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Molecular Identification of Ixodid Tick Species and their Screening for Selected Protozoan Pathogens Collected from Large Ruminants of Azad Kashmir, Pakistan

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MUH, AR and AS planned and designed the research experiment. AM executed the research trials and wrote the initial draft of manuscript. MAASH, SUR and MK analyzed the data. AAF, AR and AZ critically revised the manuscript and approved the final version.

Key words
Ixodid ticks, Ectoparasites, *Theileria* sp., *ITS 2, Rhipicephalus* sp., *Hyalomma* sp., *Haemaphysalis* sp., *Babesia* sp.,

ABSTRACT

Ticks (Acari: ixodid) are notorious blood sucking ecto-parasites of wide range of animals which serve as vector of different types of pathogens like viruses, bacteria, rickettsia and protozoa and cause mortality in humans and animals. This study was focused on morphological and molecular identification of ixodid ticks, using morphological keys and an internal transcribed spacer *(ITS-2)* Deoxyribonucleic acid (DNA). Moreover, the presence of *Babesia* and *Theileria* species was also investigated in ticks using *18S rRNA* gene. Identification of ticks collected from 384 cattle and 384 buffaloes screened revealed three tick genera and six tick species: *Rhipicephalus microplus, Rhipicephalus decoloratus, Rhipicephalus annulatus, Hyalomma anatolicum anatolicum, Hyalomma anatolicum excavatum* and *Haemaphysalis punctata*. Of those four species were confirmed on morphological basis, two ticks species *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum* whose morphological feature were overlapping with other identified species, were confirmed through molecular tools amplifying *ITS-2* gene. Ticks DNA were then examined by PCR employing a genetic marker that target (*18S rRNA gene*), for the presence of *Babesia* and *Theileria* species in identified ticks. The most common pathogen species observed in ticks was *Theileria annulata*. This study exposed different hard tick species are prevalent in the study area and these ticks are playing major role in transmission of protozoa (*Theileria annulata*). On the basis of finding of present study an area-wise control strategy for ticks and ticks borne protozoan species have been suggestive.

INTRODUCTION

Ecto-parasite acts as a double edge sword, on the one hand they are vector of many diseases and on the other hand they are incurring economic loss to the farmers as these highly compromise the health status of animals and hide quality. Hard ticks are obligatory blood imbibing arthropods, external parasite of animals and humans (Jongejan and Uilenberg, 2009). They have a major effect on the husbandry and productivity of livestock such as reduced weight gain, loss of milk production, blood loss and damage to quality of hide. They are vectors for many diseases including tick-borne protozoan diseases (e.g. *theileriosis* and *babesiosis*) and rickettsial diseases (e.g. *anaplasmosis* and heart water or *cowdriosis*). Tick-borne pathogens affect 80% of the world cattle population and the estimated annual global loss due to ticks is between US$ 13.9 billion and US$ 18.7 billion (Hussain *et al.*, 2021; Estrada-Pena and Salman, 2013). Among hard-tick taxa on the taxonomic and phylogenetic interactions

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there is a need for detailed studies and for this purpose morphological methods have been extensively used but relying merely on morphological approaches in some species may be questionable (Guglielmone *et al*., 2014). Molecular identification methods, which focus on DNA sequence variations, seem to provide a better tool for the evaluation of differences within and among tick species (Cruickshank, 2002). In the tick genome as compared to coding regions there is a rapid assessment of the internal transcribed spacer 2 segments *(ITS-2)* and to discriminate between closely related species *(ITS-2)* regions has been used (Hillis and Dixon, 1991). Many molecular studies on ticks have used *ITS-2* gene for the identification of different tick species (Brahma *et al*., 2014; Lempereur *et al*., 2010). Molecular technique such as PCR has been widely used in veterinary parasitology in recent years to identify several parasitic pathogens including blood protozoa. Several studies documented that PCR is more specific and sensitive than conventional techniques in determining infectious agent carriers (Salih *et al*., 2015) therefore; testing ticks for the presence of pathogens using polymerase chain reaction-based methods provides distinct advantages over conventional detection methods. Only a single study of Sultana *et al*. (2015) is available on the topic from this study area earlier to present study associated with certain limitations. On the basis of review of literature and current scenario in the study area present research work has been planned with following objectives: Molecular and morphological identification of ticks infesting bovine (cattle and buffalo), and screening of ticks carrying protozoan pathogens (*Babesia* and *Theileria* spp.) in order to devise control measure against ticks in the study area.

