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Original Article

Occurrence of ticks and tick-borne mixed parasitic microbiota in cross-bred cattle in District Lahore, Pakistan

Ocorrência de carrapatos e microbiota parasitária mista transmitida por carrapatos em bovinos mestiços no distrito de Lahore, Paquistão

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Abstract

The present study was focused on the incidence of ticks and tick-borne diseases (TTBD) in cross-bred cattle (Friesian x Sahiwal) of two farms (n = 2548) in district Lahore, Pakistan. We collected total of 572 ticks (adults and nymphs) and blood samples (10 ml) for microscopic i.e., blood smear test – Giemsa Stain (BST) and molecular analysis; Reverse Line Blot-General Primer-PCR (RLB-PCR) and Specie Specific Primer PCR (SP-PCR) from infested cattle (n = 100) from months of April to September. Results: The tick specie identified was *Rhipicephalus microplus* at both farms, with significant difference in infestations rate amongst both farms (p< 0.0001). The cross-bred cattle having higher ratio of Friesian blood and lower ratio of Sahiwal blood were mostly infested by ticks (p < 0.0458) and haemoparasites (p <0.474) and vice versa. The SP-PCR showed higher number of haemoparasites infection than BST, which revealed 16% *T. annulata* (p < 0.0001 and k value 0.485, 0.0001), 51% *B. bigemina* (p < 0.0001 and k value 0.485, 0.0001) and 15% *A. marginale* (p < 0.001 and k value 0.207, 0.001), respectively. The single infection with *B. bigemina* was 34% (n = 34/100) and *A. marginale* 6% (n = 6/100). The double infection with *T. annulata/B. bigemina* was 8% (n = 8/100) *and B. bigemina/A. marginale* 1% (n = 1/100). Whereas the triple infection with *T. annulata/B. bigemina/A .marginale* was 8% (n = 8/100). The phylogenetic study of isolated sequence of *T. annulata* revealed close homology to isolates from Iran (87%), *B. bigemina* to isolates from Cuba (94 to 100%) and *A. marginale* with isolates from Pakistan (99 to 98%).

Keywords: *Rhipicephalus microplus*, polymerase chain reaction, *Theileria annulata, Babesia bigemina, Anaplasma marginale*.

Resumo

O presente estudo foi enfocado na incidência de carrapatos e doenças transmitidas por carrapatos (TTBD) em bovinos mestiços (Friesian x Sahiwal) de duas fazendas (n = 2.548) no distrito de Lahore, Paquistão. Foram coletados 572 carrapatos (adultos e ninfas) e amostras de sangue (10 ml) para microscopia, ou seja, esfregaço sanguíneo – coloração de Giemsa (BST) e análise molecular; Reverse Line Blot-General Primer-PCR (RLB-PCR) e Specific Primer PCR (SP-PCR) –, de bovinos infestados (n = 100) nos meses de abril a setembro. Resultados: A espécie de carrapato identificada em ambas as fazendas foi *Rhipicephalus microplus*, com diferença significativa na taxa de infestação nos dois locais (p < 0,0001). Os bovinos mestiços Friesian, com maior proporção de sangue, e Sahiwal, com menor proporção de sangue, foram principalmente infestados por carrapatos (p < 0,0458) e hemoparasitos (p < 0,474), e vice-versa. O SP-PCR mostrou maior número de infecção por hemoparasitos do que a BST, revelando 16% de *Theileria annulata* (p < 0,0001; k valor 0,485; 0,0001), 51% de *Babesia bigemina* (p < 0,0001; k valor 0,485; 0,0001) e 15% de *Anaplasma marginale* (p < 0,001; valor de k 0,207; 0,001). A infecção única com *B. bigemina* foi de 34% (n = 34/100), e com *A. marginale*, de 6% (n = 6/100). A dupla infecção com *T. annulata/B. bigemina* foi de 8% (n = 8/100), e com *B. bigemina/A. marginale*, de 1% (n = 1/100). Já a tripla infecção com *T. annulata/B. bigemina/A. marginale* foi de 8% (n = 8/100). O estudo filogenético da sequência isolada de *T. annulata* revelou estreita homologia com isolados do Irã (87%), de *B. bigemina* com isolados de Cuba (94 a 100%) e de *A. marginale* com isolados do Paquistão (98 a 99%).

