Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.063

RESEARCH ARTICLE

Tick Taxonomy and Nucleotide Sequence Analysis by Internal Transcribed Spacer 2 (ITS 2) in Large Ruminants of Pothohar, Pakistan

Farkhanda Jabeen[1], Muhammad Mushtaq[1], Mazhar Qayyum[1], Murtaz ul Hasan[2*], Muhammad Arif Zafar[3], Aayesha Riaz[2] and Farooq Nasir[4]

[1]Department of Zoology, PMAS Arid Agriculture University, Rawalpindi, Pakistan; [2]Department of Parasitology and Microbiology, Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University, Rawalpindi, Pakistan
[3]Department of Clinical Studies, Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University, Rawalpindi, Pakistan; [4]Department of Entomology, PMAS Arid Agriculture University, Rawalpindi, Pakistan
*Corresponding author: murtazhassan@yahoo.com

ARTICLE HISTORY (22-174)

Received: May 19, 2022
Revised: August 18, 2022
Accepted: August 19, 2022
Published online: September 20, 2022

Key words:
Hard ticks
Ruminants
Prevalence
*ITS*2
Phylogenetics

ABSTRACT

Ectoparasites (insect and arachnids) are a worldwide problem, which are responsible for up to 80% losses to the livestock economy. Among these, the Ixodids (hard ticks) are the most important and also transmit diseases like babesiosis, theileriosis and anaplasmosis. The present study was aimed to identify prevalence and distribution of the hard tick species using morphological and molecular keys in large ruminants of Pothohar region of Pakistan. Morphological investigations of ticks from 967 cattle and 665 buffaloes revealed the genera (3) and species (13) of various Ixodid ticks mainly *Rhipicephalus (R), Hyalomma (Hy),* and *Dermacentor (D)* comprised of *R. microplus, R. annulatus, R. australis, R. eversti, R. turanicus, R. decloratus, Hy. dromedarii, Hy. scupens, Hy. rufipes, Hy. truncatum, Hy. excavatum, Hy. detritum* and *D. atrosignatus.* Tick invasion was statistically higher in cattle (53.48%) than buffaloes (40.70%). Among these 10 species identifications were confirmed through morphological identification, however, molecular characterization of tick analysis (using partial *ITS2* gene) confirmed prevalence of *R. microplus, Hy. detritum* and *D. atrosignatus*. This study provides the basic genetic identification tool for investigating the tick species and their phylogenetic relationship in tick species commonly found in humans and animals. The precise identification unquestionably can play a dynamic role in employment of appropriate control measurements, as different genera/species cause different diseases in livestock. Different tick species identified on the basis of morphological features and molecular characterization, will add value to the existing knowledge in identification of ticks in the Pakistan, as well as helpful in various disease diagnosis spread due to these hard tick species.

To Cite This Article: Jabeen F, Mushtaq M, Qayyum M, Hasan MU, Zafar MA, Riaz A and Nasir F, 2022. Tick taxonomy and nucleotide sequence analysis by internal transcribed spacer 2 (ITS 2) in large ruminants of Pothohar, Pakistan. Pak Vet J, 42(4): 554-560. http://dx.doi.org/10.29261/pakvetj/2022.063

INTRODUCTION

Pakistan being an agri-based country contributes 18.2% to the national gross domestic product (GDP), where livestock shares 60.1% in agriculture and 11.5% in GDP (Pakistan Economic Survey 2020-2021). Government of Pakistan recent investment initiative in livestock sector, playing a key role in Pakistan economy by gross value addition of Rs1,505 billion from Rs 1,461 billion (2019-2020), an increase of three percent (Pakistan Economic Survey 2020-2021). Globally, livestock community is threatened by the ectoparasites affecting 80% of the cattle (Raut *et al*., 2008) and so the Pakistan. These ectoparasites and endoparasites are also considered to be a major obstacle in livestock productivity (Durrani and Shakoori, 2009). In a survey of 2015 by Nawaz and Faraz, the prevalence of blood parasites was estimated to be (18.1%) in cattle, (14.4%) in buffaloes, (12.6%) in camels, (11%) in sheep, and (10%) in goats. Tick infestation has been reported throughout the world but restricted to tropical and subtropical regions including Pakistan. Ticks also spread diseases to human, recently blood nose fever by tick in middle east in animal farm workers (Brites-Neto, 2015).

A considerable loss of animal population has been recorded due to diseases associated with ticks (Kabir *et al*., 2011). Climatic conditions in Pakistan are conducive for propagation and expansion of ticks (Durrani and Kamal, 2008).

In past, the morphological identification method was preliminary procedure for ticks but comes with critical infirmities due to complicated morphological characters in the studied species which may lead to misidentification. In particular, it was hard to recognize the specimen which are physically impaired, engorged with blood meal, at immature life cycle stages, even for cryptic or sibling species (Lv *et al*., 2014). The modern biotechnological tools present an excellent solution to these problems. The molecular markers *ITS* 2, *COI*, 12SrDNA and 16S rDNA are suggested for identification of species for DNA sequences (Lv *et al.,* 2014). Various studies had employed genetic markers i.e COX 1, 16S rRNA and *ITS* 2 for correct molecular identification, also for phylogenetic relationships of different organisms including hard ticks (Lv *et al.,* 2014).

