000U Vit-D-375000U Vit-E-2.5gm	As such	Sub-cut administration 1 mL/50 kg live-body weight
E (10 goats)	Fipronil 0.25g in each 100 mL	As such	Spray on animals 20 mL/animal
F (10 goats)	Control (Water)	-	-

Table 2. *In-vitro acaricide efficacy trial against ticks collected from goats*		
Medicine	**Petri Dish #**	**Concentration**
Cypermethrine 25%	A1	5 mL/liter (0.125 g/mL)
	A2	4 mL/liter (1 g/m)
Deltamethrin 2.5% W/V (100 mL)	B1	4 mL/liter (0.1 mg/mL)
	B2	3 mL/liter (0.075 mg/mL)
Trichlorphon 98% W/W, Dimethylester of (2,2,2- trichloro-1 hydroxy-ethyl phosphoric acid) (100 gm)	C1	2 mg/mL
	C2	1.5 mg/mL
Fibronil 0.25g in each 100 mL	D 1	0.0025g /100 mL
	D2	0.002 g/100 mL
Control	E	Water

scale of infestation as recommended [23]. The raw data of the ticks count were transformed in a natural logarithm of 10 (count +1). The data were analyzed using the analysis of variance test (ANOVA) followed by least significant difference (LSD) test for means comparisons. The level of significance used was P≤0.05. The threshold of 90% reduction in the counts of ticks in treated goats compared to untreated ones was considered as of acceptable efficacy for tick control agents, as recommended [23].

The data of five acaricides efficacy in *in vivo* trail were initially analyzed by descriptive statistics (mean, standard error) using Statistix 8.1 program. The efficacy was determined as follows:

Efficacy (%) = C-T/C X 100

Where: C = present mean number of ticks per animal in the control group and T = mean number of ticks per animal in the treatment group.

The data of acaricides efficacy in *in vitro* trail were as follows:

Efficacy (%) = N_0- N/N_0* 100

Where N_0 is the number of ticks prior to acaricidal treatment and N is the number of ticks recorded post-treatment [21].

Results

In vivo Experiment

The experimental goats were infested with four tick genera i.e. *Hyalomma*, *Rhipicephalus*, *Ixodes* and *Haemaphysalis*. The *in vivo* post-treatment quantitative assessment of tick burden revealed that both cypermethrine and ivermectin treated groups resulted in significantly lower (P<0.05) tick counts relative to other medicines and controls on all post-treated days. The finger counts were significantly higher (P=0.00) in group A (cypermethrine -treated group) than in group D (ivermectin treated), as shown in *Table 3*. From day 0 (pre-treatment) to day 1 (post-treatment), the reduction in the mean number of ticks was not significant (P>0.05) in all treatments. The maximum reduction in mean number of ticks in the CYM and IVM treated group was recorded from day 3 to 4, followed by complete shedding of ticks on day 5. However, deltamethrin, trichlorphon-dimethylester and fipronil showed 100% efficacy on the sixth day. The ticks in control group almost remained the same with no significant (P>0.05) changes during the experimental period.

In vitro Experiment

The results of *in vitro* efficacy trail showed that fipronil recorded 100% tick's mortality within 18th h post-treatment

Table 3. *Mean (±SD) and percentage reduction (%) in tick burden against different acaricidal treatments in goats*							
Acaricide	**Pre-treatment**	**Post-treatment Days**					
		1st	**2nd**	**3rd**	**4th**	**5th**	**6th**
Cypermethrine	117.7±4.2 (0.4)	117.3±4.1a (33.6)	75.9±1c (73.3)	18.9±3.4c (81.91)	4.4±1.4c (87.12)	0.9±0.3b (100)	
Deltamethrin	118.9±6.12 (0.7)	118.1±6.16a (21)	92.1±3.2b (67.4)	29.8±4.8bc (66.7)	8.2±0.9c (72.1)	2.1±1.1b (95.8)	0.1±0.0b (100)
Trichlorphon Dimethylester	116.7±1.81 (0.3)	116.3±1.94a (22.1)	91±9.2bc (65.6)	29.4±2.3bc (69.2)	8.5±0.83c (78.6)	1.9±0.6b (95.5)	0.2±0.0b (100)
Ivermectin	113.9±2.67 (0.3)	113.5±2.3a (22.3)	87.9±6.6b (70.8)	25.5±4.2bc (73.3)	6±1c (96.2)	0.2±0.6b (100)	
Fibronil	118±3.46 (0.8)	117±3.17a (14.6)	99.9±4b (63.6)	36±5.5b (59.4)	15.6±8.5b (90.8)	1.3±0.4b (56.7)	0.5±0.4b (100)
Control	118.1±0.63 (0.9)	117.2±1.13a (0.1)	116.3±1.42a (-0.6)	116.9±1.4a (0.2)	117.6±0.8a (0.2)	117.5±0.8a (-1.4)	118.8±0.4a (0.2)
P- Value		0.90	0.00	0.00	0.00	0.00	0.00

Parenthesis indicates the tick's mortality percentage; Mean with different letters are significantly different (P<0.05)

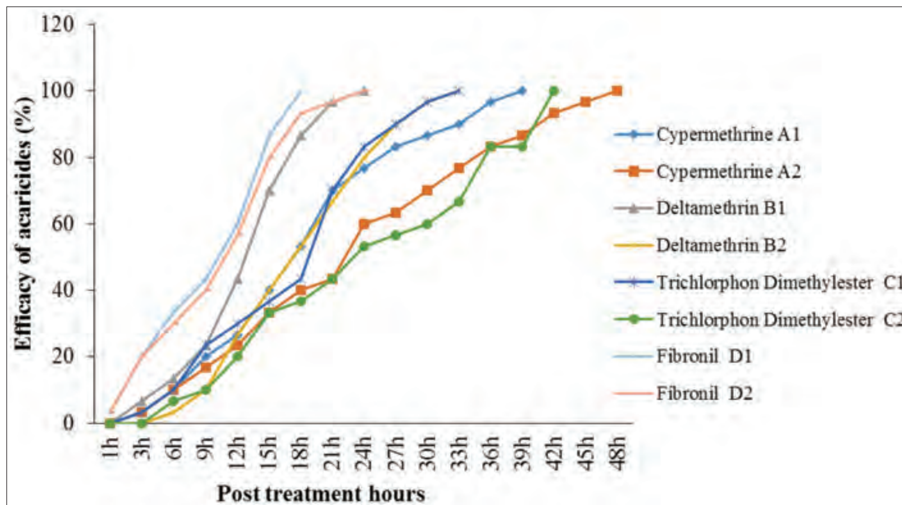

Fig 1. *In-vitro* percentage reduction of ticks on goats against two different concentrations of five commercial acaricides

with higher concentration (0.25 g/100 mL) and 24 h with lower concentration (0.2 g/100 mL). Deltamethrin, trichlorphon + dimethylester and cypermethrine were on 2nd, 3rd and 5th ranked based of their 100% efficacy within 24-33 h, 33-42 h and 39-48 h, respectively *(Fig. 1)*. However, all the four acaricides i.e., trichlorphon+ dimethylester, deltamethrin and cypermethrine showed 100% tick mortality within 48 h of post application. The cypermethrine and trichlorphon+ dimethylester treated group (A2 and C2) showed lowest efficacy, as 83.3% after 36th h.

Discussion

The application of acaricides may significantly reduce the abundance of the tick species [24] and help to mitigate the risk of tick-borne diseases [25]. However, application of acaricides may lead to development of tick resistance to several chemical compounds [26], which needs regular monitoring of acaricides. The present investigation was designed to measure the comparative efficacy of five different formulations of acaricides. The current *in vivo* trials showed 100% tick mortality with cypermethrin and ivermectin on the 5th day of post-treatment. Similar findings were recorded on larval stages of different species of ticks *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* with cypermethrin [27]. *Ixodes ricinus* showed 100% mortality at the 9th day of ivermectin post-treatment [28], while another study reported even longer period of 21 days against *R. microplus* [29]. The resistance of ivermectin against *I. ricinus* was also reported [30,31]. Comparative to the present study, a higher efficacy of cypermethrine as 50% tick's mortality was recorded within 10 min and 100% in 30 min with the dose rate of 1.0 mg/mL or 10.0 mg/m [32]. In contrast to these results, lower mortality (92% and 96.7%) was recorded with cypermethrin application on unfed female of *R. sanguineus* and engorged females, respectively [33]. The differences among the mortality rates may dependent on the dose formulation, mode of application and the type of tick species.

In current research, trichlorphon showed complete reduction of ticks on the 6th day of post-treatment. Several studies recorded lower efficacy, resistance, and reinfection to tick populations after trichlorphon treatment [29,34,35]. *In vitro* trichlorphon concentrations 2 mg/mL and 1.5 mg/mL resulted 50% ticks' mortality within 9th and 24th h and 100% at 18th and 24th h, respectively [36].

The post treatment efficacy of deltamethrin was 100% at the 6th day in the present investigation, which is not consistent with the previous findings [35,37]. Lower efficacy of 13.2%, 12.3% and 16.2% was observed at 3, 7, and 14 days of post-treatment for immature ticks, respectively [38]. Deltamethrin produced about 52.8% reduction of semi-engorged females at 3 days post-treatment and lower percentages were observed at 7 and 14-days post-treatment. The present deltamethrin trials with two concentrations i.e., 0.1 mg/mL and 0.075 mg/mL caused 50% tick's mortality in 12th and 18th h and 100% in 24th and 33rd h, respectively. A previous study on *R. microplus* and *H. anatolicum* ticks showed both susceptibility and resistance to deltamethrin [39]. The deltamethrin (0.0025) tested for *R. sanguineus* engorged female showed low sensitivity [40]. However, resistance with deltamethrin concentration of 0.1 mg/mL was 86.7% (26/30) [34] and for commercial preparation of 1.25% against *R. microplus* was 63% [41]. The possible reasons for differences in results are inconsistent experimental conditions, route of administration, formulation, sampling and analytical methods.

The fipronil *in vivo* formulation presented acaricidal efficacy of 90.8% and 100% on day 4 and 6, respectively in the current study, which agrees with the study recorded maximum efficacy (99.39%) against *R. microplus* female after nine days post-treatment [41]. The mean efficacy of fipronil at a dose of 1 mg/kg in cattle on adults, nymphs and larvae of *R. microplus* female was 74.96%, 92.24% and 80.13%, respectively [41]. Longer period of 17 days of 100% effectiveness was also recorded for fipronil against *R. sanguineus* [42]. However, a study on tick's counts of dogs calculated efficacy of fipronil on weekly basis (2nd, 4th, 8th, 12th) and were 97.6%, 93.8%, 100% respectively [43]. These difference in the effectiveness of fipronil may be dose dependent, as higher dosage caused mortality of both adults and larva of *R. microplus* [42].

The study concluded that application of the tested compounds can reduce the abundance of successive generations of four tick genera namely: *Hyalomma, Rhipicephalus, Ixodes and Haemaphysalis*, which may contribute to reduction of population of tick species. Further-more, experimentation on acaricidal efficacy testing with different formulations of other members of major acaricidal classes needs to be standardized.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author's contribution

K.A. and A.R. designed the study. A.M. performed the experiment. A.R., Z.F., M.H. advised on methods, experimentation and interpretation of findings. A.M., K.A., S.F. and A.R. conducted literature search, data analysis and manuscript preparation. K.A. and S.F. reviewed the manuscript. All authors participated in the study and concur with the submission and subsequent revisions submitted by the corresponding author.

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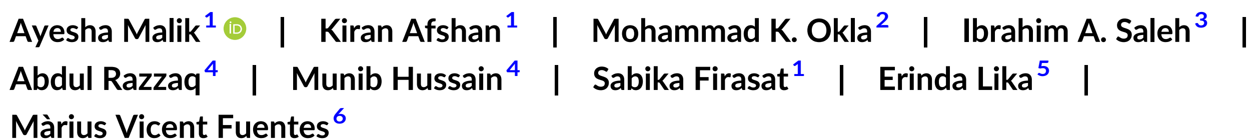
Revealing tick diversity: Chemical profiling and dynamics in scanning microscopy and molecular phylogenetics



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Abstract

This study presents a comprehensive investigation into the evolutionary trajectories of *Rhipicephalus* ticks (Ixodidae) through the interpretation of molecular phylogenetics, elucidating their chromatographic spectrum. The use of advanced chromatographic tools in this study explored the dynamics chemical profiling, providing valuable insights into the evolutionary history and ecological adaptations. Prevalence of *Rhipicephalus* ticks was 4.5% in sheep and 3.9% in goats. The ITS2 sequence of the *Rhipicephalus sanguineus* (OK642408) and *Rhipicephalus microplus* (OK642409) form a distinct clade with sequences from other countries. The 16S rRNA sequences of *R. sanguineus* (OK560870) clustered with sequences form three lineages, tropical, temperate, and south-eastern. The Cox I gene-identified *Rhipicephalus turanicus* (OK623472) and *R. microplus* (OK623463) form separate clades with sequences. The HPLC chromatogram of tick samples reveals a diverse array of identified hydrocarbons, explained the complex chemical composition of their exoskeletons. This analytical approach provides valuable insights into the specific hydrocarbon profiles, allowing for potential applications in species differentiation, ecological studies, and a deeper understanding of the functional roles played by hydrocarbon compounds in tick physiology. The findings revealed the potential of applying molecular phylogenetics tools with chromatography not only to enhance our understanding of tick evolution but also to inform strategies for disease control and management in regions where *Rhipicephalus* ticks (Ixodidae) are endemic.

Research Highlights

- Chemical mapping utilizing advanced chromatographic techniques.
- Scanning microscopic insights high-resolution scanning tool to observe structural and morphological features of ticks at a molecular level.
- Molecular phylogeny data elucidate the evolutionary relationships among tick species.

KEYWORDS

chromatogram, chromatography, hydrocarbons, Ixodidae, microscopy

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1 | INTRODUCTION

A high-performance liquid chromatography (HPLC) study on *Rhipicephalus* ticks, a genus of arthropods known for its significance as disease vectors, holds immense promise in unraveling the complex biochemical composition within these insects. HPLC, with its precision in separating and analyzing compounds, offers a powerful tool to identify and quantify various molecular components present in *Rhipicephalus* ticks (Pažoutová et al., 2013). This analytical method can reveal the existence of bioactive metabolites biomarkers, providing useful information about the ticks ecophysiological characteristics. HPLC study may contribute to our understanding of the molecular basis of host-pathogen interactions and guide the development of targeted strategies for controlling tick-borne diseases, thereby enhancing our overall knowledge of these medically and veterinary relevant insects (Magni et al., 2018).

Worldwide, ticks are associated with significant medical and veterinary challenges (Brites-Neto et al., 2015), which cause tissue injury, body paralysis, and anemia in heavy infestation (Hurtado & Giraldo-Ríos, 2018) and transmit numerous pathogenic microorganisms including bacteria, fungi, protozoa, rickettsia, spirochetes, and viruses (Anderson & Magnarelli, 2008). The economic losses related to the tick burden have been estimated to be US $498.7 million annually in Asian countries (Minjauw & McLeod, 2003). Arthropods have evolved a wide range of defensive systems to combat microbial diseases. Antimicrobial substances, lysozymes, protease inhibitors, and lectins are the active chemicals identified among tick species (Bouchon et al., 2016). Ticks, belonging to the suborder Ixodida, are infamous ectoparasites and carriers of human and animal pathogens, surpassing other blood-feeding arthropods in the transmission of a wide range of infectious agents. The tick parasites inflict considerable harm on their hosts, causing significant physical damage such as blood loss and toxicosis (Gulia-Nuss et al., 2016).