Palavras-chave: *Rhipicephalus microplus*, reação em cadeia da polimerase, *Theileria annulata, Babesia bigemina, Anaplasma marginale.*

1. Introduction

Ticks are known for their negative impact on animal and human health through infestation and are capable of transmitting a wide range of pathogens including protozoan, viruses and bacteria. The ticks during summer exceed all other arthropod parasites in the number and varieties of diseases (Castro and Newman, 2003). Ticks are

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widely distributed in different ecological and geographical regions of Pakistan and reported to transmit bovine theileriosis, babesiosis and anaplasmosis in livestock. The Sahiwal breed exhibits a high degree of resistance to ticks (Ashfaq and Razzak, 2000). The exotic livestock breed is highly susceptible to ticks and tick-borne diseases even kept in very controlled environment (Young et al., 1988). The overall prevalence of *Theileria annulata*, *Babesia bigemina* and *Anaplasma marginale* in a cattle population can be as 22.0%, 19.33% and 10.6%, respectively (El-Dakhly et al., 2020). *T. annulata* can be found in both erythrocytes and lymphocytes of their host and is transmitted by ticks of *Hyalomma* (*H. anatolicum anatolicum*, *H. anatolicum excavatum*, *H. detritum* and *H. marginatum marginatum* (Sayin et al., 2003). *B. bigemina* is presently considered as one of the most important constraints in production of livestock and transmitted by *Rhipicephalus* ticks (Makenov et al., 2021) and mainly infects the red blood cells (Ristic, 1981). *A. marginale* is caused by a group of obligate intracellular bacteria and are transmitted by *Rhipicephalus* ticks (Yan et al., 2020; Galay et al., 2021), and observed within erythrocytes (Jurkovic et al., 2020). These haemoparastes can be seen in thick and thin smears of blood prepared with Giemsa's stain (Darghouth et al., 1996). The PCR test is more specific and sensitive in the diagnosis of tick-borne diseases including Theileriosis, Babesiosis and Anaplasmosis. The subject study has been carried out to find the prevalence of ticks and tick-borne diseases (TTBD) in various crosses of cross-bred cattle (Friesian x Sahiwal) in two mid-sized commercial farms in Lahore, Pakistan.

2. Materials and Methods

2.1. Animal data

During field study, the reference population of cross-bred cattle (Friesian x Sahiwal) was selected from two mid-sized commercial farms of city of Lahore, Pakistan (31.5204° N, 74.35887° E). These farms are named as Centre Farm (CF) and Branch Farm (BF). These farms are in the heart of city and dependent on each other on various animal management and veterinary practices. CF is a milk producing farm and holds large no of animals (n = 1459). BF is basically rearing and breeding farm (n = 1089) and holds heifers and dry adult cattle. The breeding policy of these farms are to cross the Friesian breed (sire) with Sahiwal breed (dam) to achieve blood and genetic ratio between 50-95% Friesian blood in offspring to get max potential of milk production as well as to avoid the disease proneness of pure exotic cattle. Hence, six types of offspring (Friesian x Sahiwal) are found at these farms and termed in ratios as 1/2 (50:50), 5/8 (62.5:37.5), 3/4 (75:25), 7/8 (87.5:12.5), 15/16 (93.75:6.25) and 31/32 (96.875:3.125), respectively. These farms follow a strict regime of management and veterinary practices.

2.2. Tick collection

The ticks for this study was collected from April to September. The data of animals found infested with ticks were also recorded. These included the age, type, breed, month of sampling, number and location of ticks on each animal. Ticks removed and placed in tubes containing 70% ethanol. The ticks were examined under stereo microscope (Olympus SZ40, Japan) and morphologically identified (Estrada-Peña et al., 2004).