MATERIALS AND METHODS

Study area

The study was conducted in District Poonch of Azad Kashmir. Tick specimens were collected from three Tehsils (Rawalakot, Abbaspur, Hajeera) of District Poonch. The study area is located at an approximate geographic coordinate of 33º–36º North latitude and 73º–75º East longitude. The occupied Poonch District of central Kashmir bond the region on East, Rawalpindi city is located on the West, Tatta pani and Kotli on the South and Suddhen Gali Muzaffarabad on the North side of the study area (Sultana *et al*., 2015).

Sampling and tick identification

A total of 865 tick samples were collected. A total of 768 household animals, (cattle 384 and 384 buffalo) were examined for tick infestations. Of the 768, 325 (42%) were infested with ticks. Samples were randomly collected from different body parts of animal body and preserved in 70% ethanol. Illustrated taxonomic keys of Walker *et al*. (2014) were used for identification of ticks.

Molecular identification of ticks

The DNA of 50 tick samples of morphologically closely related species was extracted using DNA extraction kit [The WizPrep™gDNA Mini Kit (Cell/Tissue)] Wizbio solutions following the manufacture's protocol for molecular identification of ticks whose physical characters were overlapping and detection of selected protozoan pathogen in ticks.

ITS 2 region of the extracted DNA was PCR amplified using the pair of previously published specific degenerative primers (Abdigourdarzi *et al*., 2011).

ITS-F 5′-YTGCGARACTTGGTGTGAAT-3′and
ITS-R 5′TATGCTTAARTTYA GSGGGT-3′

The 40 µL of total PCR reaction mixture consisted of 8 µl of distilled water, 100 ng/µl of genomic DNA as the template, 1 µl of each primer (10 pmol/µL) and 20 µl PCR master mix (The WizPure™ PCR 2X, Wiz Bio Solutions). The amplification conditions for *ITS 2* region are as follows: initial denaturation at 94°C for 2.5 minutes (min), denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min and final Extension at 72°C for 30 min. Nuclease free water was used as negative control. PCR amplifications were performed in T-100 thermocycler (Applied Biosystems Veriti 96 wells 2720 thermocycler Germany).

Molecular identification of pathogens

A pair of previously published primers designed for *18S rRNA* gene amplification of pathogens was used i.e Forward 5′CACAGGGAGGTAGTGACAAG3′and Reverse 5′AAGAA TTTCACCTATGACAG-3′ (Motavalli-Haghi and Fakhar, 2013). The PCR conditions for *18S rRNA* gene amplification were set as follows: Initial denaturation at 95°C for 5 min, denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 45 sec, final extension at 72°C for 10 min.

PCR product of tick *ITS 2* gene ranging in size from 800-1500 bp was separated on 1% agarose gel, while that of pathogen *18S rRNA* gene ranging in size from 380-430 bp was separated on 1.5% agarose gel. Ladder from BioLabs® inc (1kb and 100 bp) was used as a size marker. PCR products were later run on horizontal gel electrophoresis system (Wide mini sub® cell GT, Bio-Rad, Pakistan) and visualized under Gel Imaging System (JY04S-3C, Beijing, China).

Sequencing and phylogenetic analysis of ITS 2 *and* 18S rRNA *genes*

The positive amplified PCR products and gel bands of tick (6 samples) and pathogen species (2 samples) were investigated and then preceded for sequencing and phylogenetic analysis. For gene sequencing, the samples were sent to Macrogen® Korea for Sanger sequencing using ABI 3730 XL, the standard DNA sequencer. The sequences obtained from the present study were further submitted to GenBank for accession numbers. Sequences derived from this study and other sequences present in GenBank database were aligned using NCBI Blast. A comparison was made among the sequences of *ITS 2* gene and *18S rRNA* gene from this study with similar gene sequences of other studies. For phylogenetic analysis the gene sequence results were analyzed and the contig file was generated using Geneious prime tool (http://www.geneious.com). The contig file was generated using assemble and align tool available in program. A consensus was generated using chromatogram. The phylogenetic trees were constructed with the help of partial *ITS-2* and *18S rRNA* gene sequence data. The evolutionary history was inferred by using the Neighbor-joining method (Saitou and Nei, 1987) and evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004). The distances were computed mean-wise and overall using MEGA 10. Sequences were subsequently analyzed with neighbor joining to construct the phylogenetic tree (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Tick identification