Livestock plays a central role in the economy of Pakistan, making up 55.4% of the total value of the farm sector, respectively, adding 11.9% to the gross domestic product of the country. In Pakistan, 30–50 million people are directly involved in livestock rearing as supplementary work, and each farming family owns 5–6 sheep/goats. A total of 31.2 million sheep and 78.2 million goats are present in the country (Ashfaq et al., 2014). Livestock raising has provided food security, a source of funding, and a position of privilege. Tick and tick-borne ailments have a significant influence on the country's cattle sector due to a lack of veterinary care and poor facilities (Nieto et al., 2012). Recently, six ixodid ticks (*Rhipicephalus haemaphysaloides*, *Rhipicephalus microplus*, *Rhipicephalus turanicus*, *Haemaphysalis* (*Hs.*) *punctata*, *Hs. sulcata*, and *Hyalomma anatolicum*) were reported from small ruminants based on molecular characterization from the federally administered tribal area. Therefore, it is desirable to also use a molecular approach to determine the diversity of ticks infesting small ruminants in the other agroecological zones of Pakistan (Ghafar, Khan, et al., 2020).

The morphological characters are frequently used for tick identification (Nava et al., 2009). However, the morpho-taxonomical differentiation has been challenged in the past few years on the identification of *R. microplus* because of the existence of its four closely related taxa; *Rhipicephalus australis*, *Rhipicephalus annulatus*, *R. microplus* in clade A and *R. microplus* in clade B (Barker & Walker, 2014). Molecular approaches are the most ideal methods to study species identity and diversity in *Rhipicephalus* ticks (Burger et al., 2014). The molecular studies confirmed that 16S rRNA and mitochondrial cytochrome oxidase I (Cox I) and the second internal transcribed spacer (ITS2) of the nuclear ribosomal genes are powerful genetic markers to define species identification, interspecies variations, and phylogenetic relationship (Low et al., 2015). Genetic characterization also provides as an alternative approach for species differentiation. Recent phylogenetic analysis of mitochondrial genome sequences has revealed the presence of a species complex within *R. microplus*. This complex encompasses *R. annulatus*, *R. australis*, and two distinct clades of *R. microplus*: clade A, or *Rhipicephalus microplus*, which includes ticks originating from South America, Africa, and Southeast Asia, and clade B, comprising ticks from China and Northern India. Notably, in clade B are more closely related to *R. annulatus* than to *R. microplus* (Roy et al., 2018).

The scanning electron microscopy (SEM) is an advanced type of electron beam microscope applied for the examination of surface stratification about tissues, intracellular surfaces, isolated organelles, and molecular structures such as individual proteins, viruses, nucleoprotein complexes, and nucleic acids (Abdel-Shafy et al., 2019). The growing demands for high-resolution SEM featured field emission electron sources (FE-SEMs), it has become possible to image molecular organization details at fine resolutions (0.4 nm), a proximity to the capabilities of transmission electron microscopes, depending upon the sample and its preparation methods.

The overlapping of scanning morphological traits of *Rhipicephalus* ticks within the Ixodidae family serves as a fundamental precursor on genetic variants and chromatographic studies. The understanding of comparative genetic morphological similarities among *Rhipicephalus* ticks is essential to analyze their complex evolutionary history and ecological adaptations. However, chromatographic separations can provide valuable information on the biochemical elements present in ticks shedding light on potential bioactive metabolites and their roles in host–parasite interactions. The integration of genetic variant analysis and chromatography not only offers a holistic perspective on the molecular landscape of *Rhipicephalus* ticks but also holds significant implications for disease transmission dynamics and vector control strategies. This interdisciplinary approach is justified by its potential to reveal novel insights into the intricate relationship between genetics, morphology, and chemical composition, fostering a more nuanced understanding of these vectors and opening avenues for targeted research in public health and veterinary sciences.

This study aims to elucidate the genetic variants within *Rhipicephalus* ticks (Ixodidae) using scanning microscopic morphological traits providing insights into the species' genetic diversity and potential implications for their ecological adaptation. Furthermore, the investigation seeks to characterize the tick chemical composition using HPLC to better recognize their molecular mechanisms and their physiological and ecological functions.

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2 | MATERIALS AND METHODS

2.1 | Study area topography

The study area comprised three ecological zones of Pakistan: Punjab (Lahore, Jhelum, Attock, and Rawalpindi), Baluchistan (Quetta), and Azad Kashmir (Muzaffarabad) (Figure 1). These ecological regions have diverse climates: tropical, semiarid, hot, and subtropical monsoon type in Punjab, continental semiarid in Quetta, and humid subtropical climate in Muzaffarabad. The rainfall pattern varies markedly across the regions.

2.2 | Tick collection

A total of 4488 animals (2184 sheep and 2304 goats) were examined for tick infestation from April to August 2019. The animals were managed under natural grazing and stall-feed. Five farms were randomly selected from each sampling site and visited twice a month, all animals (sheep and goats) at the farms were physically examined for the presence of ticks. Relevant information regarding tick infestation, age, breed, sex, and site of infestation was recorded at the time of sampling.

The tick collection protocol required inspection of the head area with special attention given to the ears, thorough examination of the neck-chest area and legs. Selected ticks were removed using a tweezers, ensuring that the mouthparts remained intact and attached to the tick. In followed experimentation, a maximum of five ticks per animal were gathered for permanent slide preparation and DNA extraction. These ticks were then placed in separate tubes containing 70% ethanol, and each tube was appropriately labeled with the animal code and the date of collection (Kirvar et al., 2000).

2.3 | Morphological identification

Ticks were morphologically identified ($n = 450$) using dichotomous keys (Walker, 2003). The standard morphological measurements are presented in mean and standard error values. All ticks were carefully examined, and characters were noted for taxonomical variation of ticks as previously described (Beati & Keirans, 2001).

2.4 | PCR amplification, ribosomal and mitochondrial DNA sequence analysis

In this protocol, only up to four adult ticks, morphologically identified as belonging to the *Rhipicephalus* genus, were selected from infected sheep ($n = 25$ ticks) and goats ($n = 24$ ticks) from each of the agro-ecological study zones and processed for molecular identification. Genomic DNA was extracted from a total of 49 ticks (male = 22 and female = 27) using a commercial DNA extraction kit (Bio-Rad Lab, Inc. USA) according to the manufacturer instructions. The second internal transcribed spacer (ITS2) of rDNA and two mitochondrial loci (Cox I

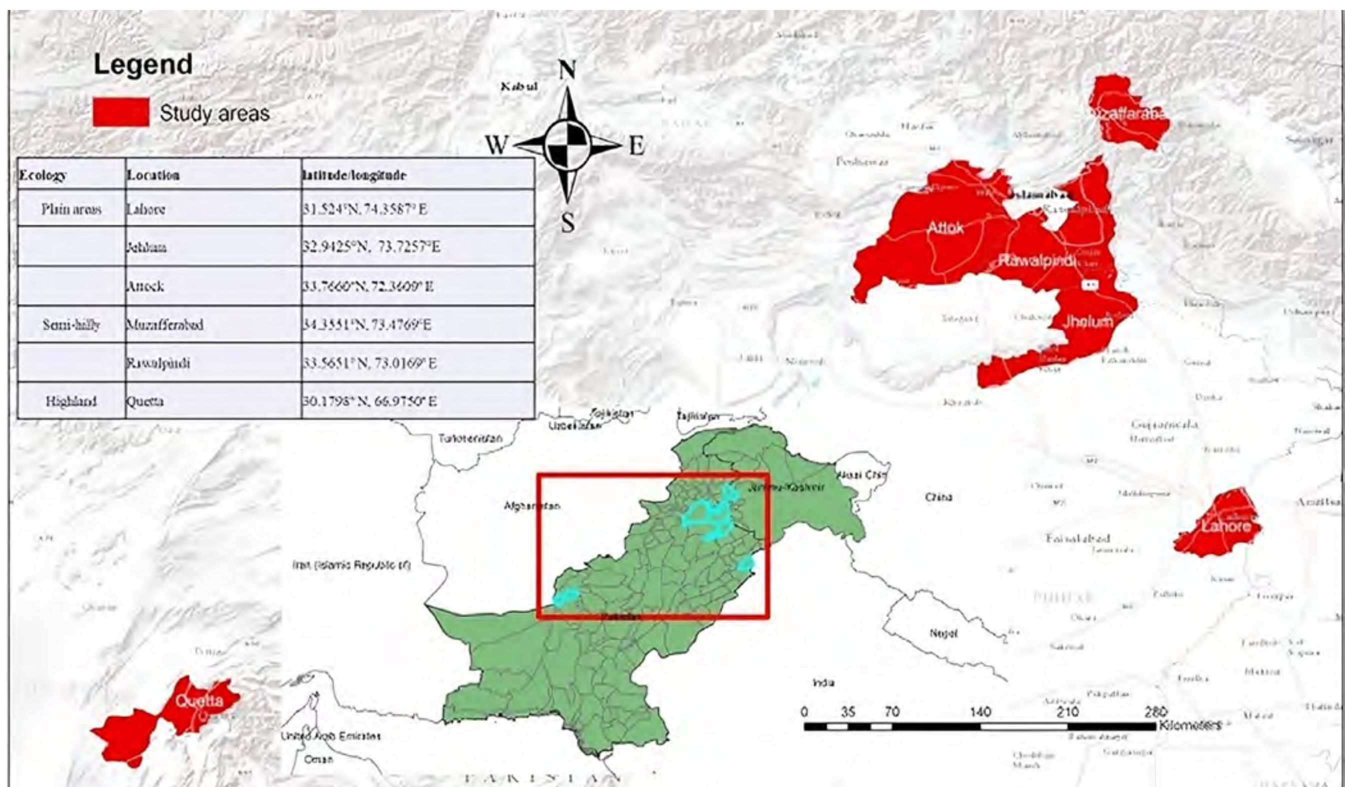

FIGURE 1 Map of Pakistan indicating six tick sampling areas within three agroecological zones.

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TABLE 1 Primer sequences for the amplification of *Rhipicephalus* species ITS2, 16S, and Cox1 regions.

Primer name	Sequences (5′-3′)	Annealing temperature	References
ITS2-F	CGGATCACATATCAAGAGAG	59°C	Csordas et al., 2016
ITS2-R	CCCAACTGGAGTGGCCCAGTTT		
16S-F	CTGCTCAATGAATATTTAAATTGC	61°C	Black and Piesman, 1994
16S-R	CGGTCTAAACTCAGATCATGTAGG		
Cox1-F	CTTCAGCCATTTTACCGCGA	56°C	Csordas et al., 2016
Cox1-R	CTCCGCCTGAAGGGTCAAA		

and 16S) were amplified using previously reported primers (Table 1). The 25 μL PCR reaction mixtures preparation, amplicon purification, and sequencing were performed (Khan et al., 2021). Multiple sequence alignment was performed with CLUSTALW (https://www.genome.jp/tools-bin/clustalw), sequences were edited and submitted to GenBank, and accession numbers were obtained.

2.5 | Molecular phylogeny of rDNA (ITS2) and mitochondrial loci (16S and Cox I)

The molecular phylogeny of ITS2 and Cox I sequence data was inferred by using the maximum likelihood method and the Kimura 2-parameter model (Kimura, 1980), while the Hasegawa-Kishino-Yano model was used for 16S sequence data (Hasegawa et al., 1985). The trees with the highest log likelihood (ITS2 [−1011.33], Cox I [−1547.30], 16S [−1251.19]) are shown. The bootstrap test (1000 replicates) values were clustered; the associated taxa are presented next to branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with a superior log-likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites for ITS2 (4 categories [+G, parameter = 13.9487]), 16S (4 categories [+G, parameter = 2.0585]), and Cox 1 (4 categories [+G, parameter = 0.2590]) sequence data. Complete deletion options were used to remove gaps and missing data. The sequences used in comparison and out groups (*Haemaphysalis longicornis* [EU604103]; *Argas persicus* [MK555333; FN394341]) to construct trees were downloaded from the NCBI GenBank database. Evolutionary relationship was conducted in MEGA X (Kumar et al., 2018).

2.6 | Scanning electron microscopy

The protocol for SEM identification of tick species involves several key steps. First, collected tick specimens are fixed in a suitable solution, such as buffered formalin, to preserve their morphological features. Thereafter, the specimens undergo a series of dehydration steps using various concentrations of ethanol. After dehydration, critical point drying is performed to ensure the removal of residual water from the specimens. The dried ticks are then mounted on SEM stubs using a conductive adhesive and coated with a thin layer of gold or gold–palladium alloy to enhance surface conductivity. Finally, the prepared specimens are examined under the SEM at appropriate magnifications for detailed visualization (Kwak, 2017).

2.7 | High performance liquid chromatography

The HPLC method for analyzing tick body parts species involves initial preparation of homogenized samples from distinct anatomical regions. The collected tick specimens are dissected, and specific body parts, such as the midgut or salivary glands, are isolated. Subsequently, the isolated tissues are homogenized. Extraction of target compounds is carried out using an appropriate solvent system, followed by filtration to remove particulate matter. The resulting extract is then injected into the HPLC system equipped with a suitable column and mobile phase (Amer et al., 2023). The chromatographic separation is performed, and detection is achieved through UV or fluorescence detectors, depending on the nature of the compounds being analyzed. This HPLC protocol enables the identification and quantification of specific chemical components within different tick body parts, contributing to a comprehensive understanding of the species physiological and biochemical characteristics (Boulghobra et al., 2022).

2.8 | Statistical analysis

The prevalence of ticks and their relationship with animal breed, age, and sex were investigated using the chi-square test (SPSS 20.0 statistical software). The *t*-test statistic was applied to find the differences among morphometric measurements of the *Rhipicephalus* group. Significance was defined as $I \leq 0.05$.

3 | RESULTS

3.1 | Prevalence of ticks

A total of 2184 sheep and 2304 goats were examined, among which 514 (23.5%) sheep and 462 (20.1%) goats were infested with ticks (Table 2). All ticks collected were identified as adults, males and

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TABLE 2 Prevalence and tick infestation rate in sheep and goats with respect to geographical location, host, sex, age, and breed.

	Number of animals			Ticks infestation				
Characteristics	Examined	Infested	Prevalence (%)	*Hyalomma*	*Rhipicephalus*	*Haemaphysalis*	*Ixodes*	Chi-square
Sheep sites								
Lahore	384	60	15.6	33	13	4	6	$\chi^2 = 7.22$, $p = .99$* NS
Jhelum	384	154	40.1	106	29	9	12	
Attock	384	110	28.7	71	20	13	9	
Muzaffarabad	264	124	46.97	77	23	11	13	
Rawalpindi	384	27	7.0	13	5	3	4	
Quetta	384	39	10.2	22	8	6	4	
Goats sites								
Lahore	384	49	12.8	27	13	6	5	$\chi^2 = 14.6$, $p = .79$* NS
Jhelum	384	146	38.0	65	43	25	12	
Attock	384	112	29.2	55	9	15	18	
Muzaffarabad	384	103	26.8	67	16	15	15	
Rawalpindi	384	20	5.2	14	4	3	2	
Quetta	384	32	8.3	25	4	1	3	
Host (type)								
Sheep	2184	514	23.5	322	98	46	48	$\chi^2 = 0.82$; $p = .93$* NS
Goats	2304	462	20.1	253	89	65	55	
Age (years)								
Sheep								
1 year and below	1092	281	25.7	181	50	20	26	$\chi^2 = 3.35$, $p = .99$* NS
Above 1 year	1092	233	21.3	141	48	26	22	
Goats								
1 year and below	1152	240	20.8	135	45	40	21	$\chi^2 = 3.35$, $p = .99$* NS
Above 1 year	1152	222	19.3	118	44	25	34	
Sex								
Sheep								
Male	1092	266	24.4	160	46	27	23	$\chi^2 = 4.02$, $p = .94$* NS
Female	1092	248	22.7	162	52	19	25	
Goats								
Male	1152	197	17.1	121	22	34	20	$\chi^2 = 4.02$, $p = .94$* NS
Female	1152	265	23.0	132	67	31	35	
Breeds								
Sheep								
Balochi	192	34	17.7	32	4	9	6	$\chi^2 = 21.6$, $p = .79$* NS
Balkhi	708	192	27.1	127	40	13	19	
Shinwar-white	384	132	34.4	71	20	9	9	
Non-descriptive	900	156	17.3	92	34	15	14	
Goats								
Beetal	896	217	24.2	129	41	38	29	$\chi^2 = 21.6$, $p = .79$* NS
Lehri	192	14	7.3	11	2	1	2	
Taddy	128	38	29.7	20	5	6	5	
Non-descriptive	1088	193	17.7	93	41	20	19	

*NS, not significant ($p > .05$).