2.3. Blood collection

Screening of animals found infested by ticks were done by thick blood smear examination and PCR. Ten milliliter blood collected from juggler vein of cattle in disposable syringes and transferred five milliliter each to two vacutainers (EDTA) under aseptic condition.

2.4. Thick blood smears

The dried blood smears stained by Giemsa's Staining Technique studied under microscope for presence of protozoans (Soulsby, 1982; Moretti et al., 2010).

2.5. PCR tests

The blood collected in vacutainers stored at -20°C in freezer till further processing. The DNA extraction of blood was carried out by using DNA extraction kit (GeneAll® Type G, ExGene) as per manufacturer's protocol (Handbook for DNA purification version 3.3). The DNA extracted were placed in -70°C freezer till further processing. The concentration of DNA was estimated by Spectrophotometric analysis. In this procedure 10 µl of DNA was mixed with 90 µl of autoclaved distilled water. The quantity of DNA was calculated by using 260 nm and 280 nm wavelength ratio. The primers used in subject study is as shown in Table 1.

The PCR analysis of samples were done by using ready to use Master Mix of Bioshop®, Canada. The concentration used for tests were 10 µl Master Mix, 2 µl each forward and reverse primers, 4 µl denucleased water and 2 µl DNA extract of samples. Amplification of 20 µl samples were performed for specified number of cycles in a thermocycler SimpliAmp™, Applied Biosystems, Thermo Fisher Scientific. Known positive and negative samples were also included in each PCR test. The condition of PCR cycles for each primer set is as shown in Table 2. The amplified products of each PCR run (6 µl) was examined on 1% (w/v) agarose gel added with SYBR® Safe DNA Gel Stain, Invitrogen, Thermo Fisher Scientific, USA and subsequently photographed using a GelDoc system of BioRad, USA and Digital Camera.

2.6. Phylogenetic studies

For each samples found positive on gel electrophoresis the gel extracted and DNA purified using GeneJET Genomic DNA purification kit by Thermo Scientific, Lithuania, using manufacturer's guidelines. The purified DNA along with primers were submitted to 1st BASE DNA Sequencing Company, Singapore for DNA sequencing and identification of protozoan. The processed sequences were then compared with the already published sequences in the National Centre for Biotechnology Information (NCBI) using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to get the specific identity of individual organism/ protozoan.

Table 1. Primer used in PCR tests along with target gene and predicted amplicon size.

Primer	Primer Sequence	Target Genome	Predicted amplicon size	References
Primer Set-A Forward Reverse	Reverse Line Blot (RLB) General primer 5′- GAC ACA GGG AGG TAG TGA CAA G - 3′ 5′- CTA AGA ATT TCA CCT CTG ACA GT - 3′	18s rRNA gene	430 bp	Gubbels et al., 1999
Primer Set-B Forward Reverse	*T. annulata* specific 5′- CAA ATG AGC TTC TGG GGA GC - 3′ 5′- TTC CTG CCA TTG CCA AAA GTC - 3′	Cytochrome b gene	475 bp	Bilgic et al., 2010
Primer Set-C Forward Reverse	*B. bigemina* specific 5′- GAC GAA TCG GAA AAG CCA CG - 3′ 5′- AGA GGG ACT CCT GTG CTT CA - 3′	18s rRNA gene	321 bp	Umber et al., 2020
Primer Set-D Forward Reverse	*B.bovis* specific 5′- AAT ATG GGT TGG GCA ATG CG - 3′ 5′- CCA CCC AAA ACA AGA GCA ACT - 3′	Cytochrome b gene	269 bp	Umber et al., 2020
Primer Set-E Forward Reverse	*A. marginale* specific 5′- CCT TAT GGG GTG GGC TAC AC - 3′ 5′- CCC GAG AAC GTA TTC ACC GT - 3′	16s rRNA gene	178 bp	Designed in current study

Table 2. The conditions of PCR used for each primer set.