Three genera (*Rhipicephalus, Hyalomma* and *Haemaphysalis*) and six tick species (*Rhipicephalus decoloratus*, *Rhipicephalus annulatus*, *Rhipicephalus microplus*, *Hyalomma anatolicum excavatum, Hyalomma anatolicum anatolicum* and *Haemaphysalis punctata*) were identified during present study on the basis of their physical characters (Table I). *Rhipicephalus* (71.67%) was found to be most prevalent, followed by *Hyalomma* (21.84%) and *Hemaphysalis* (6.4%). Table II shows specie wise distribution of ticks. Similar reports on the topic have been published earlier from other parts of the world (Khan *et al*., 2022; Ali *et al*., 2016; Gudina *et al*., 2016; Sultana *et al*., 2015). The morphologically identified species during the present study were found similar to tick species reported by other researchers (Jabeen *et al*., 2022; Mossad *et al*., 2021; Ramzan *et al*., 2020; Ghaffar *et al*., 2020; Hosseni *et al*., 2013; Patel *et al*., 2013). However, other studies (Jobir and Gure, 2021; Batool *et al*., 2019; Ramadan *et al*., 2016) also reported different ticks species found infesting bovines. This difference in results of prevalence percentage of different species in present and other studies are due to climate and topography of the study area, farmer's knowledge about ticks, husbandry practices, grazing pattern, treatment and control measures. This statement also endorsed several other studies (Iqbal *et al*., 2014; Ali *et al*., 2013; Greenfield, 2011; Sajid *et al*., 2009). On the basis of morphological features numerous species belongs to genus *Rhipicephalus* and *Hyalomma* are easy to distinguish. However, *Rhipicephalus microplus,* and *Hyalomma anatolicum anatolicum* are closely related to other species of the same genus (Abdigoudarzi *et al*., 2011). The bodily features of *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum* were overlapping with other species having nearly similar physical appearance so they were further confirmed using molecular tools (Jabeen *et al*., 2022).

The PCR products for tick *ITS-2* gene were subjected to sequencing. BLAST queries of the resulted sequenced nucleotides indicated the sequence identity with *ITS-2* gene of *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum*. For comparative purposes, the sequences of *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum* were aligned from NCBI database. The four nucleotide sequences of *Rhipicephalus microplus* were submitted to GenBank and assinged numbers were: (Genbank: MZ 458595.1, MZ458596.1, MZ452632.1 and MZ458594.1). Two nucleotide sequences of

Table I. Identification characteristics of different tick species.

Features	*Rhipicephalus microplus*	*Rhipicephalus decoloratus*	*Rhipicephalus annulatus*	*Hyalomma anatolicum*	*Hyalomma excavatum*	*Haemaphysalis punctata*
Palps	Short	Short	Short	Long	Long	Short
Mouth parts	Anterior/short	Anterior/short	Anterior/short	Protruding out wards/large	Protruding out wards/large	Small/short
Festoons	Absent	Absent	Absent	Reduced in number	Reduced in number	11 in number
Legs	No pale rings	No pale rings	No pale rings	Patchy marbled pale rings	Distinct pale rings	No pale rings
Hyposomal teeth	4+4 in column	3+3 in column	4+4 in column	--	--	--