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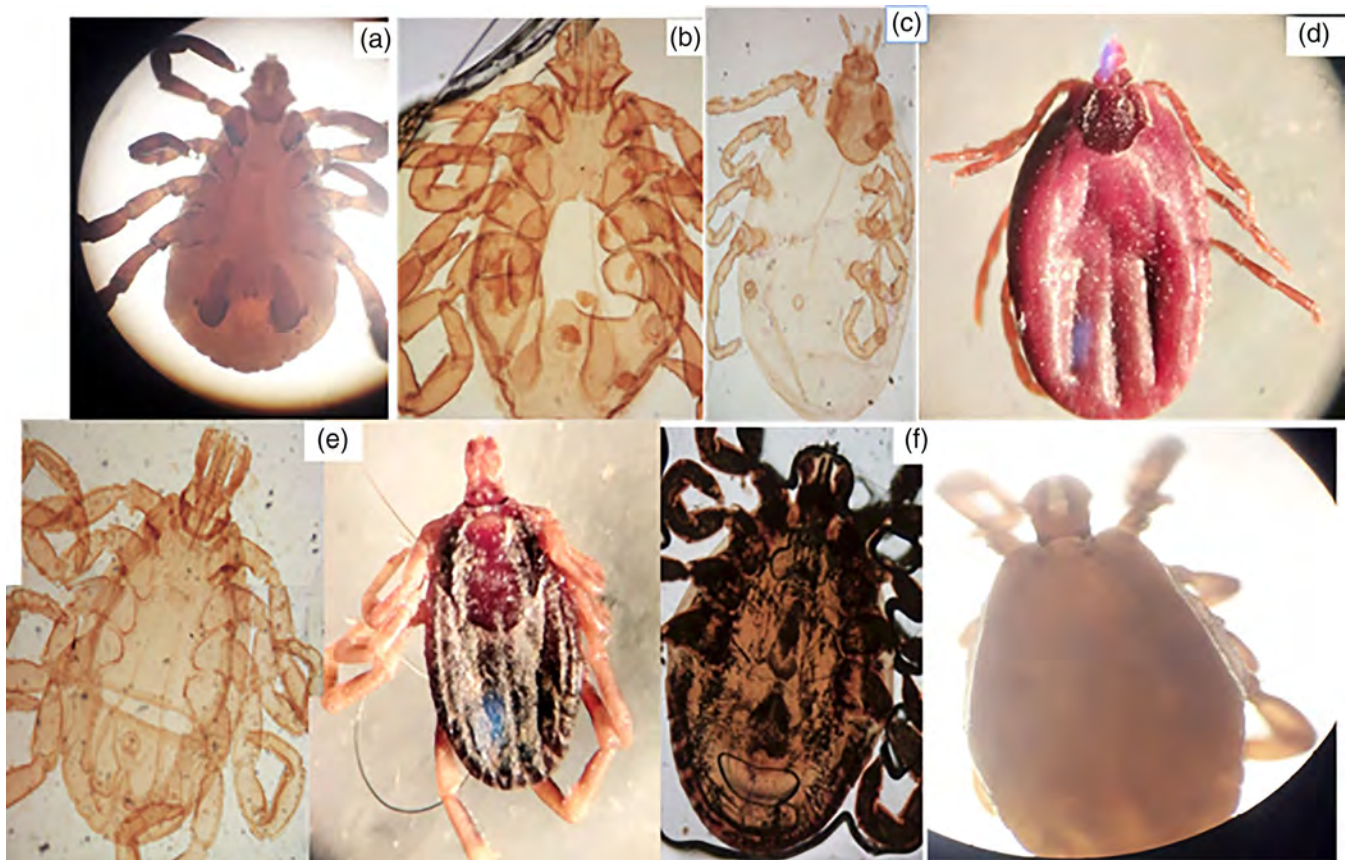

FIGURE 2 Ventral view of (a) *Rhipicephalus sanguineus*, (b) *Rhipicephalus turanicus*, and (c) *Rhipicephalus microplus*. (d) Dorsal view of *Ixodes*. (e) *Hyalomma* ventral and dorsal view. (f) *Haemaphysalis* ventral and dorsal view.

females, or as immature stages belonging to four tick genera: *Rhipicephalus*, *Hyalomma*, *Haemaphysalis*, and *Ixodes*. Their prevalence in sheep and goats was recorded. Tick prevalence was slightly higher in males 23.4% (266/1092) when compared with females 22.7% (248/1092) in sheep, whereas in goats, females had a higher prevalence, 23.0% (265/1152), than males, 17.1% (197/1152). Tick prevalence was similar across the study zones ($p = .79$), with sheep in Muzaffarabad showing a slightly higher prevalence (47.0%; 124/264); and goats in Jhelum showing a somewhat lower prevalence (38.0%; 146/384). The prevalence was higher in sheep and goats younger than 1 year (25.7% and 20.8%, respectively) and did not vary statistically ($p = .99$) from sheep and goats older than 1 year. Finally, the Shinwar-White sheep breed 34.4% (132/384) and Taddy goats 29.7% (38/128) were noticeably more infested with ticks when compared with other sheep and goat breeds; however, the difference was not significant ($p = .79$).

3.2 | Morphological identification

The prevalence of *Hyalomma* species was the highest recorded at 14.7% and 11.0%, followed by *Rhipicephalus* 4.5% and 3.9%, *Haemaphysalis* 2.1% and 2.8%, and *Ixodes* 2.2% and 2.4% in sheep and goats, respectively (Figure 2). Tick specimens from each species were morphologically measured for data analysis (Table 3). Intraspecific morphological variations among *Rhipicephalus* species were apparent, mainly in terms of size, scutal pattern, female genital opening, spiracular plate length, and male adanal plate. Regarding morphological parameters, only the basis capituli length/width ratio of *R. sanguineus* was significantly ($p = .001$) different between *R. turanicus* and *R. microplus*. The length/width ratio of the male *R. sanguineus* was significantly ($p = 0.001$) different from male *R. microplus*.

3.3 | Ticks molecular identification

The ITS2 rDNA sequences of each of the 49 ticks (male = 22 and female = 27) were aligned for intraspecific variations. The phylogenetic analysis is based on ITS2 sequences generated in this study along with 20 available sequences from GenBank (Figure 3). Ticks identified herein as *R. sanguineus* Pakistan (OK642408) and *R. microplus* Pakistan (OK642409) formed a distinct subclade. The identified species blast results showed 88.19%–89.76% identity with reference *R. sanguineus* and 97.3%–97.8% similarity with reference *R. microplus* sequences from different countries.

Phylogenetic analysis of the 16S sequences data set involved 31 reference nucleotide sequences from the NCBI GenBank database (Figure 4). The 16S *R. turanicus* (OK560873) sequences acquired in this study were clustered into clade II, which included three sequences from Afghanistan, Kyrgyzstan, and Israel. It revealed

TABLE 3 Morphological measurements of *Rhipicephalus* ticks presented in millimeters (mm).

Characters	*Rhipicephalus sanguineus*		*Rhipicephalus turanicus*	*Rhipicephalus microplus*	
	Male ($n = 50$)	Female ($n = 50$)	Male ($n = 50$)	Male ($n = 50$)	Female ($n = 50$)
Body L	3.66 ± 0.07	3.85 ± 0.09	3.85 ± 0.19	5.2 ± 0.0	4.42 ± 0.15
Body W	1.82 ± 0.03	1.90 ± 0.13	1.81 ± 0.20	3.52 ± 0.0	2.82 ± 0.11
Ratio BL/BW	2.01	2.03	2.13	1.48	1.57
Adanal plates L	0.81 ± 0.03	0.82 ± 0.06	0.93 ± 0.06	1.1 ± 0.0	0.81 ± 0.04
Adanal plates W	0.30 ± 0.01	0.33 ± 0.04	0.57 ± 0.20	0.97 ± 0.01	0.399 ± 0.04
Basis capituli L	0.36 ± 0.05	0.39 ± 0.16	0.26 ± 0.04	0.33 ± 0.02	0.28 ± 0.02
Basis capituli W	0.59 ± 0.07	0.51 ± 0.10	0.75 ± 0.07	0.99 ± 0.01	0.59 ± 0.05
Ratio BCL/BCW	0.61	0.76	0.35	0.33	0.47
Distance between porose areas	0.26 ± 0.01	0.35 ± 0.05	0.23 ± 0.02	0.29 ± 0.0	0.18 ± 0.011
Idiosoma L	2.99 ± 0.09	3.09 ± 0.06	3.23 ± 0.17	4.9 ± 0.0	3.48 ± 0.16
Idiosoma W	1.80 ± 0.04	1.90 ± 0.13	1.81 ± 0.20	3.52 ± 0.02	2.82 ± 0.11
Palp L	0.31 ± 0.007	0.35 ± 0.02	0.29 ± 0.03	0.46 ± 0.01	0.32 ± 0.01
Palp W	0.15 ± 0.01	0.13 ± 0.006	0.18 ± 0.002	0.16 ± 0.00	0.15 ± 0.008
Spiracular area L	0.31 ± 0.04	0.40 ± 0.10	0.38 ± 0.08	0.35 ± 0.0	0.28 ± 0.01
Spiracular area W	0.20 ± 0.02	0.20 ± 0.009	0.14 ± 0.01	0.26 ± 0.00	0.18 ± 0.005
Posterior grooves L	0.54 ± 0.03	0.47 ± 0.04	0.68 ± 0.02	0.86 ± 0.0	0.57 ± 0.03
Posterior grooves W	0.23 ± 0.02	0.37 ± 0.10	0.37 ± 0.03	0.46 ± 0.0	0.32 ± 0.02
Accessory adanal shield L	0.35 ± 0.04	0.32 ± 0.02	0.35 ± 0.03	0.45 ± 0.0	0.25 ± 0.008
Accessory adanal shield W	0.17 ± 0.02	0.15 ± 0.02	0.19 ± 0.02	0.21 ± 1.0	0.16 ± 0.005
Genital aperture L	0.19 ± 0.01	0.16 ± 0.02	0.22 ± 0.01	0.25 ± 0.0	0.18 ± 0.005
Genital aperture W	0.17 ± 0.01	0.15 ± 0.02	0.17 ± 0.01	0.26 ± 0.0	0.15 ± 0.006
Palp article I L	0.09 ± 0.009	0.13 ± 0.03	0.07 ± 0.008	0.13 ± 0.01	0.09 ± 0.004
Palp article I W	0.10 ± 0.007	0.11 ± 0.008	0.08 ± 0.004	0.10 ± 0.01	0.06 ± 0.005
Palp article II L	0.09 ± 0.004	0.10 ± 0.003	0.09 ± 0.005	0.16 ± 0.00	0.11 ± 0.004
Palp article II W	0.11 ± 0.008	0.13 ± 0.004	0.014 ± 0.02	0.13 ± 0.01	0.09 ± 0.005
Palp article III L	0.11 ± 0.01	0.10 ± 0.01	0.14 ± 0.02	0.190 ± 0.0	0.11 ± 0.006
Palp article III W	0.10 ± 0.007	0.09 ± 0.01	0.11 ± 0.02	0.11 ± 0.00	0.07 ± 0.003
Scutum L	2.73 ± 0.20	2.25 ± 0.41	3.17 ± 0.17	2.15 ± 0.05	0.72 ± 0.024
Scutum W	1.76 ± 0.06	1.60 ± 0.03	1.81 ± 0.20	2.10 ± 0.0	0.80 ± 0.012
Ratio SL/SW	1.55	1.41	1.75	1.02	0.90
Hypostome L	0.74 ± 0.04	0.85 ± 0.03	0.94 ± 0.05	0.94 ± 0.00	0.52 ± 0.043
Hypostome W	0.18 ± 0.01	0.19 ± 0.003	0.22 ± 0.02	0.42 ± 0.00	0.18 ± 0.014
Coxa I L	0.44 ± 0.02	0.38 ± 0.08	0.50 ± 0.01	0.98 ± 0.02	0.43 ± 0.015
Coxa I W	0.36 ± 0.03	0.44 ± 0.03	0.30 ± 0.03	0.75 ± 0.0	0.26 ± 0.014
Coxa II L	0.50 ± 0.02	0.44 ± 0.05	0.63 ± 0.02	1.15 ± 0.05	0.47 ± 0.023
Coxa II W	0.42 ± 0.02	0.51 ± 0.06	0.35 ± 0.02	1.35 ± 1.5	0.39 ± 0.023
Coxa III L	0.43 ± 0.02	0.41 ± 0.04	0.48 ± 0.02	0.91 ± 0.0	0.41 ± 0.027
Coxa III W	0.48 ± 0.03	0.58 ± 0.02	0.36 ± 0.03	0.85 ± 0.0	0.37 ± 0.021
Coxa IV L	0.41 ± 0.01	0.41 ± 0.007	0.51 ± 0.02	0.96 ± 0.0	0.40 ± 0.020
Coxa IV W	0.50 ± 0.03	0.60 ± 0.03	0.33 ± 0.05	0.65 ± 0.0	0.35 ± 0.015
Cornua L	0.28 ± 0.08	0.47 ± 0.21	0.20 ± 0.007	0.35 ± 0.00	0.15 ± 0.009
Cornua W	0.13 ± 0.009	0.30 ± 0.16	0.20 ± 0.006	0.22 ± 0.0	0.215 ± 0.03
Tibia L	0.66 ± 0.03	0.66. ± 0.09	0.73 ± 0.02	0.69 ± 0.0	0.35 ± 0.018
Tibia W	0.26 ± 0.01	0.26 ± 0.02	0.25 ± 0.02	0.22 ± 0.01	0.146 ± 0.009
Tarsus L	0.58 ± 0.04	0.69 ± 0.09	0.63 ± 0.03	0.87 ± 0.02	0.38 ± 0.016

(Continues)

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TABLE 3 (Continued)

	Rhipicephalus sanguineus		*Rhipicephalus turanicus*	*Rhipicephalus microplus*	
Characters	Male ($n = 50$)	Female ($n = 50$)	Male ($n = 50$)	Male ($n = 50$)	Female ($n = 50$)
Tarsus W	0.22 ± 0.03	0.15 ± 0.01	0.20 ± 0.01	0.18 ± 0.0	0.139 ± 0.007
Anal groove L	1.00 ± 0.03	0.82 ± 0.21	0.91 ± 0.05	0.98 ± 0.02	0.675 ± 0.064
Anal groove W	0.47 ± 0.04	0.70 ± 0.17	0.38 ± 0.03	0.69 ± 0.0	0.469 ± 0.032
Anus L	0.18 ± 0.01	0.26 ± 0.06	0.22 ± 0.01	0.22 ± 0.00	0.174 ± 0.004
Anus W	0.19 ± 0.01	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.00	0.16. ± 0.007

Abbreviations: L, length; W, width.