The Pothohar region of Pakistan has not been studied extensively in recent and studies must be performed regularly to keep the tick data and diversity information updated. These up to date periodic surveys may help in designing, employment of promising control measures. Therefore, the present study was aimed for identification of hard tick fauna, their prevalence among large ruminants, and identify the significant risk factors in Pothohar, Pakistan.

MATERIALS AND METHODS

Study area and sampling method: Pothohar region of Pakistan comprises of Jhelum, Chakwal, Attock, Rawalpindi and Islamabad. Four season i.e summer (May to August), spring (March to April), autumn (September, October), winter (November to February) prevails in this region. Pothohar plateau exhibits extremely hot summer and winter with cold condition with an average annual rainfall of 812 mm spanning from July to September. However, the highest temperature sustains till June and minimizes along the rainfall spell while the observed coldest month is January (14.6 to 18.7°C), (Pakistan Meterological Department 2018-2019).

In the present study, the formula for the sample size calculation was followed as described by Thursfield (2007).
$N = 1.962 \times P\ exp\ (1\text{-}Pexp)/d^2$
Whereas, N = the required sample size; P exp = the Expected prevalence; and d = Desired absolute precision (0.05). Sampling was done conveniently during the study. Total 3788 tick samples were collected from local/cross bred cattle (n = 967) and buffaloes (n = 665) from the study area. The forceps were used for tick sampling without damaging their mouthparts (Soulsby, 1982), after the thorough examination of different body parts such as ears, udder, neck and tail etc. A questionnaire was prepared to collect preliminary data from the study area, which contained the protocols for tick sample collection and host species. Commercial farms were selected for this study those having animals between 15 - 40 heads and with mixed/separate farming patterns, cemented/non-cemented and with/without boundary conditions.

Storage and tick identification: The tick samples collected in clean and dry labelled plastic caps bottles for taxonomic studies, were preserved in 70% ethanol (Riaz and Ullah, 2015) and stored in the laboratory at Department of Parasitology and Microbiology, Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University, Rawalpindi, Pakistan. Tick samples were run through morphological identification using stereomicroscope (Meiji techno, UK) with key characteristics i.e mouth parts, legs coloration, scutum, conscutum, genital aperture, adanal plates, adanal accessory plates and body colour pattern up to genus and species level (Walker *et al*., 2014). Various risk factors/associated factors i.e gender, age group, health status of cattle and buffalo were studied and body condition scoring was done. Tick distribution in various parts of host animals and their seasonal prevalence also ruled out during the study.

DNA extraction and *ITS*2 amplification: Three ticks which were not identified and confirmed by morphological identification procedure, processed for molecular characterization. The DNA was extracted from tick samples by following protocol by Wiz prep gDNA kit Cell/Tissue (WIZBIO SOLUTIONS South Korea). The DNA concentration of each sample was estimated using a Nanodrop ND1000. A pair of specific degenerate primers (Abdigoudarzi *et al*., 2011) designed for ITS2 amplification was used i.e forward 5´-YTGCGARACTTG GTGTGAAT-3´ and reverse 5´- TATGCTTAARTTYA GSGGGT-3´. To perform the PCR reaction, master mix Taq 2X (New England Biolabs, UK) was selected. The approximate quantity of extracted DNA was measured to be around 100 ng/µl in reaction mixture of 50µl for the PCR (Applied Biosystems Veriti 96 wells 2720 thermocycler Germany), along with reverse and forward primers (2µl each). The PCR conditions was set as initial denaturation for at 94°C for 2.5 minutes, followed by 35 cycles (denaturation at 94°C for 30 seconds, annealing for 1 min at 51°C, extension at 72°C for 1 minute) and final extension at 72°C for time of 30 minutes. Nuclease free water was used as negative control.

The PCR processed products were cleaned up by Wiz Prep Gel/PCR cleanup kit protocol (Wizbio Solutions South Korea) as described by Kasi *et al*. (2020). The PCR products were separated on 1% agarose gels which was 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).

Sequence and phylogenetic analysis: The positive gene amplification and gel bands were investigated, then proceeded for sequencing as well as phylogenetic analysis. For the gene sequencing, samples were dispatched to Macrogen® Korea for sequencing using ABI 3730 XL, the standard DNA sequencer. The phylogenetic trees were constructed with the help of partial *ITS*2 sequence data. All the sequences obtained during study and reference sequences deposited in the GenBank were then aligned by multiple alignment program CLUSTAL_W procedure in the Seaview® software (2014). The evolutionary history was inferred by using the Neighbor-joining method (Saitu and Nei, 1987) and evolutionary analyses were conducted in MEGA X

(Kumar *et al*., 2018), whereas, evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

All distances were then computed mean-wise, however overall used the MEGA-X®. The sequences were later analyzed by NJ (neighbor joining) method to assemble the phylogenetic tree (Kumar *et al*., 2018).