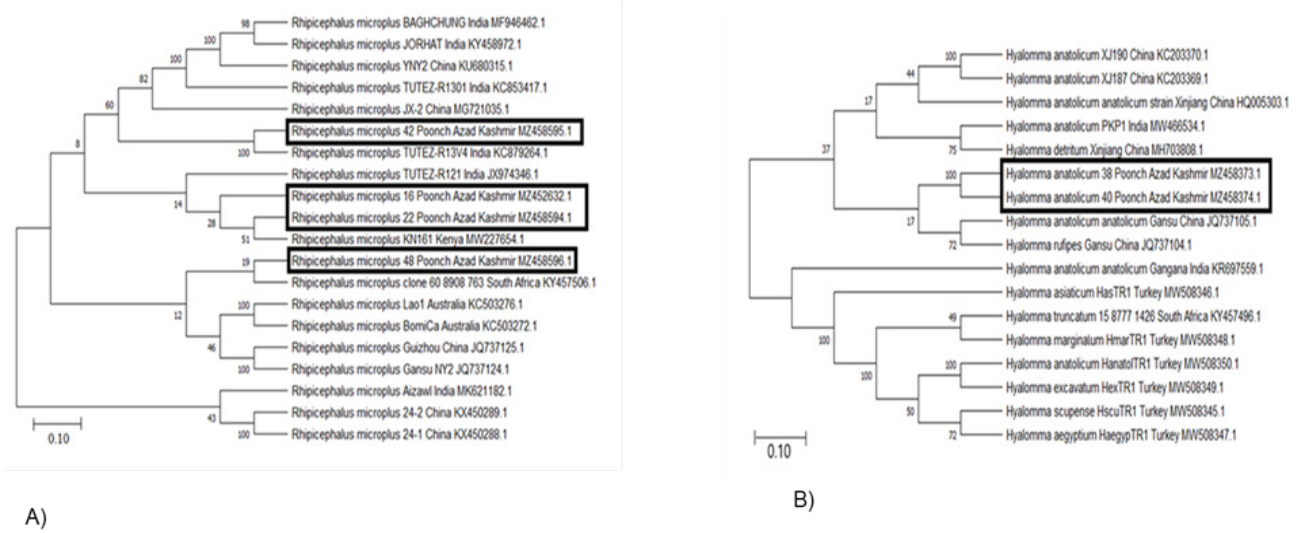

Fig. 1. (A) Phylogenetic tree showing evolutionary relationship of *Rhipicephalus microplus.* (B) Phylogenetic tree showing evolutionary relationship of *Hyalomma anatolicum.*

Table II. Species wise distribution of tick species.

Tick species	District Poonch	
	Frequency	%
Haemaphysalis punctata	56	6%
R. decoloratus	86	9%
Hy. anatolicum	93	10%
Hy. anatolicum excavatum	96	11%
R. annulatus	210	24%
R. microplus	324	37%

Hyalomma anatolicum anatolicum were also submitted to Genbank and assigned accession numbers were (GenBank: MZ458373.1 and MZ458374.1). The sequences of *ITS-2* gene of *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum* were compared with sequences reported by other researchers. A phylogenetic tree was constructed based on alignment with those sequences retrieved from NCBI database that showed high homology with our sequences (Fig. 1). Phylogenetic analysis demonstrated that the sequence of *ITS-2* gene obtained in the present study were showing 85 to 100% homology with most of the *ITS-2* gene sequences of *Rhipicephalus* and *Hyalomma* ticks' worldwide. The similarity of *Rhipicephalus microplus* with the reference strains of the China (Genbank: KU680315.1) is 99% and with the reference of India (Genbank: KC85341.71) is 100%. The similarity of *Hyalomma anatolicum anatolicum* with the reference strains of the India (Genbank: KR697559.1) and China (Genbank: HQ005303.1) ranged between 99 % and 100%.

Our study supports that *ITS-2* gene is a reliable tool for discriminating different genera and species of Ixodidae family members. Molecular identification of *Rhipicephalus microplus* and *Hyalomma anatolicum anatolicum* in our study is in parity with the findings of other researchers (Ghaffar *et al*., 2020; Rehman *et al*., 2017; Chhillar *et al*., 2014; Ganjiali *et al*., 2014; Baker and Walker, 2014; Abdigoudarzi *et al*., 2011; Lempurer *et al*., 2010) who confirmed similar tick species on the basis of *ITS-2* gene. On the basis of results obtained in present study, it can be concluded that *ITS-2* is a suitable molecular marker for distinguishing different genera as well as species of ticks' including *Hyalomma and Rhipicephalus* (Labruna *et al*., 2009; Dergousoff and Chilton, 2007; Kawther *et al*., 2005).