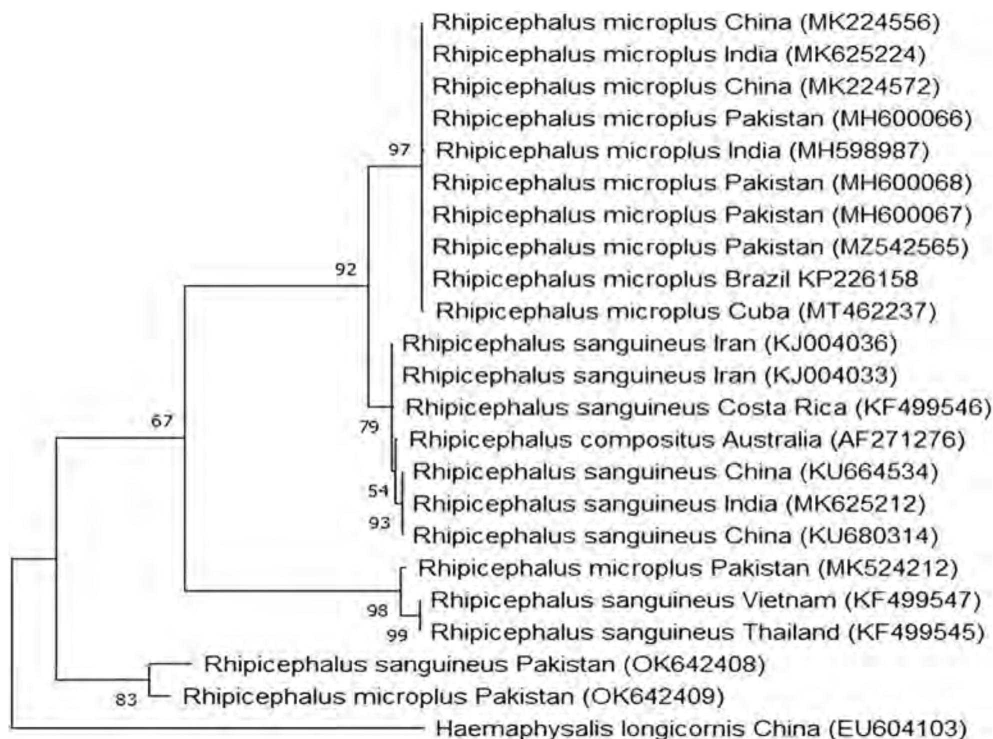

FIGURE 3 Phylogenetic relationships of partial sequences of the ITS2 of the nuclear ribosomal DNA of *Rhipicephalus microplus* and *Rhipicephalus sanguineus* ticks.

98.5%–99.3% similarity with sequences from Pakistan and Afghanistan. The identified *R. sanguineus* (OK560870) was in clade II along with closely related species from China, France, Israel, Greece, and Romania. Blast results showed a 94.4%–98.2% similarity to *R. sanguineus* from other countries.

The Cox I sequence of sufficient quality was generated for 34 tick samples. Cox I sequence was aligned with 12 nucleotide sequences available on the public NCBI GenBank database. The resulting phylogram based on the Cox I gene identified the *R. turanicus* (OK623472) and clustered it in clade with sequence from Pakistan, China, and Kazakhstan (Figure 5). The *R. microplus* (OK623463) clustered with sequences from China, Bangladesh, Pakistan, and India.

3.4 | Scanning microscopic identification of ticks exoskeleton

The scanning electron microscopic identification of *Rhipicephalus* ticks exoskeletons (Ixodidae) revealed intricate details crucial for accurate species discrimination (Figure 6). High-resolution images captured under SEM distinctive morphological features, such as the scutum shape, festoons along the posterior margin, and the spiracular plate. The meticulous examination of structural elements allowed for a precise identification of the *Rhipicephalus* species under study. Furthermore, the SEM images provided valuable insights into the fine surface topography, enabling a detailed analysis of setae distribution and other minute structures. This approach not only facilitated the variation among closely related species within the *Rhipicephalus* genus but also contributed to a comprehensive understanding of the exoskeletal characteristics specific to each identified tick species.

SEM findings coupled with a comparison with existing taxonomic literature and databases, strengthened the validity of the identification process. This study emphasizes the significance of using advanced microscopy techniques for arthropod taxonomy, particularly in distinguishing closely related species with prominent morphological differences. The detailed SEM documentation serves as a valuable resource for future research implications in tick identification, contributing to a more refined systematic biodiversity within the Ixodidae

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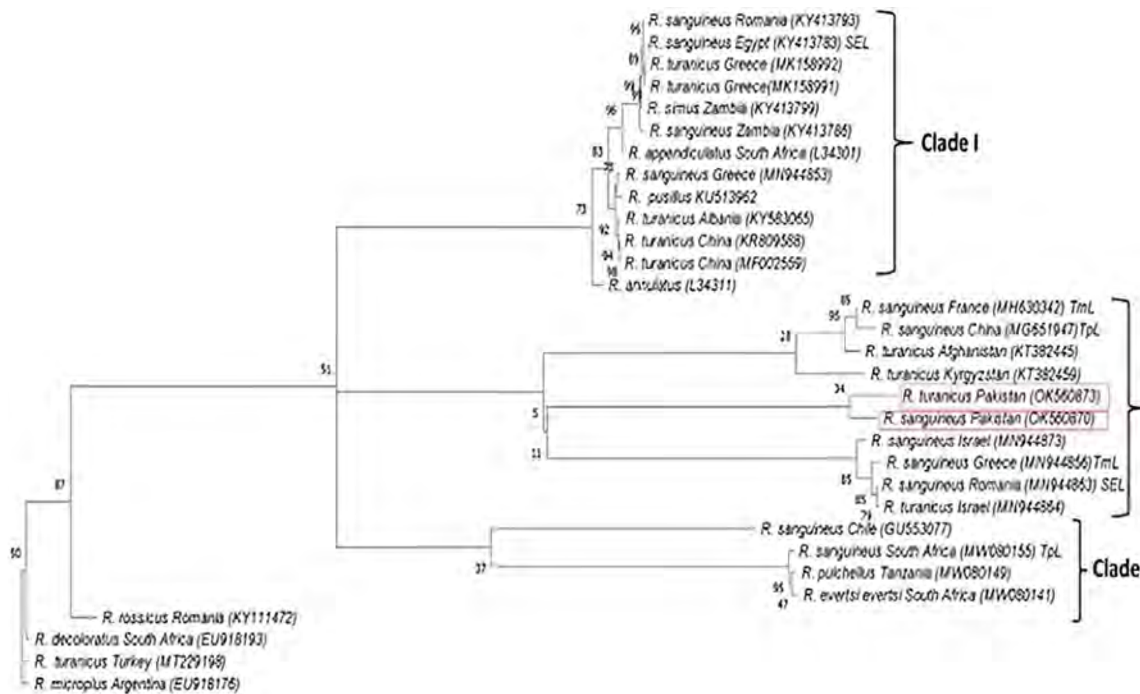

FIGURE 4 Phylogenetic relationships of partial 16S rRNA sequences of *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* ticks.

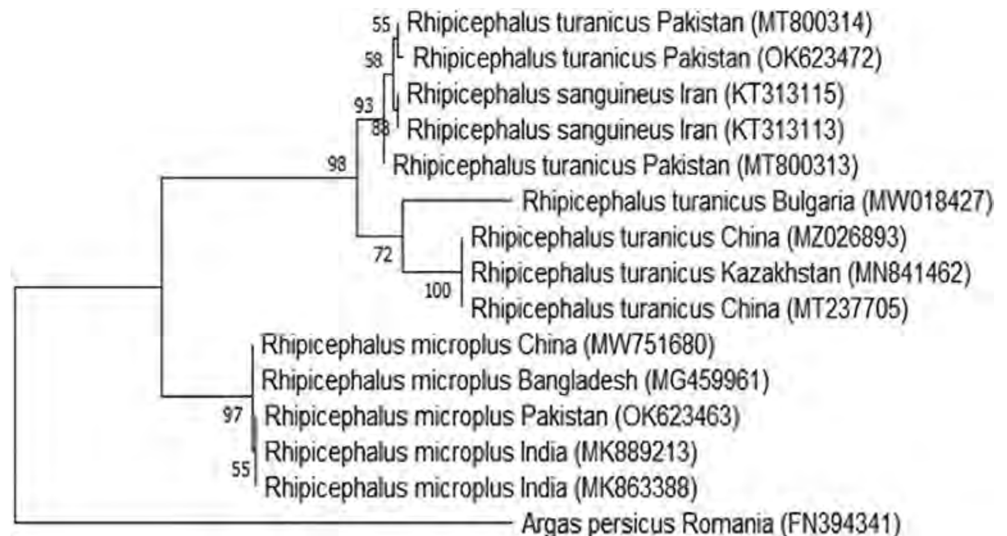

FIGURE 5 Phylogenetic relationships of partial Cox I sequence of *Rhipicephalus microplus* and *Rhipicephalus turanicus*.

family. Moreover, these outcomes provide a foundation for collaborative efforts with experts in entomology and tick taxonomy, ensuring the accuracy and reliability in the identification of *Rhipicephalus* species.

3.5 | HPLC chromatogram description of ticks exoskeleton

The HPLC chromatogram analysis of tick exoskeleton provides valuable information into the chemical composition of arthropod structures. The chromatogram described prominent peaks conforming the identified cuticular hydrocarbons present in the exoskeleton (Figure 7 and Table 4). The separation and identification of organic hydrocarbon compounds are crucial to explain the biochemical composition and potential bioactive substances within the tick exoskeleton. Peaks on the chromatogram represent specific molecules, 3-methylheptadecane, 3-methylpentatriacontane, tritriacontane, dotriacontane, 4,8-dimethyl-1,3,7-nonatriene, and octacosane each contributing to the overall functionality of the exoskeleton. The retention peak times offer information about the relative abundance, and concentration of biochemical compounds explained the exoskeleton microstructural and physiological traits.

Furthermore, the HPLC chromatogram allows for the comparison of exoskeletal compositions across different tick species or

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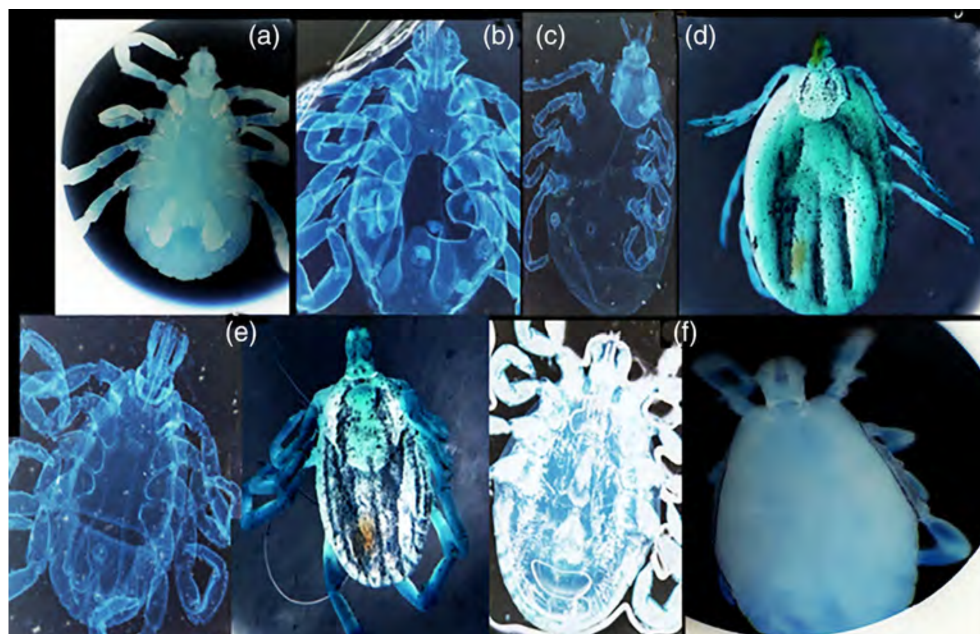

FIGURE 6 Scanning microscopic surface-visualized images: ventral view of (a) *Rhipicephalus sanguineus*, (b) *Rhipicephalus turanicus*, and (c) *Rhipicephalus microplus*; (d) dorsal view of *Ixodes*; (e) *Hyalomma* ventral and dorsal view; (f) *Haemaphysalis* ventral and dorsal view.

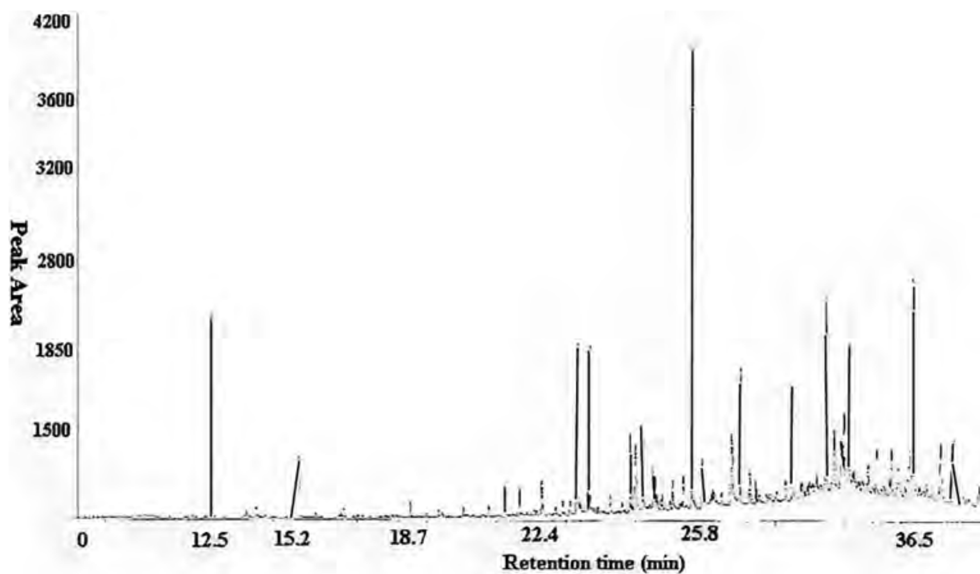

FIGURE 7 Revealing the HPLC chromatogram showcasing the detected peaks of bioactive hydrocarbons in *Rhipicephalus* species. HPLC, high-performance liquid chromatography.

TABLE 4 HPLC chromatographic peaks identified cuticular hydrocarbons in *Rhipicephalus* ticks species.

				Rhipicephalus sanguineus	*Rhipicephalus turanicus*	*Rhipicephalus microplus*
Hydrocarbons	Chemical formula	PubChem CID	Molecular weight (g/mol)	RT (min)	Peak area	Concentration (mg/mL)
3-Methylheptadecane	$C_{18}H_{38}$	94321	254.5	12.5	3200	0.25
3-Methylpentatriacontane	$C_{36}H_{74}$	518892	506.97	36.5	3600	0.28
Tritriacontane	$C_{33}H_{68}$	12411	464.9	15.2	1850	0.15
Dotriacontane	$C_{32}H_{66}$	11008	450.9	18.7	4200	0.3
4,8-Dimethyl-1,3,7-nonatriene	$C_{11}H_{18}$	12589924	150.26	22.4	1500	0.1
Octacosane	$C_{28}H5_{8}$	12408	394.8	25.8	2800	0.2

Abbreviation: RT, retention time.