Data analysis: The data regarding prevalence of hard tick infestation was calculated by the following formula (Thrusfield, 1995).

$P=d/n \times 100$

Where, P =prevalence, d=number of animals found positive, n=total number of animals sampled

Data concluded for various tick species prevalence and linked determinants by Pearson's Chi-square (χ2) test which was statistically analyzed, through (SPSS) version 20. ANOVA (two-way) test and Regression model analysis were also performed during study.

RESULTS

Based on our study in the Pothohar region of Pakistan Table 1 summarizes the percent prevalence of tick identified in all districts of sampled areas. The distribution of tick genera revealed the dominant genera-*Rhipicephalus*. Total, three tick genera i.e *Rhipicephalus* (71.60%), *Hyalomma* with 27.16% prevalence and *Dermacentor* genera with 1.2% prevalence identified in Pothohar region. While, a total of 13 tick species were identified,10 species were identified on the basis of clearly distinguished morphological features including *Rhipicephalus annulatus, R. australis, R. decloratus, R. turanicus, R. eversti, Hyalomma dromedarii, Hy. scupens, Hy. rufipes, Hy. truncatum, Hy. excavatum,*3 hard tick species i.e *Rhipicephalus microplus*, *Hy. detritum* and *Dermacentor atrosignatus* were identified genetically, their respective prevalence is given in Table 1.

Various risk factors associated with tick infestation were studied. The cattle were found to be significantly more infested 53.48%, than the buffaloes 25.88%. In gender wise distribution, females in both categories were significantly more infested than the males. In cattle, females were 78.73% significantly more infested than male host (37.15%). While in buffalo, females were significantly more infested (41.52%) than the male host animal with 17.75% (p=0.00) prevalence (Table 2). Percent prevalence has been reported to be significantly higher in cattle calves (56.87%) and buffalo calves (66.01%) in contrast to young cattle (43.12%), young buffaloes (33.83%), as depicted in (Table 2). As extremely slim host animals with protruding dorsal spines, enabled to touch and the evident transverse processes so that finger could be easily pushed, were categorized as poor body condition. The medium (good) body condition scoring was done for animals with visible ribs along with minute fatty cover and hardly noticeable dorsal spines. Then the scoring of very good body condition was assumed for animals with fatty cover visible in critical parts and could be sensed as well as absence of the transverse processes (Kemal *et al*, 2016). Cattle and buffalo of very good body condition had significantly higher tick infestation, followed by host animals with apparently good (medium) body conditions, whereas the least infestation was observed in poor body conditioned host animals as given in Table 2. Two-way ANOVA analysis showed significant results (Table 3) for hard tick preferred attack on various body parts (udder, neck, ear, tail, leg, brisket) of cattle and buffalo. The preferred attacking sites and number of *Rhipicephalus* collected were (275, 260) from udder, tail (210,170) for *Hyalomma* and ear (13,12) for *Dermacentor* in cattle and buffalo, respectively.

The regression analysis of seasonal distribution of ticks with risks factors i.e temperature and humidity variation in the summer, spring, autumn and winter season, had significant influence on the tick proliferation and distribution. In summer season, cattle tick infestation was high as 48.94%, than buffaloes 48.92%, which decreased in the spring in cattle (28.25%) and in buffalo (24.71%). The incidence further reduced in ~~rainy~~ autumn season i.e. 11.89% and 10.11% in winter season, in cattle and buffalo, respectively. The lowest infestation was documented during winter 10.90% (cattle) and 9.08% (buffalo) host animals (Table 4).

Hard tick identification by PCR, Gel electrophoresis and molecular characterization: DNA was extracted from the tick samples and utilized for the amplification of *ITS*2 gene. Positive PCR products yielded bands of approximately 1100- 1300 bp, Fig. 1(a, b, c) shows the amplified products with their respective band size obtained from the gel electrophoresis.

The sum of all sequence analysis showed similarity index of 93%-100% with the sequences containing *ITS*2 gene deposited in GenBank. Six nucleotide sequences were later deposited in the GenBank and assigned accession numbers i.e. MW580928, MW584975, MW584976, MW580866, MW580713, MW580878. The all sequences were then edited and blasted in GenBank, got the identification through sequence comparison with the closely linked sequences of *ITS*2 gene (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The higher similarity index of sequences with the study sequences proved to be the best matches, and so downloaded for the phylogenetic study analysis. Based on BLAST (NCBI) findings, *ITS*2 gene similarity with the other study sequences suggests that *ITS*2 gene appeared as conservative for tick species.