PCR Protocols for Heamoparasites						
Primers	**Cycles Number**	**Initial Denaturing**	**Denaturing**	**Annealing**	**Extension**	**Final Extension**
RLB	40	95°C for 4 min	94°C for 35 seconds	51°C for 35 seconds	72°C for 35 seconds	72°C for 10 min
T.annulata	35	95°C for 4 min	95°C for 30 seconds	58°C for 30 seconds	72°C for 1 min	72°C for 10 min
B.bigemina	37	95°C for 1 min	95°C for 30 seconds	57°C for 30 seconds	72°C for 30 seconds	72°C for 5 min
B.bovis	40	94°C for 5 min	94°C for 5 seconds	60°C for 45 seconds	72°C for 45 seconds	72°C for 10 min
A.marginale	35	95°C for 5 min	94°C for 30 seconds	55°C for 30 seconds	72°C for 1 min & 30 seconds	72°C for 5 min

The current reference sequences were downloaded from NCBI. For *T. annulata* the evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-1566.88) created. The percentage of trees in which the associated taxa clustered together were shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Biological Neighbour Joining (BioNJ) algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. There was a total of 903 positions in the final dataset. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis across Computing Platforms software (MEGA X) (Kumar et al., 2018). In case of *B. bigemina*, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree was created. The tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 18 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 705 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The evolutionary history of *A. marginale* was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree created. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using

the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 175 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.7. Statistical analysis

Statistical analysis performed by using the software program of SPSS text analysis @2004 SPSS 25.0 (California, USA) for windows as well as GraphPad Prism version 5.0 (La, Jolla, USA). The prevalence of tick was calculated by number of animals found infested with ticks divided by total number of animals and numbers in each age group of animals. The Chi-square and One-Way ANOVA tests were used to compare the prevalence of ticks and protozoan in each farm, age group, season of collection and breed. A *p*-value of <0.05 was considered as statistically significant.

3. Results

3.1. Prevalence of ticks

A total of 572 ticks (including 292 males, 235 females and 45 nymphs) of *R. microplus* were collected from 3.92% (100/2548) cattle in this study. Interestingly, no other tick genus was found at both farms. At CF 3.63% (53/1459) and at BF 4.31% (47/1089) cattle were found infested, hence statistically significant difference in tick infestation ($p < 0.0001$). The animals belonging to all age groups were found infested in both farms. The overall prevalence of ticks in both farms in calves was 0.51% (n = 13/2548), heifers 1.26% (n = 32/2548) and adults 2.16% (n = 55/2548). However, at CF 5.3% (13/245) of calves and 3.53% (40/1131) of adult cattle, whereas in BF 3.9% (32/820) of heifers and 5.57% (15/269) adults were found infested. No heifer in CF (n = 83) was found infested during the study. There was a significant difference of tick infestation between three age groups ($p < 0.0001$). The month-wise distribution of infested animals for April was 9% (n= 9/100), May 11% (n = 11/100), June 17% (n= 17/100), July 24% (n = 24/100), August 25% (n = 25/100) and September 14% (n = 14/100). There was significant difference statistically between month of infestation between three animal groups ($p < 0.0001$).

3.2. Tick Infestation and Genetic makeup of animals

The number of cross-bred cattle (Friesian x Sahiwal) having higher concentration of Friesian blood and lower concentration of Sahiwal blood were mostly infested by ticks both in CF and BF. The cattle with genetic makeup of 7/8 were highly infested at 34% (n = 34/100) followed by 15/16 at 38% (n = 38/100) and 31/32 at 12% (n = 12/100) infestation rates, whereas animals with 1/2 at 2% (n = 2/100), 5/8 at 8% (n = 8/100) and 3/4 at 6% (n = 6/100) were least infested by ticks. The same pattern was observed in calves, heifers and adults. Statistically significant difference was found ($p < 0.0458$) (see Figure 1).