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developmental stages. Variations in peak patterns and retention times may indicate species-specific differences or developmental changes in the exoskeleton. This analytical technique serves as a powerful tool for researchers studying tick biology, ecology, and physiology, providing a detailed chemical profile that complements traditional morphological analysis. The HPLC chromatogram in conjunction with other molecular and morphological data contributes to a comprehensive complex nature of tick exoskeletons and their functional roles in adaptation and survival.

4 | DISCUSSION

The morpho-chromatographic authentication of *Rhipicephalus* ticks through the integration of HPLC chromatograms and morphological (SEM) traits unveils intriguing overlaps. This multidimensional approach not only underscores the potential for comprehensive species differentiation but also prompts further exploration into the correlation between chemical profiles and morphological characteristics, offering valuable data for tick identification and bioactive compounds studies.

The present research provides the distribution and morphological and molecular diversity of hard ticks, mainly *Rhipicephalus* species, from small ruminants in Punjab, Baluchistan, and Azad Kashmir, Pakistan. Numerous researchers have identified, in several areas of Pakistan, ticks that carry numerous pathogens of veterinary and medical significance, such as *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Hepatozoon*, *Rickettsia*, and *Theileria* (Ghafar, Gasser, et al., 2020).

Previously, tick infestation in small ruminants study from tribal areas of Pakistan identified the occurrence of *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus* species (Khan et al., 2019), consistent with the findings from the present study. However, the present work provides the first detailed morphological and genetic evidence for *R. sanguineus* from small ruminants of Pakistan.

In current study, the prevalence of *Hyalomma* was highest, followed by *Rhipicephalus* in sheep and goats, consistent with previous studies from the same agroecological zones of Pakistan and neighboring countries (Sultana et al., 2015). *Hyalomma* ticks are a potential vector for the transmission of *Theileria* in Pakistan. The current findings showed the presence of three *Rhipicephalus* species: *R. sanguineus*, *R. turanicus*, and *R. microplus* based on morpho-molecular evidence among small ruminants from the three agroecological zones of Pakistan. Among *Rhipicephalus* species, *R. microplus* was the dominant tick species in the northern part of Punjab (Rehman et al., 2017). The variations in temperature and climatic conditions may be the cause of the variability in tick infestation between research areas (Iqbal et al., 2014). The data regarding the prevalence of ticks in different hosts and breeds of animals revealed the highest prevalence in Shinwar-White sheep (34.4%) compared with all goat breeds studied. The low tick infestation in goats could be explained by their feeding and grooming behavior, which helps to prevent the attachment of ticks (Malla et al., 2021). The tick prevalence might be linked to habitats, diverse vegetation patterns, and exposure to the same community grazing space (Sajid et al., 2009). The results showed that animals aged 1 year and below were more heavily infested with ticks (24.7% in sheep and 21.8% in goats) compared with older animals, consistent with reports from a previous study. This might be due to the pliant and soft skin of young animals, which enables easier attachment of the ticks (Kabir et al., 2011).

The morphological and molecular-based methods are valued for enabling accurate species differentiation between various tick genera (Balinandi et al., 2020; Estrada-Pena et al., 2017). This study identified the differences between body length/width and the basis capituli length/width ratio, which might be partially interpreted as geographically related intraspecific morphological variations (Li et al., 2017). However, the morphological discrimination of *R. sanguineus* and *R. turanicus* is difficult because of the similarity in the form of spiracle plates, scutum shape, punctuations, and anal accessory shields (Estrada-Peña et al., 2004).

Our analysis of ITS2, 16S, and Cox I loci sequences of *R. sanguineus*, *R. turanicus*, and *R. microplus* with sequences from the public database showed consistent interspecific variations. The ITS2 molecular marker, which also displayed interspecific changes, corroborated the differences between *R. microplus* (OK642409) and *R. sanguineus* (OK642408). The low percentage similarity values shown by these identified species to corresponding accession sequences of *R. microplus* and *R. sanguineus* species could be associated with the cryptic hybridization factor or geographical separations which results in nucleotide substitutions (Taberlet et al., 1992). Accurate species description relies on the intraspecific and interspecific variations in the selected markers. The tick species identification based on ITS2 should be the most efficient marker, but ITS2 has no advantage over Cox I, 16S rDNA, and 12S rDNA in its resolution power of species identification (Lv et al., 2014).

The 16S sequence of identified *R. sanguineus* form a clade with three lineages, tropical, temperate, and south-eastern lineages, consistent with identification revealed in earlier studies (Chitimia-Dobler et al., 2017), and major differences might be due to the ecology, vector competence, crossbreeding, and other biological attributes (Zemtsova et al., 2016). Our study confirms that some ticks of the *R. sanguineus* complex in Pakistan belong to more than one entity and are comprised of different closely related species.

Likewise, the Cox I sequence of *R. turanicus* (OK623472) and *R. microplus* (OK623463) forms a separate clade and showed similarity with previously reported species from Pakistan and China. Similar to the present work, *R. microplus* was genetically confirmed in the northern part of Punjab and FATA, Pakistan (Rehman et al., 2017).

The HPLC chromatogram of tick exoskeleton hydrocarbons reveal a complex mixture of compounds, providing valuable insights into the chemical composition of this arthropod outer covering (Dupraz et al., 2022). The chromatographic peaks represent various hydrocarbons present in the exoskeleton, likely comprising a combination of alkanes, alkenes, and possibly alkynes. The identification of specific hydrocarbons can contribute to our understanding of the tick cuticular lipids, which play crucial roles in functions such as waterproofing, protection, and communication (Villar et al., 2020). Moreover, the chromatogram may exhibit variations in hydrocarbon profiles across different tick species or developmental stages, offering a potential avenue for taxonomic differentiation and ecological studies.

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5 | CONCLUSIONS

The combined morpho-chromatographic approaches in the authentication of *Rhipicephalus* ticks represent a comprehensive strategy to examine their biological traits. This characterization using HPLC chromatography and morpho-genetic SEM traits offers a perspective on the complex biochemical and morphological features of ticks within the Ixodidae family. The methodology followed not only enhances the accuracy of species identification but also provides valuable insights into the bioactive hydrocarbons present in *Rhipicephalus* ticks. The correlation between chromatographic profiles and morpho-genetic traits establishes a multidimensional framework for accurate authentication, contributing to the modification of taxonomic classifications and ecological studies. Furthermore, this study shows the importance of utilization of diverse phylogenetic tools in ticks taxonomy describing the gap between molecular and morphological interrelationship. The findings have implications not only to signify the ecological roles of *Rhipicephalus* ticks but also for advancing methods in tick-borne disease and control. The complex landscape of tick taxonomy and biology, the morpho-chromatographic authentication in context of phylogenetic hierarchical approach represent a valuable asset for future surveys into medically and economically significant arachnids.

AUTHOR CONTRIBUTIONS

Ayesha Malik: Writing – review and editing; writing – original draft; methodology. **Kiran Afshan:** Supervision; software; visualization; writing – review and editing. **Mohammad K. Okla:** Writing – review and editing; investigation; funding acquisition. **Ibrahim A. Saleh:** Investigation; conceptualization; validation. **Abdul Razzaq:** Writing – review and editing; formal analysis; software. **Munib Hussain:** Data curation; resources; investigation. **Sabika Firasat:** Conceptualization; writing – review and editing; supervision. **Erinda Lika:** Writing – review and editing; methodology; software. **Màrius Vicent Fuentes:** Conceptualization; investigation; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INFORMED CONSENT

Data have been provided with information about the purpose, procedures, and potential risks and benefits of this study. The data or findings resulting from your participation may be used in publications, presentations, or other scholarly activities.

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ORIGINAL ARTICLE

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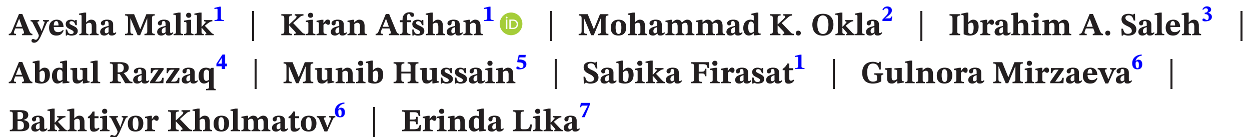
Insights into tick dynamics and anaplasmosis in ruminants: A microscopic and molecular perspective



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Abstract

Ticks are blood-sucking parasite and transmit pathogens to humans, domestic and wild animals. Ticks are seriously damage the production of sheep and goats, resulting in heavy economic loss to farmers. The present study investigated microscopic and molecular identification of ticks and tick-borne anaplasmosis infesting sheep and goats in three agro-ecological zones of Pakistan. The collected ticks were identified at species level based on their morphological features through microscopy by using taxonomic keys. Four *Rhipicephalus (R.)* species namely: *R. guilhoni Rh. geigyi*, *R. annulatus*, *R. pulchellus*, two *Haemaphysalis (Hae)* spp. *Hae. sulcate*, *Hae. punctate*, one *Ixodes ricinus and Hyalomma anatolicum* were identified. The morphological variations in their body sizes, scutal pattern, female genital opening, spiracular plate length and male adanal plate were recorded. Furthermore, we assessed the effectiveness of utilizing the 16S rRNA gene sequence to differentiate *Anaplasma* samples at the species level. Microscopically suspected positive blood samples for *Anaplasma* were subjected to DNA extraction. *Anaplasma* was observed in the blood of 14 animals. The sequence similarity between *Anaplasma marginale* and the reference genome were 99.25%, while below this value indicating a distinct species. Phylogenetic analysis revealed close similarity with *A. marginale* isolates from Iran, Vietnam and Pakistan This study contributes to the epidemiology of ticks and TBPs in the KP province and highlights the importance of proactive TBP surveillance in livestock.

KEYWORDS

anaplasmosis, microscopy, molecular identification, ruminants, taxonomy, ticks

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1 | INTRODUCTION

Ticks are arthropods which belong to the phylum: Arthropoda; class: Arachnida; Order: Acari and three major families viz: Ixodidae, Argasidae and Nuttalleillidae, with approximately 899 species (Mehlhorn & Armstrong, 2010). Ticks are prevalent worldwide from Asia (Singh & Rath, 2013), Africa (Elghali & Hassan, 2012), North and South American (Lohmeyer et al., 2011), Australia (Kamau et al., 2011) and Europe (Scharlemann et al., 2008). Worldwide, tick-borne diseases are a persistent example of problems at the One Health interface between humans, wildlife and agriculture. The hard ticks can play a crucial role in economic losses by decreasing of milk production and weight loss, paralysis, anaemia, skin irritation and transmission of wide range of pathogens including protozoa, bacteria, rickettsiae, spirochetes and viruses (Anderson & Magnarelli, 2008). Detection and diagnosis of many tick-borne infections in humans is also challenging, given the broad clinical presentation of many tick-borne diseases, current availability of reliable diagnostic tests and multi-tiered approaches needed to confirm pathogens (Bush & Vazquez-Pertejo, 2018; Fatma et al., 2017). The financial impact of the tick burden in Asian countries has been approximated to be around US $498.7 million per year (Minjauw & McLeod, 2003). Several studies have been found in small ruminants regarding prevalence of ticks ruminants but mainly focused on the morphological characterization (Ahmed et al., 2012; Ali et al., 2013; Khan et al., 2019; Sajid et al., 2011). Five tick genera were reported including *Hyalomma anatolicum*, *Haemaphysalis*, *Rhipicephalus*, *Ornithodoros* and *Argus* (Rehman et al., 2017). However, the data on microscopic morphological details of these tick is very limited. The morphological characters used for tick identification includes sclerotized scutum, gnathosoma located apically (Hajdušek et al., 2013), shape of scutum and its ventral structure.

Tick-borne anaplasmosis is a bacterial disease transmitted by ticks, primarily caused by *Anaplasma marginale*. This Gram-negative bacterium infects red blood cells and belongs to the order Rickettsiales (Ashraf et al., 2021; Tana-Hernández et al., 2017). The primary mode of transmission of this bacterial agent belonging to the Rickettsiales order to animal hosts is through ticks. Extensive research has identified 20 tick species as efficient vectors of *A. marginale* (Kocan et al., 2004). Among the commonly reported tick species involved in *A. marginale* transmission are Ixodes spp., *Dermacentor* spp., *Rhipicephalus* spp. and *Amblyomma* spp. (Hairgrove et al., 2015). Clinical symptoms commonly observed in cattle suffering from anaplasmosis include fever, progressive anaemia, jaundice, loss of appetite, depression, decreased milk production, abortion in pregnant animals and a higher risk of mortality, particularly in exotic breeds (Camus & Uilenberg, 2010). Although anaplasmosis is considered a zoonotic concern, with the potential to infect humans, *A. marginale* has not been found to infect humans (Kumar et al., 2015).

To control tick born disease several diagnostic techniques were applied. The main methods for detecting *A. marginale* involve serological and molecular diagnosis, which have high sensitivity and specificity, enabling accurate detection of the pathogen (Rafael et al., 2021). Polymerase chain reaction (PCR) method provides a reliable and sensitive approach to identify the presence of the *A. marginale* and efficacy of the method extends to the testing of intricate blood samples containing haemolysed and coagulated bovine blood (Ben et al., 2018).

Here, we described microscopic identification of various hard tick genera infesting sheep and goats in Pakistan. In addition, molecular characterization of tick-borne anaplasmosis was performed by using *16S* ribosomal RNA genetic marker.

2 | MATERIALS AND METHODS

2.1 | Ticks collection

A total 4488 animals were observed for tick infestation belongs to three ecologies of Pakistan comprised six sites like Rawalpindi, Jehlum, Attock, Lahore, Muzafferabad and Quetta (Figure 1) during April to August 2019.

2.2 | Taxonomic identification of ticks

A standard tick collection methods described by Soulsby (1982) was fallowed. The collected ticks (in larvae, nymph and adult stages) were processed by the method describe by Shah et al. (2015) for the identification at genus and species levels on the basis of their morphological features through conventional taxonomic keys (Walker et al., 2003) under stereomicroscope using ocular and stage Lense 0.1 mm. Briefly, removed ticks preserved in ethyl alcohol to a tube containing 10%KOH solution for 24 h. After washing with water placed in 5% aqueous solution of glacial acetic acid for 30 min. Again, washed with water and stained with acid fuchsin, dehydrated and cleared with xylene before mounting (Gutierrez, 1985).