The study sequence FK 35(accession number MW580866) clustered with the reference sequences (KC503276, KX450289, MF946462, MK621182, KY458972) deposited in the GenBank and found to be 78-95% similar. Study sequence FK 31 *(*accession number MW584976) showed 96% similarity with reference sequences (KC503264, KX450287, MG721035, MK224560) deposited in the GenBank. The study sequence FK 40 (MW580878) showed 100% similarity with sequences (MH703808, KC203390, KC203391, JX845149, KC203394) deposited in GenBank. The study sequence FK41 (MW580866) found to be 91% similar to the reference sequences (AF271279.1), submitted in GenBank. The study sequence FK 44 (MW584975) showed 100% similarity with the reference sequences i.e MT297636.1, KF956798.1, KF956796, KF566802, KF956796.

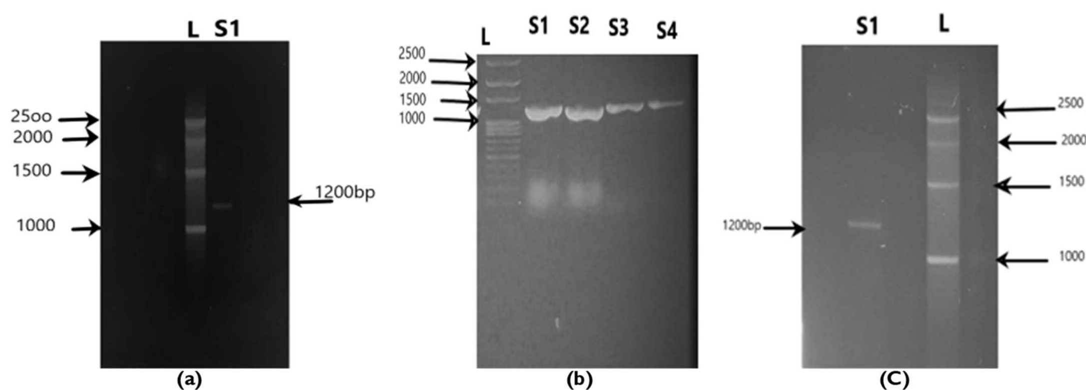

Fig. 1: Amplified products of tick samples with *ITS2* primer, L (1000 bp), (a) *S1(Dermacentor atrosignatus* 1200 bp), (b) *S1(R. australis* 1100bp*), S2(R. annulatus* 1200bp*), S3 (R. eversti* 1300bp*), S4 (R. microplus* 1400bp), (c) *S1(Hyalomma detritum* 1200 bp).

Table 1: Tick species identified and percent prevalence of hard tick species in all districts of Pothohar region

Tick spp.	Jhelum	Attock	Chakwal	Rawalpindi	Islamabad	% prevalence
R. microplus	245	165	180	95	70	20.12
R. australis	220	150	170	71	55	17.75
R. annulatus	218	140	162	50	30	15.99
R. decloratus	120	30	80	15	10	6.79
R. turanicus	80	15	115	25	15	6.66
R. eversti	60	-	20	50	30	4.26
Hy. dromedari	18	13	32	110	90	7.01
Hy. detritum	14	11	22	120	93	6.93
Hy. rufipes	30	8	29	104	89	6.93
Hy. truncatum	27	4	19	35	17	2.71
Hy. excavatum	9	11	7	15	12	1.43
Hy. scupens	13	12	8	29	18	2.13
D. atrosignatus	46	-	-	-	-	1.22

R=*Rhipicephalus*, Hy= *Hyalomma*, D= *Dermacentor*.

Table 2: Risk factors/ associated factors for tick infestation on host animals in Pothohar region

Study variables	Animal gender & Categories		Animal examined/animal infested	% prevalence	p-value
Cattle	Gender	Total	1808/967	53.48	0.00
		Female	710/559	78.73	0.01
		Male	1098/408	37.15	
	Age	Calves	967/550	56.87	0.03
		Young	967/417	43.12	
	Health status	Poor	967/100	10.34	0.01
		Good	967/308	31.85	
		Very good	967/559	57.80	
Buffalo	Gender	Total	2569/665	25.88	0.00
		Female	879/365	41.52	0.01
		Male	1690/300	17.75	
	Age	Calves	665/439	66.01	0.03
		Young	665/225	33.83	
	Health status	Poor	665/67	10.07	0.01
		Good	665/259	38.94	
		Very good	665/339	50.97	

Pearson's Chi-square test, p-value=0.00-0.03.

Table 3: Two-way ANOVA of tick distribution in various parts of the cattle and buffalo

Categories	df	Sum of sq	Mean sq	F value	Pr (> F)
Cattle					
Body parts	5	77253	15451	3.584	0.0407
Tick genera	2	388273	194137	45.029	9.97e-06*
Residuals	10	43114	4311		
Buffalo					
Body parts	5	77521	15145	3.5654	0.0309
Tick genera	2	388312	19471	45.011	9.97e-05*
Residuals	10	43165	4121		

Significant. codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ' ' 1.

Phylogenetic analysis: The phylogenetic tree (Fig. 4) i.e *D. atrosignatus* (FK 44) clustered together with sequences deposited in Genbank and showed 99% similarity. The *Hy. detritum* (FK 40) phylogenetic analysis (Fig. 3) was clustered along with the reference sequences and shared similarity value of 100%. The phylogenetic analysis (Fig. 2) *R. microplus* (FK35) was clustered with reference sequences and shared similarity up to 100%.