3.3. Blood smear and PCR test

The erythrocytes on blood smears test (BST) of animals infested with ticks (n = 100) were examined for presence of intracellular piroplasm/organism of the *Theileria*, *Babesia* and *Anaplasma* species. The results revealed incidence of theileriosis as 4% (n = 4/100), babesiosis 25% (n = 25/100) and anaplasmosis 2% (n = 2/100). The RLB-PCR test for 18s rRNA gene amplified 430bp size band as depicted in agarose gel electrophoresis (see Figure 2). The RLB-PCR showed 59% (n = 59/100) infection rate (CF n = 29/100 and BF n = 30/100) of haemoparasites. The SP-PCR for *T. annulata* (cytochrome b gene), *B. bigemina* (18s rRNA gene) and *A. marginale* (16s rRNA gene) produced PCR product of 475bp, 321 bp and 175 bp, respectively (see Figure 3A, 3B and 3C). The SP-PCR showed that 57% cattle (57/100; CF n = 29/53 and BF n = 28/47) showed presence of haemoparasites. The incidence of *T. annulata* was found 16% (n = 16/100; CF n = 10/53 and BF n = 6/47), *B. bigemina* at 51% (n = 51/100; CF n = 23/53 and BF n = 28/47) and

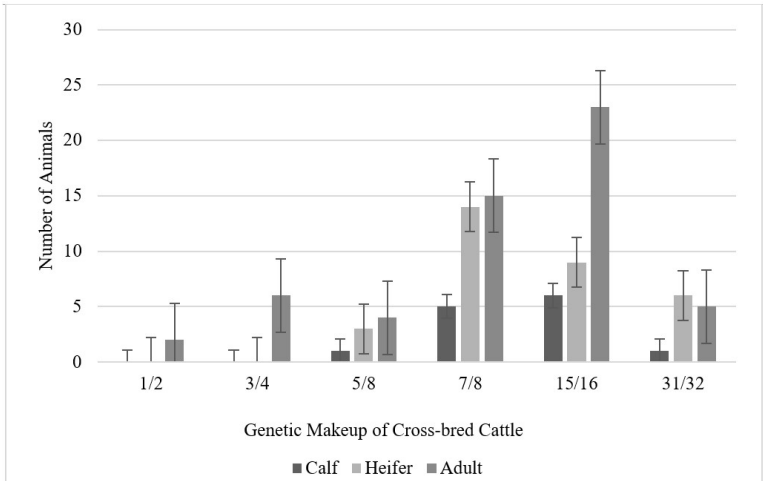

Figure 1. Prevalence of ticks in various groups of cross=bred (Friesian x Sahiwal) cattle.

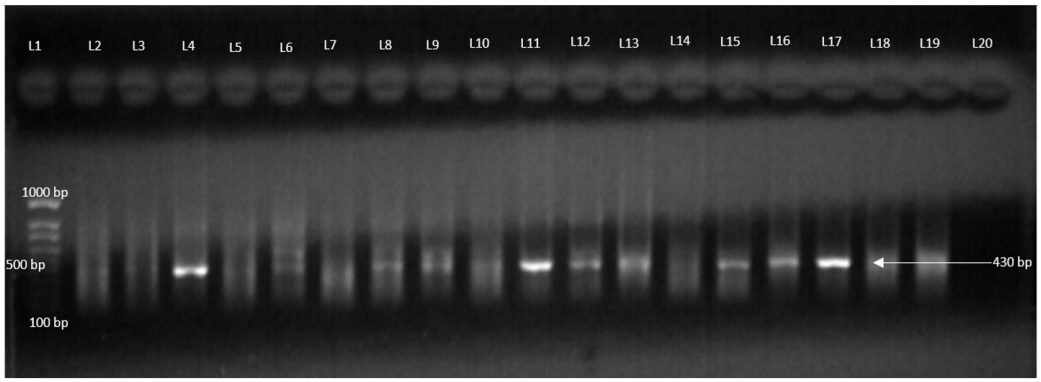

Figure 2. RLB specific primer PCR detection of DNA in cross-bred Cattle (Friesian x Sahiwal). L1 100bp ladder. L2 & L3 negative control, L4 PCR positive control. L6, L8, L9, L11, L12, L13, L15, L16, L17, L18, L19 positive for protozoan specific to primers.