2.3 | Tick born pathogen: Anaplasmosis

2.3.1 | Sample collection, screening and DNA extraction

Blood samples were taken from jugular veins of tick-infested animals ($n = 24$) only if they were symptomatic. The

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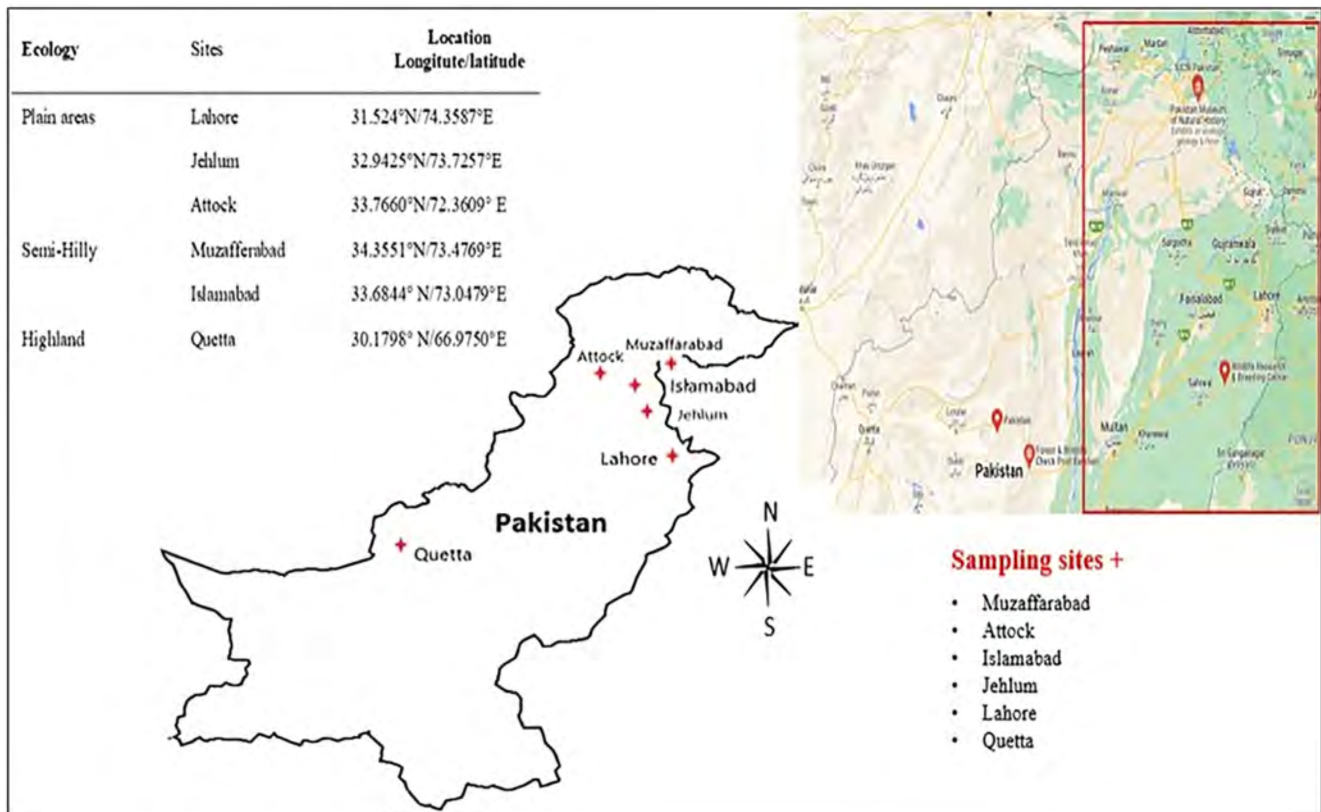

FIGURE 1 Map of Pakistan indicating six tick sampling areas within three agro-ecological zones.

TABLE 1 Animal information detected positive for microscopy and used in PCR analysis.

Code	Host animal	Location	Blood type
A1	Goat	Abbotabad, KP	Whole Blood
A2	Cattel	Serdheri, KP	Whole Blood
A3	Cattel	Charsadda, KP	Whole Blood
A4	Sheep	Mardan, KP	Whole Blood
A5	Goat	Mardan, KP	Whole Blood
A6	Goat	Mardan, KP	Whole Blood
A7	Goat	Mardan, KP	Whole Blood
A8	Goat	Mardan, KP	Whole Blood
A9	Goat	Serdheri, KP	Whole Blood
A10	Goat	Serdheri, KP	Whole Blood
A11	Goat	Serdheri, KP	Whole Blood
A12	Cattel	Charsadda, KP	Whole Blood
A13	Cattel	Abbotabad, KP	Whole Blood
A14	Cattel	Abbotabad, KP	Whole Blood

study was approved by the Animal Ethics Committee of the Quaid-i-Azam University, Islamabad. The ethical approval No. DFBS/2017.765 Dated: April 17, 2017. Approximately 4–5 mL of blood was collected of each animal in EDTA tube, a thin blood smear was created stained with Giemsa. Microscopically suspected positive blood samples for were subjected to DNA extraction. A total of 14 blood samples for *Anaplasma* were subjected to molecular characterization (Table 1). DNA was extracted by using the standard phenol-chloroform protocol (Sambrook et al., 1989).

2.3.2 | Standard PCR for the *16S* rRNA

The characterization of the *Anaplasma* species detected in blood samples of 14 animals were carried out standard PCRs for the *16S* rRNA of *Anaplasma* spp with Pan-primers designed for the study (Forward: 5′ GCCACTA GAAATGGTGGGTAATA and Reverse: 3′ AAAGCAGCT CCAGGGTTAAG). The 25 μL PCR reaction mixtures consisted of 2 μL of PCR buffer (1×) (Thermo Fisher Scientific, USA), 2 μL MgCl2 (25 mM), 2 μL of 2.5 mM dNTPs, 0.7 μL of the primer mix (10 pmol/μl final concentration of each primer), 2 μL of gDNA and 0.3 μL of Taq DNA polymerase (5 U/μl) (Thermo Fisher Scientific, USA), and 16 μL ddH20. The thermocycler conditions were set at 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension procedure of 72°C for 10 min. The amplified products were visualized by electrophoresis in 2% agarose gel and cleaned with WizPrepTM Gel/PCR Purification Mini kit (Seongnam 13209; South Korea) as mentioned in Table 1.

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2.3.3 | Data analysis

To align the sequences, CLUSTALW was employed as a tool (accessible at https://www.genome.jp/tools-bin/clustalw). The evolutionary tree was inferred using the Maximum Likelihood method and the Kimura 2-parameter model (Kimura, 1980). 1000 replicates were used to create a bootstrap consensus tree, which shows the evolutionary relationships between the taxa under consideration (Felsenstein, 1985). A discrete Gamma distribution with four categories (+G, parameter = 2.4272) was used to account for changes in evolutionary rates between sites. 11 nucleotide sequences made up the data set, and any gaps or incomplete data were fully removed (full deletion option). The final data set included 177 places in it. Using MEGA X, evolutionary analysis was carried out (Kumar et al., 2018). The phylogenetic tree was generated utilizing the interactive tree of life (ITOL) tool, available at https://itol.embl.de/upload.cgi. The 28 reference sequences used for comparison were acquired from NCBI, with information provided in Table 2.

3 | RESULTS

3.1 | Morphological identification of tick species

A total of 600 adult ticks were morphologically measured collected from 4488 infected animals. Morphologically, eight species of ticks belonged to four genera, namely *Rhipicephalus* and *Haemaphysalis*, *Hyalomma and Ixodes* were characterized. The tick species with the highest prevalence was *Hyalomma anatolicum* 6.33% followed *Rhipicephalus* spp. *namely R. guilhoni Rh. geigyi*, *R. annulatus*, *R. pulchellus* with 3.9%. The prevalence of *Haemaphysalis* (*Hae.*) *punctate* was 2.07%, *Hae. sulcata* 0.56% and *Ixodes ricinus* 4.6%.

The morphological measurements of eight microscopically identified species are given in Table 3 and Figure 2. The *Rhipicephalus* species were identified based on intraspecific morphological variations in their body sizes, scutal pattern, female genital opening, spiracular plate length and male adanal plate. The difference

TABLE 2 Reference sequences downloaded from NCBI GenBank for comparison.

Organism	Host	Country	Accession no.
Anaplasma bovis	Tick	China	KP314249
	Tick	Mauritania	MZ476211
Anaplasma camelii	Camel	Pakistan	MZ411410
Anaplasma capra	Sheep	Pakistan	ON238131
Anaplasma centrale	Cattle	Kyrgyzstan	MW672119
Anaplasma marginale	Cattle	China	KX279690
	Sheep	Pakistan	OL307000
	Goat	Pakistan	OL305725
Anaplasma odocoilei	Deer	China	JX876644
Anaplasma ovis	Tick	Russia	MW600413
	Tick	China	KJ410245
	Goat	China	KX279688
	Goat	Russia	MW600407
Anaplasma phagocytophilum	Tick	Ireland	OL519484
	Tick	Ireland	OL519483
	Cat	Pakistan	ON796007
Anaplasma platys	Dog	Pakistan	MW019671
	Dog	Brazil	KC989957
	Dog	Egypt	LC632659
	Dog	Egypt	MZ068099
	Isolate WH	China	MN630836
	Dog	Egypt	MT053461
Anaplasma sp.	Dog, South	South Africa	AY570538
	Sheep	Senegal	MN317253
	Procapra gutturosa	China	KM227008
	Goat	China	FJ389575
	Cattle	Mongolia	MK575506
Ehrlichia ruminantium (out group)		South Africa	KF786045

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TABLE 3 Morphological measurements of eight hard ticks species are presented in values of Mean ± SD, all measurement were taken in millimetres (mm).

	R. guilhoni		*Rh. geigyi*	*Rh. annulatus*		*R. pulchellus*	*Hae. punctata*	*Hae. sulcata*	*H. anatolicum*		*I. ricinus*	
Characters	**M (*n* = 50)**	**F (*n* = 50)**	**F (*n* = 50)**	**M (*n* = 50)**	**F (*n* = 50)**	**M (*n* = 50)**	**F (*n* = 50)**	**M (*n* = 50)**	**M (*n* = 50)**	**F (*n* = 50)**	**M (*n* = 50)**	**F (*n* = 50)**
Body L	3.66 ± 0.10	1.82 ± 0.02	4.43 ± 0.19	4.42 ± 0.15	4.83 ± 0.31	4.70 ± 2.71	1.98 ± 0.08	2.21 ± 0.01	2.33.7 ± 9.1	2.58 ± 0.05	2.21 ± 0.01	4.25 ± 0.25
Body W	1.76 ± 0.11	1.07 ± 0.02	2.73 ± 0.17	2.82 ± 0.11	3.49 ± 0.30	1.53 ± 0.88	0.85 ± 0.07	0.98 ± 0.12	1.08.0 ± 3.5	1.33.5 ± 0.05	0.98 ± 0.12	2.46 ± 0.05
Adanal plates L	0.80 ± 0.02	0.64 ± 0.005	0.63 ± 0.03	0.81 ± 0.04	0.80 ± 0.11	1.09 ± 0.63	0.47 ± 0.02	0.11 ± 0.01	0.42.7 ± 0.9	0.42 ± 0.009	—	—
Adanal plates W	0.27 ± 0.01	0.18 ± 0.00	0.42 ± 0.04	0.39.9 ± 0.04	0.49 ± 0.08	3.8 ± 0.22	0.23 ± 0.01	0.25 ± 0.08	0.14.3 ± 1.2	0.16 ± 0.01	—	—
Basis capituli L	0.24 ± 0.01	0.15 ± 0.005	0.28 ± 0.01	0.28 ± 0.02	0.29 ± 0.04	0.39 ± 0.22	0.36 ± 0.004	0.10 ± 0.00	0.20.7 ± 0.9	0.21 ± 0.009	0.11 ± 0.01	0.14 ± 0.005
Basis capituli W	0.72 ± 0.02	0.54 ± 0.005	0.73 ± 0.06	0.59 ± 0.05	0.46 ± 0.07	0.93 ± 0.53	0.17 ± 0.004	1.91 ± 0.08	30.7 ± 0.3	0.33 ± 0.008	0.25 ± 0.08	0.38 ± 0.03
Distance b/w Porose areas	0.24 ± 0.009	0.18 ± 0.005	0.22 ± 2.1	0.18 ± 0.011	0.26 ± 0.02	0.31 ± 0.18	0.12 ± 0.009	0.98 ± 0.12	16.3 ± 0.9	0.18 ± 0.01	0.10 ± 0.00	0.19 ± 0.03
Idiosoma L	3.02 ± 0.09	1.52 ± 0.01	3.64 ± 0.17	3.48 ± 0.16	3.99 ± 0.23	3.46 ± 2.00	1.39 ± 0.09	0.19 ± 0.01	1.61 ± 0.04	1.70 ± 0.04	1.91 ± 0.08	3.43 ± 0.22
Idiosoma W	1.77 ± 0.11	1.07 ± 0.02	2.68 ± 0.20	2.82 ± 0.11	3.49 ± 0.30	2.20 ± 1.27	0.85 ± 0.07	0.11 ± 0.01	1.08 ± 0.03	1.33 ± 0.05	0.98 ± 0.12	2.46 ± 0.05
Palp L	0.34 ± 0.02	0.29 ± 0.00	0.38 ± 0.01	0.32 ± 0.01	0.47 ± 0.09	0.48 ± 0.28	0.38 ± 0.01	0.14 ± 0.03	0.24 ± 0.009	0.25 ± 0.16	0.19 ± 0.01	0.38 ± 0.03
Palp W	0.48 ± 0.17	0.14 ± 0.00	0.14 ± 0.007	0.15 ± 0.008	0.31 ± 0.08	0.23 ± 0.13	0.16 ± 0.01	0.10 ± 0.01	0.09 ± 0.006	0.09 ± 0.006	0.11 ± 0.01	0.15 ± 0.01
Spiracular area L	0.37 ± 0.02	0.33 ± 0.02	0.44 ± 0.001	0.28 ± 0.01	0.39 ± 0.05	0.53 ± 0.31	0.29 ± 0.01	0.07 ± 0.01	0.14 ± 0.007	0.17 ± 0.012	0.14 ± 0.03	0.28 ± 0.03
Spiracular area W	0.19 ± 0.01	0.12 ± 0.005	0.28 ± 0.01	0.18 ± 0.005	0.30 ± 0.07	0.26 ± 0.15	0.20 ± 0.004	0.10 ± 0.005	0.26 ± 0.006	0.23 ± 0.01	0.10 ± 0.01	0.17 ± 0.01
Posterior grooves L	0.55 ± 0.02	0.51 ± 0.005	0.41 ± 0.005	0.57 ± 0.03	0.55 ± 0.08	0.59 ± 0.34	0.53 ± 0.03	0.05 ± 0.01	0.42 ± 0.01	0.30 ± 0.028	—	0.44 ± 0.01
Posterior grooves W	0.29 ± 0.02	0.24 ± 0.005	0.31 ± 0.003	0.32 ± 0.02	0.35 ± 0.08	0.40 ± 0.23	0.30 ± 0.23	0.06 ± 0.02	0.21 ± 0.006	0.20 ± 0.008	—	0.28 ± 0.03
Accessory Adanal Shield L	0.36 ± 0.02	0.30 ± 0.00	0.25 ± 0.005	0.25 ± 0.008	0.24 ± 0.01	0.31 ± 0.18	0.235 ± 0.012	0.09 ± 0.01	0.11 ± 0.006	0.19 ± 0.01	—	0.29 ± 0.005
Accessory Adanal Shield W	0.19 ± 0.01	0.18 ± 0.005	0.21 ± 0.003	0.16 ± 0.005	0.26 ± 0.03	0.18 ± 0.10	0.22 ± 0.004	0.10 ± 0.00	0.14 ± 0.01	0.16 ± 0.01	—	0.20 ± 0.005
Genital aperture L	0.16 ± 0.009	0.12 ± 0.00	0.21 ± 0.003	0.18 ± 0.005	0.19 ± 0.002	0.20 ± 0.11	0.11 ± 0.006	0.07 ± 0.01	0.11 ± 0.007	0.12 ± 0.01	0.07 ± 0.01	0.21 ± 0.005
Genital aperture W	0.16 ± 0.01	0.16 ± 0.00	0.18 ± 0.004	0.15 ± 0.006	0.18 ± 0.01	0.19 ± 0.11	0.10 ± 0.006	0.08 ± 0.00	0.08 ± 0.003	0.805 ± 0.002	0.10 ± 0.005	0.19 ± 0.005
Palp article I L	0.07 ± 0.005	0.08 ± 0.00	0.08 ± 0.004	0.09 ± 0.004	0.10 ± 0.009	0.11 ± 0.06	0.10 ± 0.009	1.90 ± 0.10	0.06 ± 0.000	0.073 ± 0.004	0.05 ± 0.01	0.05 ± 0.005
Palp article I W	0.10 ± 0.004	0.12 ± 0.00	0.06 ± 0.004	0.06 ± 0.005	0.07 ± 0.009	0.10 ± 0.05	0.09 ± 0.004	0.98 ± 0.12	0.06 ± 0.000	0.07 ± 0.003	0.06 ± 0.02	0.05 ± 0.01
Palp article II L	0.09 ± 0.004	0.09 ± 0.00	0.09 ± 0.003	0.11 ± 0.004	0.142 ± 0.005	0.13 ± 0.07	0.125 ± 0.006	0.35 ± 0.06	0.12 ± 0.000	0.117 ± 0.003	0.09 ± 0.01	0.10 ± 0.005
Palp article II W	0.13 ± 0.006	0.10 ± 0.00	0.07 ± 0.005	0.09 ± 0.005	0.095 ± 0.01	0.13 ± 0.07	0.10 ± 0.005	0.09 ± 0.01	0.10 ± 0.003	0.117 ± 0.003	0.10 ± 0.00	0.09 ± 0.005
Palp article III L	0.12 ± 0.009	0.16 ± 0.00	0.11 ± 0.001	0.11 ± 0.006	0.14 ± 0.006	0.19 ± 0.011	0.12 ± 0.004	0.22 ± 0.02	0.11 ± 0.009	0.11 ± 0.006	0.07 ± 0.01	0.14 ± 0.005
Palp article III W	0.11 ± 0.007	0.12 ± 0.00	0.06 ± 0.004	0.07 ± 0.003	0.10 ± 0.01	0.09 ± 0.05	0.082 ± 0.005	0.17 ± 0.01	0.08 ± 0.003	0.083 ± 0.03	0.08 ± 0.00	0.09 ± 0.005
Scutum L	3.00 ± 0.09	0.63 ± 0.005	0.99 ± 0.11	0.72 ± 0.024	1.40 ± 0.21	3.46 ± 2.00	1.38 ± 0.10	0.19 ± 0.01	1.55 ± 0.03	0.81 ± 0.02	1.90 ± 0.10	3.40 ± 0.20
Scutum W	1.74 ± 0.10	0.65 ± 0.04	1.04 ± 0.12	0.80 ± 0.012	1.15 ± 0.19	2.20 ± 1.27	0.84 ± 0.07	0.18 ± 0.005	1.00 ± 0.03	0.89 ± 0.03	0.98 ± 0.12	2.46 ± 0.05
Hypostome L	0.80 ± 0.02	0.65 ± 0.00	0.60 ± 0.10	0.52 ± 0.043	0.51 ± 0.05	1.13 ± 0.65	0.55 ± 0.01	0.23 ± 0.04	0.60 ± 0.007	0.62 ± 0.02	0.35 ± 0.06	0.55 ± 0.04