DISCUSSION

The present study carried out on associated risk factors/prevalence and distribution of tick in various regions, various body parts and seasonal distribution presented recent trends of infestation. The trend was found within the 95% confidence interval similar as in the previous studies but the incidence of tick population was high. During the study, female cattle host species and calves age group of both host species showed higher prevalence. In both cattle and buffalo hard tick preferred udder, neck, ear, tail, leg and brisket. Female host species

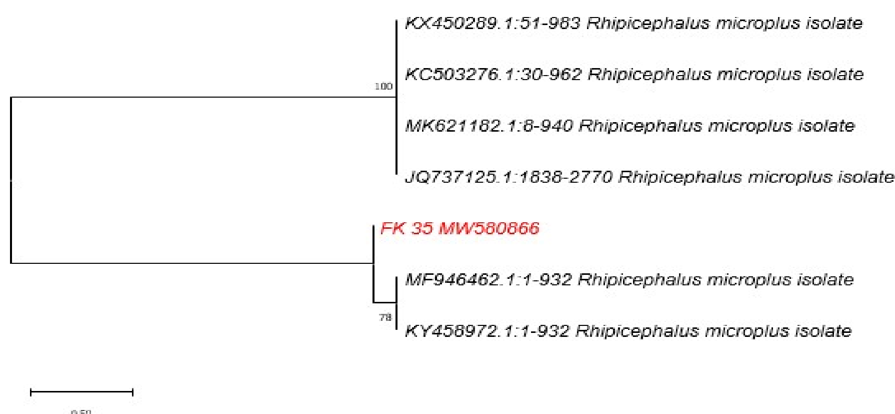

Fig 2: Evolutionary relationships of FK 35 (*R. microplus*).

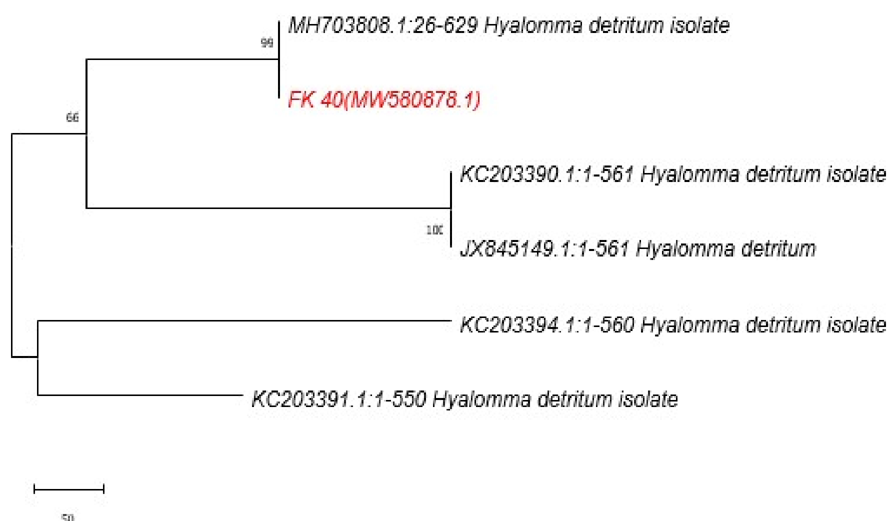

Fig 3: Evolutionary relationships of FK 40 (*Hy. detritum*).

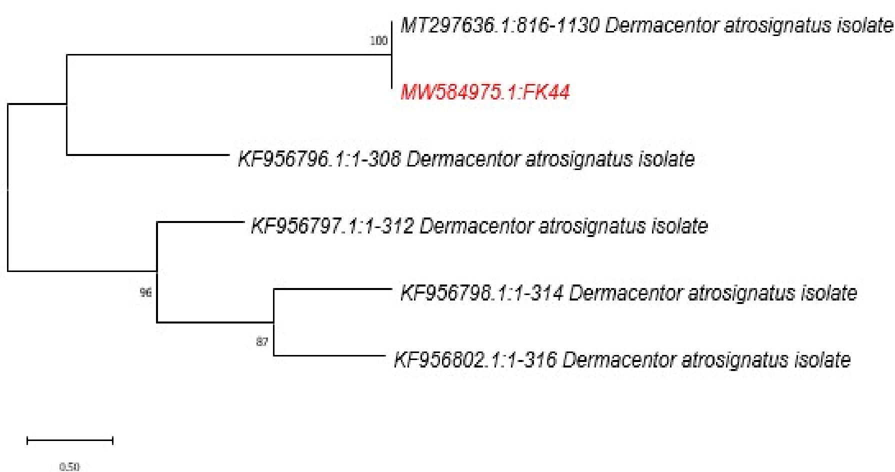

Fig 4: Evolutionary relationships of FK 44 (*D. atrosignatus*).