Molecular characterization of tick borne pathogens

Ticks were also analyzed for the presence of selected protozoan pathogens. Tick DNA samples were examined by PCR for the presence of two pathogens *Babesia* and *Theileria*. Interestingly 9 ticks out of 50 samples examined were found positive for only *Theileria*. All the ticks were found negative for *Babesia*. The base pair length for *Theileria* was estimated ≈420-430 bp according to marker size.

The PCR products for tick *18S rRNA* gene were further subjected to sequencing and phylogenetic analysis. The results of the sequence analysis showed that enquired sequences were of *18S rRNA* gene of *Theileria annulata*. The two sequences were deposited in GenBank for accession numbers and assinged numbers to the genesequences for *Theileria annulata* in present study were:

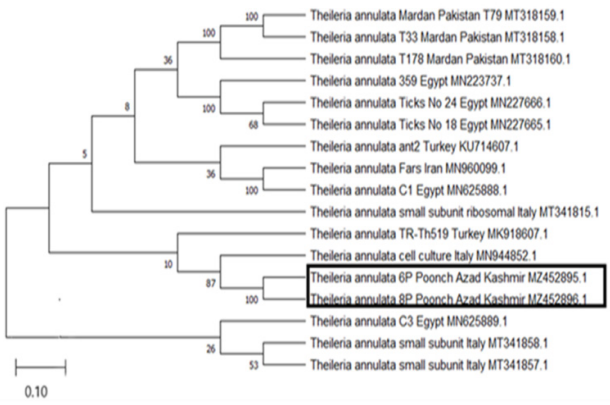

Fig. 2. Phylogenetic tree showing evolutionary relationship of *Theileria annulata.*

(Genbank: MZ452895.1 and Genbank: MZ452896.1). For comparative purposes, the sequences of *Theileria annulata* were aligned from NCBI database. The phylogenetic tree was constructed based on *Theileria annulata* sequences obtained from District Poonch Azad Kashmir with the nucleotide sequences that are 85-100% similar to the reported species (Fig. 2). The similarity index of *Theileria annulata* with the reference strains of the Mardan Pakistan (Genbank: MT318159.1) is 99% and with the reference strain of Turkey (Genbank: KU714607.1) is 100%. In present study, to evaluate presence of protozoan pathogens in tick the *18S rRNA* gene sequence was used. Our study results are in line with those detailed in other parts of world using same gene (Gargano *et al*., 2021; Ghafar *et al*., 2020; Ferrolho *et al*., 2016; Antunes *et al*., 2016; Irshad *et al*., 2010). Like our study (Tavassoli *et al*., 2011) first identified *Rhipicephalus, Hyalomma* and *Hemaphysalis* in their study and then *Theileria annulata* infection in tick vector. Similarly (Lca *et al*., 2007) reported nearly similar prevalence of *Theileria annulata* in ticks. The detection of *Theileria annulata* in ticks can be possibly due to the reason that the main vectors of the said pathogen were first encountered in this study that were further screened for pathogens presence. Microscopic and serological methods are also used for diagnosis of haemo-parasitic infections worldwide (Gubbels *et al*., 2000). However, these diagnostic methods are of limited value due to several limitations, including lower sensitivity and specificity, cross-reactivity, inability to detect carrier infections, and the requirement of expertise and time (Lew-Tabor, 2016; Igarashi and Parrodi, 2014; Mans *et al*., 2015). To overcome these limitations the use of highly sensitive molecular methods, including conventional PCR (cPCR), quantitative PCR (qPCR), nested PCR (nPCR), reverse line blotting (RLB), loop mediated isothermal amplification (LAMP), high-resolution melting (HRM) assays, high-throughput microfluidics-based real-time PCR and the next-generation sequencing (NGS) is in practice (Wang *et al*., 2019; Alessandra and Santo, 2012; Schnittger *et al*., 2004).

CONCLUSIONS

Present study confirms the existence of different tick species in the study area and ticks carry single protozoan species. The prevalence of ticks species and pathogen in the area may associate with the production losses in bovines. Therefore, it is recommended that a proper control measure plan should be formulated and implemented.

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IRB approval

The study was approved by PMAS AAUR Advanced Studies and Research Board under Notification No: PMAS AAUR- /DAS/624.

Ethical statement

Before ticks collection from infested cattle and buffalo prior consent was obtained from the owners.

Statement of conflict of interest

The authors have declared no conflict of interest.

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