(Continues)

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TABLE 3 (Continued)

Characters	*R. guilhoni* M (n = 50)	*R. guilhoni* F (n = 50)	*Rh. geigyi* F (n = 50)	*Rh. annulatus* M (n = 50)	*Rh. annulatus* F (n = 50)	*R. pulchellus* M (n = 50)	*Hae. punctata* F (n = 50)	*Hae. sulcata* M (n = 50)	*H. anatolicum* M (n = 50)	*H. anatolicum* F (n = 50)	*I. ricinus* M (n = 50)	*I. ricinus* F (n = 50)
Hypostome W	0.19 ± 0.01	0.15 ± 0.00	0.22 ± 0.01	0.18 ± 0.014	0.24 ± 0.01	0.27 ± 0.16	0.19 ± 0.01	0.18 ± 0.04	0.14 ± 0.01	0.14 ± 0.008	0.09 ± 0.01	0.28 ± 0.03
Coxa I L	0.48 ± 0.02	0.54 ± 0.00	0.41 ± 0.01	0.43 ± 0.015	0.53 ± 0.05	0.66 ± 0.38	0.42 ± 0.04	0.25 ± 0.005	0.24 ± 0.01	0.22 ± 0.007	0.22 ± 0.02	0.51 ± 0.07
Coxa I W	0.33 ± 0.01	0.31 ± 0.00	0.32 ± 0.003	0.26 ± 0.014	0.34 ± 0.04	0.40 ± 0.23	0.21 ± 0.006	0.27 ± 0.01	0.26 ± 0.006	0.25 ± 0.009	0.17 ± 0.01	0.33 ± 0.01
Coxa II L	0.53 ± 0.02	0.45 ± 0.00	0.52 ± 0.003	0.47 ± 0.023	0.56 ± 0.05	0.64 ± 0.37	0.43 ± 0.03	0.06 ± 0.00	0.34 ± 0.006	0.34 ± 0.004	0.19 ± 0.01	0.47 ± 0.02
Coxa II W	0.38 ± 0.02	0.30 ± 0.005	0.42 ± 0.007	0.39 ± 0.023	0.42 ± 0.07	0.33 ± 0.19	0.28 ± 0.02	0.15 ± 0.03	0.24 ± 0.003	0.25 ± 0.008	0.18 ± 0.005	0.44 ± 0.21
Coxa III L	0.52 ± 0.02	0.40 ± 0.005	0.52 ± 0.008	0.41 ± 0.027	0.48 ± 0.04	0.73 ± 0.42	0.50 ± 0.03	0.24 ± 0.04	0.40 ± 0.003	0.40 ± 0.003	0.23 ± 0.04	0.39 ± 0.04
Coxa III W	0.48 ± 0.02	0.42 ± 0.00	0.50 ± 0.005	0.37 ± 0.021	0.38 ± 0.06	0.35 ± 0.20	0.36 ± 0.02	0.14 ± 0.00	0.21 ± 0.003	0.21 ± 0.002	0.18 ± 0.04	0.60 ± 0.20
Coxa IV L	0.45 ± 0.10	0.39 ± 0.005	0.35 ± 0.01	0.40 ± 0.020	0.54 ± 0.03	0.84 ± 0.48	0.44 ± 0.011	0.29 ± 0.005	0.33 ± 0.006	0.32 ± 0.003	0.25 ± 0.005	0.46 ± 0.14
Coxa IV W	0.52 ± 0.02	0.41 ± 0.005	0.56 ± 0.02	0.35 ± 0.015	0.43.2 ± 0.05	0.48 ± 0.27	0.41 ± 0.15	0.09 ± 0.01	0.21 ± 0.006	0.20 ± 0.003	0.27 ± 0.01	0.47 ± 0.25
Cornua L	0.11 ± 0.007	0.09 ± 0.005	0.11 ± 0.008	0.15 ± 0.009	0.18 ± 0.01	0.21 ± 0.12	0.18 ± 0.004	0.09 ± 0.00	0.08 ± 0.003	0.08 ± 0.002	0.06 ± 0.00	0.19 ± 0.01
Cornua W	0.19 ± 0.01	0.016 ± 0.005	0.75 ± 0.03	0.21.5 ± 0.03	0.22 ± 0.07	0.20 ± 0.11	0.14 ± 0.012	0.09 ± 0.01	0.10 ± 0.003	0.10 ± 0.002	0.15 ± 0.03	0.25 ± 0.03
Tibia L	0.73 ± 0.04	0.80 ± 0.00	0.50 ± 0.02	0.35 ± 0.018	0.60 ± 0.08	0.92 ± 0.53	0.66. ± 0.03	—	0.53 ± 0.01	0.52 ± 0.01	0.24 ± 0.04	0.60 ± 0.24
Tibia W	0.34 ± 0.02	0.40 ± 0.00	0.021 ± 0.003	0.14.6 ± 0.009	0.23 ± 0.03	0.36 ± 0.20	0.355 ± 0.01	—	0.19 ± 0.006	0.18 ± 0.004	0.14 ± 0.00	0.22 ± 0.06
Tarsus L	0.62 ± 0.02	0.63 ± 0.00	0.62 ± 0.01	0.38 ± 0.016	0.63 ± 0.07	0.72 ± 0.41	0.66 ± 0.01	—	0.39 ± 0.006	0.38 ± 0.003	0.29 ± 0.005	0.60 ± 0.19
Tarsus W	0.25 ± 0.03	0.14 ± 0.00	0.13 ± 0.006	0.139 ± 0.007	0.31 ± 0.05	0.21 ± 0.12	0.15 ± 0.01	—	0.11 ± 0.006	0.10 ± 0.003	0.09 ± 0.01	0.18 ± 0.02
Anal groove L	0.84 ± 0.10	0.25.5 ± 0.005	1.34 ± 0.11	0.675 ± 0.06.4	0.42 ± 0.06	1.10 ± 0.63	0.37 ± 0.02	—	0.60 ± 0.003	0.60 ± 0.002	—	0.85 ± 0.40
Anal groove W	0.43 ± 0.05	0.18 ± 0.00	0.80 ± 0.06	0.469 ± 0.032	0.44 ± 0.03	0.52 ± 0.30	0.45 ± 0.00	—	0.49 ± 0.01	0.48 ± 0.005	—	0.67 ± 0.42
Anus L	0.19 ± 0.006	0.20 ± 0.00	0.18 ± 0.01	0.174 ± 0.004	0.18 ± 0.01	0.22 ± 0.13	0.14 ± 0.02	—	0.08 ± 0.003	0.08 ± 0.002	0.09 ± 0.00	0.25 ± 0.04
Anus W	0.19 ± 0.007	0.18 ± 0.00	0.17 ± 0.005	0.16. ± 0.007	0.18 ± 0.008	0.23 ± 0.13	0.11 ± 0.00	—	0.09 ± 0.000	0.09 ± 0.00	0.09 ± 0.01	0.24 ± 0.07
Sub Anal Plates L	—	—	—	—	—	4.70 ± 2.71	0.10 ± 0.01	—	0.05 ± 0.006	0.047 ± 0.003	—	—
Sub Anal Plates W	—	—	—	—	—	1.53 ± 0.88	0.08 ± 0.007	—	0.09 ± 0.003	0.09 ± 0.002	—	—
Central festoon L	—	—	—	—	—	1.09 ± 0.63	0.087 ± 0.003	—	0.06 ± 0.00	0.06 ± 0.00	—	—
Central festoon W	—	—	—	—	—	3.8 ± 0.22	0.07 ± 0.003	—	0.05 ± 0.003	0.055 ± 0.002	—	—

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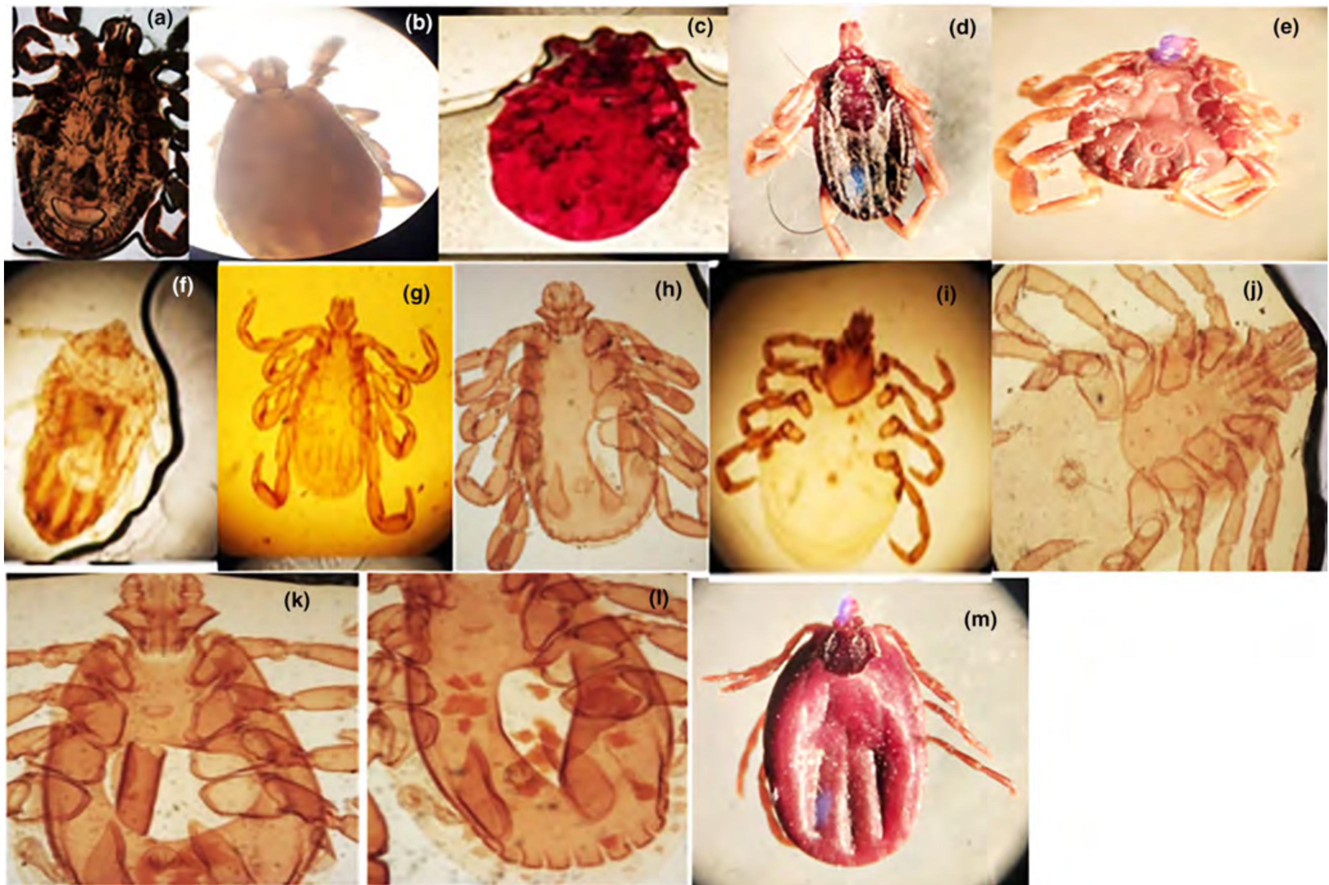

FIGURE 2 Microscopic slides: (a) *Hae. sulcate* (b) *Hae. punctate* (*Dorsal View*) (c) *Hae. punctate* (*Ventral View*) (d) *H. anatolicum* (*Dorsal View*) (e) *H. anatolicum* (*Ventral View*) (f) *R. guilhoni* (g, h) *R. geigyi* (i, j) *R. annulatus* (k, l) *R. pulchellus* (m) *I. ricinus*.

between genus *Rhipicephalus* and *Haemaphysalis* is based on capitulum (mouthparts). In *Rhipicephalus* the capitulum has angulated sides with triangular porose regions, while genus *Haemaphysalis* is not angulated on the sides but rather possesses huge porose patches that are longitudinal and distant. The *Rhipicephalus* tick species were identified based on intraspecific morphological variations in their body sizes, colour, capitulum, scutal pattern, coxae, female genital opening, spiracular plate length and male accessory adanal plate. *Hyalomma anatolicum* were characterized by genital aperture, mouth parts, presence of Coxae I-III in the ventral side and has festoons on dorsal side of the ticks. Males were characteristics by the presence of, accessory shield, anus, adanal shields, anal groove and subanal shields. The *Ixodes* tick has characteristically long mouthparts and dominant distinguish features from other genera are on the ventral surface the anus is surrounded by a U-shaped groove and has set of sharp spur-like protrusions seen proximally (coxae) in the first pair of legs. The female tick is 4.25 ± 0.25 mm long and male tick is smaller 2.21 ± 0.01 mm.