Table 4: Regression analysis for seasonal tick prevalence in cattle and buffalo in Pothohar

Host specimen	Season	% prevalence	t-value	Multiple R^2	Adjusted-R^2	p-value
Cattle	Summer	48.94	36.30	0.9987	0.997	0.04
	Autumn	11.89	30.31	0.9977	0.999	0.01
	Winter	10.90	25.15	0.9998	0.9254	0.00
	Spring	28.25	35.30	0.9978	0.9852	0.00
Buffalo	Summer	48.92	37.38	0.998	0.996	0.00
	Autumn	10.11	33.76	0.996	0.998	0.01
	Winter	9.08	24.55	0.999	0.997	0.00
	Spring	24.71	35.66	0.992	0.996	0.00

and calves were significantly more prone to infestation than the males and other age group including adult, young, old in both the categories of cattle and buffaloes (Ali *et al*., 2016). Udder showed high significance for tick attacking site in host then inner thigh part, perineum, leg area, tail, lastly neck body part, respectively, followed by

neck, the tail, perineum, inner thigh parts, area of legs, back section, and ears domain, respectively in buffaloes (Ali *et al*., 2016). The immediate parts of host body tick attacking were head, tail, udder, thorax and abdomen, where the highest tick invasion was on tail after that udder gradually had lesser tick population in abdomen, head region, while thorax was less preferred (Abbasi *et al*., 2017). Ticks prevalence as by Kakar and Kakarsulemankhel (2008), in Quetta, Pakistan and infestation was recorded to be 10.14% in cows and 6.99% in buffaloes, respectively. In, Ahmed *et al.* 2012 recorded tick infestation rate of 65.6% in cattle, and *Rhipicephalus* was found to be more prevalent than genus *Hyalomma,* while Ali *et al.* (2013) reported 70% in cattle and 34% in buffaloes. One of the reasons for higher tick prevalence in cattle might be associated with the cross breed which could be much more susceptible to tick infestation. The present study coincides with Durrani and Shakoori (2009) who showed high prevalence of genus *Hyalomma*, followed by *Haemaphysallis, and Rhipicephalus*, respectively in cattle farms in Lahore, Multan and Rawalpindi. Similarly, the genus *Hyalomma* was the major tick in buffalo and cattle of Layyah and Muzaffargarh (Sajid *et al.,* 2009).

This study provides the first insight into the molecular diversity of hard ticks from large ruminants in the Pothohar region, Pakistan. However, the present study investigated for the first time the genetic identification methodology for *Hy. detritum* and *D. atrosignatus* from Pakistan. The distribution of few *Hyalomma* spp., as well as *Rhipicephalus* spp. characterized in current study confirms the prevalence of tick as reported in the past based on morphological identification of tick in the large and small ruminants surveyed in Pakistan (Ali *et al*., 2019; Rehman *et al*., 2019; Zeb *et al*., 2020).

Our findings are in parity with the findings of (Durrani and Shakoori 2009; Ali *et al.*, 2016) who also reported high tick infestation during the summer season. The summer season investigated with $P<0.05$ significance, showed the most favorable season statistically for ticks proliferation, then winter and spring, while autumn with the lowest infestation (Ali *et al*., 2016). Cumulative tick infestation rate was significantly high $p<0.05$ (34.79%) in area of hot arid drift than hilly plane with cold which was 18.63% at $p<0.05$ in KPK, Pakistan (Khan *et al*., 2013). The results exhibited the commonality of tick infestation was related to animal species, study area and season. The highest prevalence was in summer followed by spring, autumn and winter seasons. The fluctuation of temperature and humidity from May to September were optimum climatic conditions for tick proliferation. Tick infestation was high during these months, while the prevalence of ticks in all the districts of Pothohar zone varied. These deviations in tick prevalence might be due to variation in geographic pattern, climatic conditions and temperature zones of the province, Punjab (Iqbal *et al.,* 2013).

Ticks were found to be difficult for taxonomic study and identification because of occurrence of cryptic species, damaged body parts as well as other physical impairments hard to handle for morphological characterization. Fewer tick samples observed and viewed with lost body part i.e the conscutum partially tampering with ornamentations, few morphological alterations noted on tick's body samples which may lead to incorrect identification (Abdigoudarzi *et al*., 2011). But due to phylogenetic analysis, it was thought to be quite helpful and accurate method to identify various tick genus as well as species. In the molecular-phylogenetic analysis of *Hyalomma* spp*, Rhipicephalus* spp as well as *Dermacentor* spp. the sequences were verified and supported the morphological characterization method.

The present study samples of hard tick were genetically identified then compared with various sequences from GenBank, and the *R. microplus* showed 99%*,* and *R. annulatus* 96%*, R. australis* 99%*, D. atrosignatus* 100% and *Hyalomma detritum* 93% similarity to the sequences of the reference species reported in the past. The results suggested the identified hard tick species could be regarded through genotypic identification and in phylogenetic tree analysis, all species placed to their respective monophyletic group clustering together with their reference sequences. However, the results deposited in NCBI blast revealed the considerable some nucleotide difference from earlier sequence report of the similar species, *R. microplus* (KC503264, KX450287, MG721035, MK224560, MK224573) *Dermacentor* sp., MT297636.1, KF956798.1, KF956797.1. These differences might be partially due to insubstantial accessibility of tick gene sequence. However, lower percent similarity value of 93 was showed by the *Hyalomma detritum* species with reference accession sequences already reported in GenBank, reason could be the diverse evolution due to geographical separations (Taberlet *et al*., 1992). However, it could also be justified by single nucleotide substitution mutation in two sequences due to variation arisen as a result of their widespread distribution. That was coincided with previous reports suggesting that the sequence divergence occurred because of phylogeographical units in the given species (Avise and Walker, 1999). Such similarity index could also be linked with a factor of cryptic hybridization which resulted in nucleotide substitution (Rees *et al*., 2003).

Current study results confirmed that *ITS2* marker is appropriate for genus level tick identification (*Hyalomma*, *Rhipicephalus*, *Dermacentor*), as observed in previous studies as well, and also indicated that this marker can be effectively employed for species level discrimination (Soltan *et al*., 2020). Abdigoudarzi *et al*. (2011) observed that the partial amplification of *ITS*2 region is useful in different tick species discrimination. In this research, partial sequence of *ITS* 2 region was amplified and used in tick identification and various species were identified on the basis of segment sizes. Different DNA barcoding *ITS*2, 16S rDNA, 12S rDNA and *CO*I molecular markers were used to check their utilization for identification of tick species, in all these *ITS*2 region showed the highest acceptance for inter-specific divergence criteria, however these parameter standard deviations value was large (Lv *et al*., 2014). *ITS*2 is highly acceptable DNA barcode due to its quick substitutional quality which is a good resolving power to detect the closely placed species. The upcoming molecular research should concentrate on genetic and morphological characterization of various genera of tick samples obtained from the climatically diverse areas of Pakistan.

Conclusions: Findings of the present study exhibit that numerous species of *Rhipicephalus* are easy to distinguish morphologically, however, some *Hyalomma* species as well as *Dermacentor* species had some confusing morphological identities so hard to recognize them, therefore, genetic identification was carried out to confirm their identification. *Dermacentor atrosignatus* was first time recorded from Pakistan, however, *Hyalomma detritum* was first time reported in Punjab province (Pakistan). First study in this climate zone, investigated tick species distribution, climate impact on its scattered population leading to rise in tick occupants in summer. Host specific qualities showed significant effect on tick prevalence such as different age groups, predilection site and host gender. The recent study culminates the urge for correct identification of tick species by means of molecular characterization with the use of *ITS*2 gene which is useful marker for interspecific and intraspecific species differentiation. Therefore, correct identification of ticks will be helpful to design, enhance and implement the advanced control measures as well as more extensive awareness programs for farmers welfare.

Acknowledgements: The authors are thankful to the Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University Rawalpindi, for the provision of the laboratory during the study.

Authors contribution: FJ performed experiments and wrote the manuscript. MM, MQ conceived and designed the study. MH, AR executed experiments and analyzed the sample data. FN, AZ and all authors critically revised the manuscript and approved the final version.

REFERENCES

Abdigoudarzi M, Noureddine R, Seitzer U, *et al.*, 2011. rDNA-ITS2 Identification of *Hyalomma, Rhipicephalus, Dermacentor* and *Boophilus* spp. (Acari: Ixodidae) collected from different geographical regions of Iran. Adv Stud Bio 3:221–38.

Abbasi F, Abbasi IHR, Nissa TF, *et al*., 2017. Epidemiological study of tick infestation in buffalo of various regions of district Khairpur, Pakistan. Vet World 10:688.

Ahmed S, Numan M, Manzoor AW, *et al*., 2012. Investigations into Ixodidae ticks in cattle in Lahore, Pakistan. Vet Ital 48:185-91.

Ali Z, Maqbool A, Muhammad K, *et al*., 2013. Prevalence of *Theileria annulata*-infected hard ticks of cattle and buffalo in Punjab, Pakistan. J Anim Plant Sci 23:20–6.

Ali S, Ijaz M, Durrani, AZ, *et al*., 2016. Epidemiological aspects of bovine tick infestation in the river Ravi Region, Lahore. Pak J Zool 48:2.

Ali A, Khan MA, Zahid H, *et al*., 2019. Seasonal dynamics, record of ticks infesting humans, wild and domestic animals and molecular phylogeny of Rhipicephalus microplus in Khyber Pakhtunkhwa Pakistan. Front Physiol 10:793.

Anonymous, 2020. Economic Survey of Pakistan 2020.

Avise JC and Walker D, 1999. Species realities and numbers in sexual vertebrates: perspectives from an asexually transmitted genome. Proc Nat Acad Sciences, 96:992-5.

Brites-Neto J, Daurte KMR and Martins TF, 2015. Tick-borne infections in humans and animal populations worldwide. Vet World 8:301.