3.2 | *Anaplasma* molecular characterization

Anaplasma marginale was observed in the blood of 14 samples amplified with PCR showing positive detection of *Anaplasma* with ~400 base pairs of 16S rRNA fragments on 2% gel (Figure 3). Out of these 14 sequences isolated from sheep, cattle and goats 12 *Anaplasma* sequences were of good quality and aligned with 28 NCBI GeneBank sequences of different *Anaplasma* species (Figure 3).

The genetic distance search of *Anaplasma* 16S rRNA sequences (present study) shows 99.5%–99.25% similarity to a previously identified uncultured *Anaplasma* sp. (OP297688, OP297687, OP297686 and OP297680) and *Anaplasma marginale* (MK310487, MK310488 and MH020201) (Figure 4). Further comparison showed similarities between *Anaplasma ovis* (MK855077). However, phylogenetic tree of *Anaplasma* from Pakistan (present study) revealed a close link with *Anaplasma marginale* and *Anaplasma* sp. (Figure 5). Similarly, the phylogenetic comparison showed that *A*, *bovis*, *A. ovis*, *A. platys*, *A. phagocytophilum* and *A. capra* form distinct subclades (Figure 6).

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4 | DISCUSSION

The present investigation provides insight into microscopic identification of hard ticks, mainly *Rhipicephalus*, *Hyalomma*, *Haemaphysalis and Ixodes* spp, from ruminants. These ticks carry numerous pathogens of veterinary and medical significance, such as *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Hepatozoon*, *Rickettsia* and *Theileria* (Fang et al., 2015; Kilpatrick & Randolph, 2012). In the present study, eight tick species morphological identification were refers to the process of visually identifying tick species based on their morphological characteristics, such as body shape, size, colour and pattern (Abdel-Shafy et al., 2015; Amrutha et al., 2023).This study only selected adult unengorged tick for morphological measurements, as morphological identification is insufficient in separating between related species complexes, particularly when the specimens are at immature stages, physically damaged or engorged (Paguem et al., 2023).

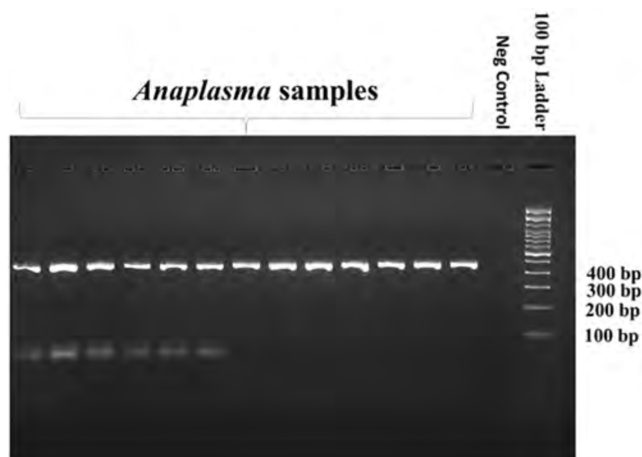
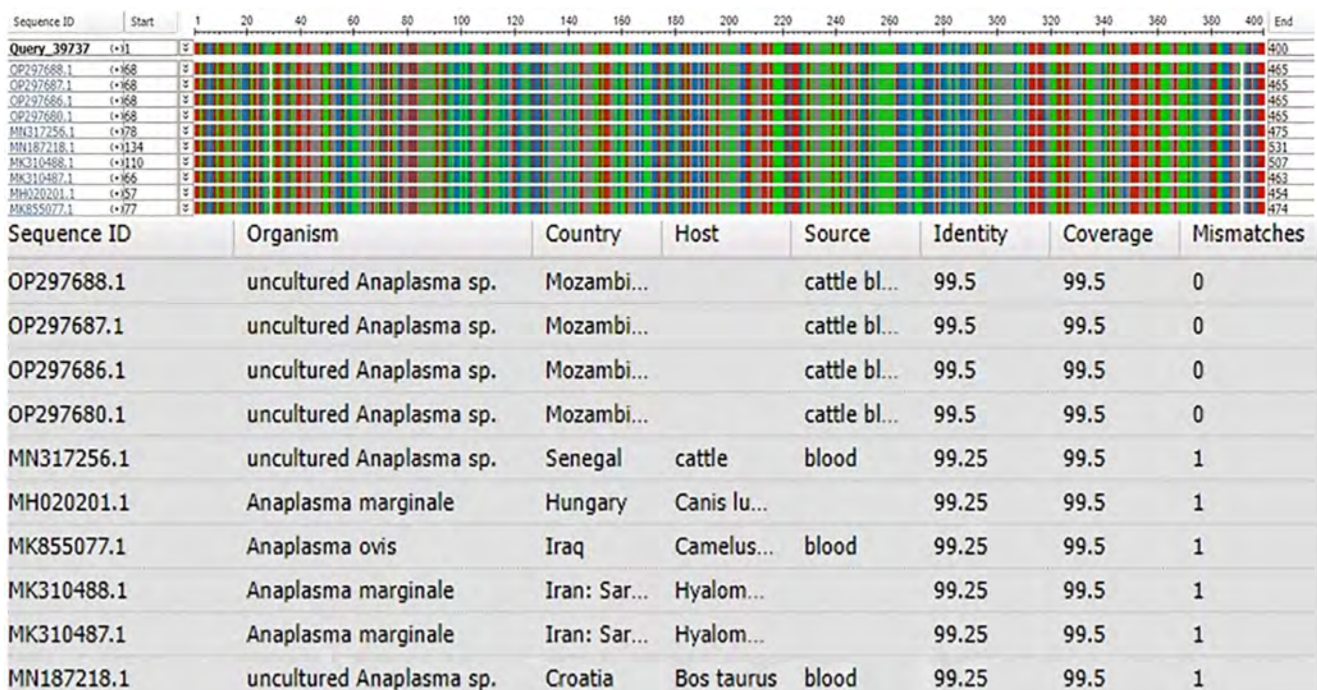


FIGURE 3 Agarose gel electrophoresis (2%) of PCR product band of 400 bp for *Anaplasma* isolates targeting 16S rRNA.

In this study four *Rhipicephalus* species were identified and showed differences between body length/width and the basis capituli length/width ratio, which might be partially interpreted as geographically related intraspecific morphological variations (Li et al., 2017). The *Rhipicephalus* and *Haemaphysalis* genera were separated based on capitulum, consistent with previous study (Intirach et al., 2023). The use of external features to differentiate between *Hyalomma anatolicum* from other ticks are consistent with previous studies (Al-Fatlawi et al., 2018; Shanan et al., 2017). The *I. ricinus* characterized in the study has prominent distinguishing features from other genera as described previously by Saari et al. (2018). Previously, studies conducted on tick infestation in small ruminants from tribal areas of Pakistan identified the occurrence of *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* species (Ghafar et al., 2020, b), consistent with the present findings. Among these tick species, few similar species were also reported by Ramzan et al. (2020) includes *Haemaphysalis punctata*, *H. anatolicum* in Multan. Irshad



Sequence ID	Organism	Country	Host	Source	Identity	Coverage	Mismatches
OP297688.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297687.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297686.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
OP297680.1	uncultured Anaplasma sp.	Mozambi...		cattle bl...	99.5	99.5	0
MN317256.1	uncultured Anaplasma sp.	Senegal	cattle	blood	99.25	99.5	1
MH020201.1	Anaplasma marginale	Hungary	Canis lu...		99.25	99.5	1
MK855077.1	Anaplasma ovis	Iraq	Camelus...	blood	99.25	99.5	1
MK310488.1	Anaplasma marginale	Iran: Sar...	Hyalom...		99.25	99.5	1
MK310487.1	Anaplasma marginale	Iran: Sar...	Hyalom...		99.25	99.5	1
MN187218.1	uncultured Anaplasma sp.	Croatia	Bos taurus	blood	99.25	99.5	1

FIGURE 4 The Blast results showed Query sequence identity with *Anaplasma marginale and Anaplasma* sp. colour lines shows different nucleotides position.

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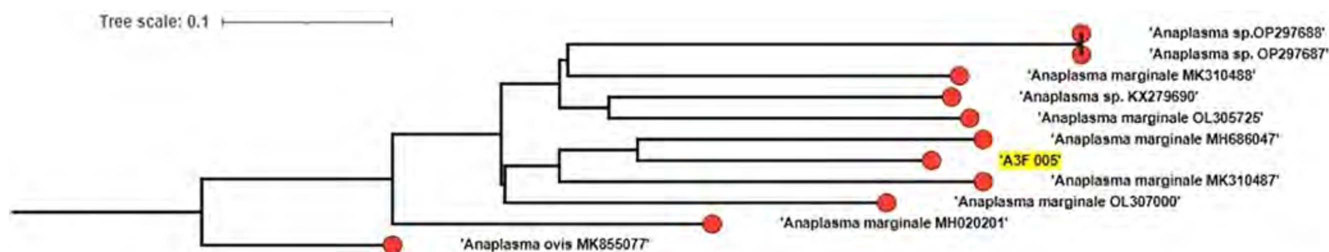

FIGURE 5 The evolutionary tree based on 16S rRNA sequence by using the Maximum Likelihood method and Kimura 2-parameter model.

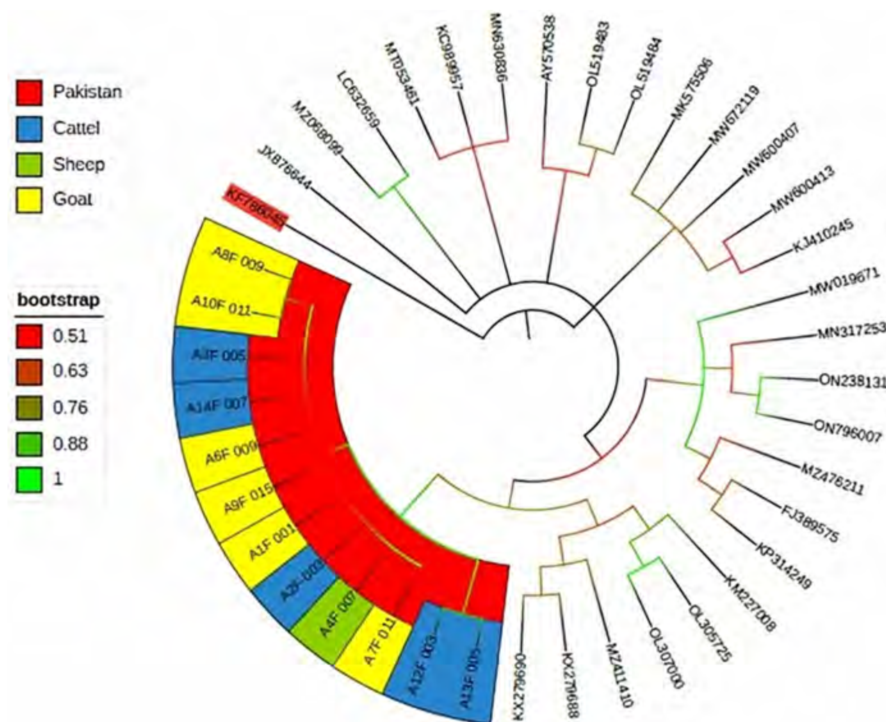

FIGURE 6 Phylogenetic tree for the 16S rRNA sequences of *Anaplasma* isolates from sheep, goats and cattle.

et al. (2010) recorded *Rhipicephalus* spp., *Haemaphysalis* spp. and *Ixodes* spp. in sheep and goats. The morphological and molecular-based methods are valued for enabling accurate species differentiation between various tick genera (Balinandi et al., 2020; Ernieenor et al., 2017; Estrada-Pena et al., 2017).

This investigation offers information about the diversity of TBPs in sheep, goats and cattle from KP, province of Pakistan. The result shows presence of *Analpasma marginale* in the blood samples. One of the most significant tick-borne infections worldwide is *Anaplasma marginale* (Palmer et al., 2000). In the present study 14 blood samples were suspected positive in microscopy for anaplasmosis and further investigated for molecular speciation. Twelve sequences were produced with good quality and blast results showed 99.25% similarity with *A. marginale* and 99.5% similarity with uncultured *Anaplasm*aa spp. from Mozambique. In current work, animals were mainly infected with *Rhipicephalus* and *Hyalomma* ticks. According to several investigations, the tick species *Hyalomma excavatum* and *Rhipicephalus annulatus* were both implicated as vectors for *A. marginale* (Samish et al., 1993; Shkap et al., 2009).

The phylogenetic analysis of present study *Anaplsama* spp. based on *16S* rRNA gene revealed close similarity with *A. marginale* isolates from Iran, Vietnam and Pakistan. And form distinct clad from other *Anaplasma* spp. circulating in other countries. The presence of comparable isolates at different locations could be due to introduction exotic species in the country, cross border or unrestricted transportation of live animals for selling. We show that the genus *Anaplasma's* available sequencing samples can be classified to the species level using 16S rRNA sequences, and *A. marginale* can be distinguished from *A. phagocytophilum*, *A. centrale* and *A. ovis*. In another study, the 16S rRNA sequence for species categorization of *Anaplasma* samples revealed identities below the 98.7% threshold, implying that two bacteria are distinct species (Caudill & Brayton, 2022). Moreover, it was observed that

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utilizing sequences that only encompass a few hypervariable regions of the 16S rRNA gene should be avoided as it may lead to misclassification of species. The intraspecies population heterogeneity for each *Anaplasma* 16S rRNA sequence remains impossible to determine rigorously, but these preliminary findings warrant further investigation.

5 | CONCLUSIONS

The present investigation provided morphological variations among various tick genera namely: *Rhipicephalus*, *Hyalomma*, *Haemaphysalis and Ixodes* spp. Further molecular characterization of *A. marginale* based on 16*S rDNA* genetic marker was investigated and phylogenetic analysis revealed close similarity with *A. marginale* isolates from Iran, Vietnam and Pakistan. Additionally, the intraspecies population heterogeneity for each *Anaplasma* 16S rRNA sequence remains impossible to determine rigorously, but these preliminary findings warrant further investigation.

AUTHOR CONTRIBUTIONS

Ayesha Malik: Conceptualization; methodology; data curation; writing—original draft preparation; supervision. **Kiran Afshan:** Conceptualization; methodology; supervision; data curation; Funding; Resources; writing—review and editing; visualization. **Mohammad K. Okla:** Formal analysis; funding; writing—review and editing. **Ibrahim A. Saleh:** Resources; writing—review and editing; project administration. **Abdul Razzaq:** Data curation; software; validation. **Munib Hussain:** Software; validation; writing—review and editing. **Sabika Firasat:** Investigation; visualization; writing—review and editing. **Gulnora Mirzaeva:** Resources; investigation; writing—review and editing. **Bakhtiyor Kholmatov:** Investigation; resources; writing—review and editing. **Erinda Lika:** Formal analysis; resources; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have declared the no potential competing interest.

CONSENT TO PARTICIPATE

Data have been provided with information about the purpose, procedures and potential risks and benefits of this study.

CONSENT TO PUBLISH

The data or findings resulting from your participation may be used in publications, presentations or other scholarly activities.

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