Durrani AZ and Shakoori AR, 2009. Study on ecological growth conditions of cattle *Hyalomma* ticks in Punjab, Pakistan. Iran J Parasitol 4:19-25.

Durrani AZ and Kamal N, 2008. Identification of ticks and detection of blood protozoa in Friesian cattle by polymerase chain reaction test and estimation of blood parameters in district Kasur, Pakistan. Trop Anim Health Prod 40:441-7.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol 39:783-91.

Iqbal A, Sajid MS, Khan MN, *et al*., 2013. Frequency distribution of hard ticks (Acari: Ixodidae) infesting bubaline population of district Toba Tek Singh, Punjab, Pakistan. Parasitol Res 112:535-41.

Kabir MHB, Mondal MMH, Eliyas M, *et al*., 2011. An epidemiological survey on investigation of tick infestation in cattle at Chittagong District, Bangladesh. Afr J Microbiol 5:346-52.

Kakar MN and Kakarsulemankhel JK, 2008. Prevalence of endo (trematodes) and ectoparasites in cows and buffaloes of Quetta, Pakistan. Pak Vet J 28:34.

Kasi KK, Von Arnim F, Schulz A, *et al*, 2020. Crimean-Congo haemorrhagic fever virus in ticks collected from livestock in Balochistan, Pakistan. Transbound Emerg Dis 67:1543-52.

Kemal J, Tamerat N and Tuluka T, 2016. Infestation and identification of ixodid tick in cattle. The case study of Arbegona District, Southern Ethopia. J Vet Med 2016.

Karim S, Budachetri K, Mukherjee N, *et al*., 2017. A study of ticks and tick-borne livestock pathogens in Pakistan. PLoS Negl Trop Dis 11:1-17.

Khan A, Mushtaq MH, Ahmad M, *et al*., 2013. Tick infestation rate in cattle and buffalo in different areas of Khyber Pakhtunkhwa, Pakistan. J Vet Anim Sci (Lahore) 3:31-5.

Kumar S, Stecher G, Li M, *et al*., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547-9.

Lv J, Wu S, Zhang Y, *et al*., 2014. Assessment of four DNA fragments (COI, 16S rDNA, ITS2 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasite Vector 7:93.

Manan A and Zabita Khan BA, 2007. Identification of ixodid tick genera in Frontier region, Peshawar. J Agric Biol Sci 2(1).

Nawaz M and Khan FM, 2015. Cross sectional survey of vector born diseases in Punjab. Livestock and dairy development Department, Government of Pakistan.

Raut PA, Sonkhusale VG, Khan LA, *et al*., 2008. Haematological changes in cattle associated with arthropods infestation. Vet World 1:338.

Rees D, Dioli M and Kirkendall R, 2003. Molecular and morphology: Evidence for cryptic hybridization in Africa *Hyalomma* (Acari: Ixodidae). J Mol Phylogenet Evol 27:131-14.

Rehman A, Conraths FJ, Sauter-Louis C, *et al*., 2019. Epidemiology of tick-borne pathogens in the semi-arid and the arid agro-ecological zones of Punjab province, Pakistan. Transbound Emerg Dis 66:526-36.

Riaz M and Ullah MZ, 2017. Epidemiological survey on diversity and seasonal distribution of hard ticks in sheep and goats in Multan, Pakistan. JEBS 10.3:50-60.

Saitou N, and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-25.

Sajid MS, Iqbal Z, Khan, MN, *et al*., 2009. In vitro and in vivo efficacies of ivermectin and cypermethrin against the cattle tick *Hyalomma anatolicum anatolicum* (Acari: Ixodidae). Parasitol Res 105:1133-8.

Soulsby EJL, 1982. Helminths. Arthropods and Protozoa of domesticated animals, 291.

Soltan AP, Ramezani Z, *et al*., 2020. Molecular characterization of Ribosomal DNA(ITS2) of hard ticks in Iran: Understanding the conspecificity of *Dermacentor marginatus* and *D. niveus*. BMC Res Notes 13:1-6.

Taberlet P, Meyer A and Bouvet J, 1992. Unusual mitochondrial DNA polymorphism in two local population of blue tit (Parus caeruleus). Mol Ecol Resour 1:27-36.

Tamura K, Nei M and Kumar S, 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Nat Acad Sci 101:11030-5.

Thrusfield M, 1995. Sampling In: Veterinary Epidemiology, 2nd Edition, Black Wellscience Ltd, London, pp:179-84.

Thrusfield M, 2007. Veterinary Epidemiology, 2nd Edition, Black Wellscience Ltd, London pp:182-98.

Walker AR, Bouattour A, Camicas JL, *et al*., 2014. Ticks of domestic animals in Africa: A guide to identification of species, Edinburgh, UK. Biosci Report pp:1-221.

Zeb J, Shams S, Din IU, *et al*., 2020. Molecular epidemiology and associated risk factors of *Anaplasma marginale* and *Theileria annulata* in cattle from North-western Pakistan. Vet Parasitol 279:109044.