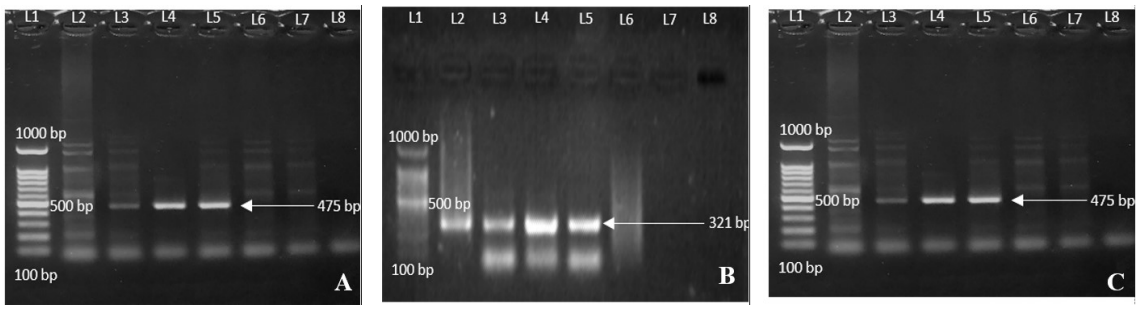

Figure 3. A: PCR detection of *T. annulata* in cross-bred Cattle (Friesian x Sahiwal). L1 100bp ladder. L2 negative control, L3 PCR positive control. L4 & L5, positive samples. B: PCR detection of *B. bigemina* in cross-bred Cattle (Friesian x Sahiwal). L1 100bp ladder. L2 PCR positive control. L3, L4 & L5 positive samples. L8 negative control. C: PCR detection of *A. marginale* in cross-bred Cattle (Friesian x Sahiwal). L1 100bp ladder. L2 PCR negative control. L3 positive control. L5, L6, L7 & L8 positive samples.

A. marginale 15% (n = 15/100; CF n = 12/53 and BF n = 3/47). It is interesting to note that in RLB-PCR 15.2% (n = 9/59) positive cases were not confirmed by SP-PCR for presence of specific protozoan. Moreover, 11.9% (n = 7/59) negative cases in RLB-PCR showed presence of protozoan in SP-PCR. The statistical difference between BST and SP-PCR for *T. annulata* was significant ($p < 0.01$ and k value 0.252, 0.001). The *B. bigemina* infection detected by BST and SP-PCR was also statistically significant ($p < 0.0001$ and k value 0.485, 0.0001). As for as *A. marginale* was concerned significant statistical difference ($p < 0.001$ and k value 0.207, 0.001) was found between BST and SP-PCR. There was no significant statistical difference between RLB-PCR test and SP-PCR tests. The infected calves, heifers and adults by *T. annulata* (n = 16/100) detected by SP-PCR were 15.4% (2/13), 3.1% (1/32) & 23.6% (13/55), respectively, which was significant statistically ($p < 0.0421$). The *B. bigemina* (n = 51) infected 38.4% (5/13), 65.6% (21/32) & 45.5% (25/55) number of calves, heifers and adults, respectively, and statistically non-significant (p value 0.120). The calves, heifers and adult infected by *A. marginale* (n = 15/100) were 30.7% (4/13), 6.2% (2/32) and 16.4% (9/55) in numbers, respectively, which was also statistically non-significant (p value 0.103).

Interestingly, single, double and triple protozoan infection were found in same cattle. The SP-PCR confirmed single infection with *B. bigemina* as 34% (n = 34/100) and *A. marginale* 6% (n = 6/100). The double infection with *T. annulata/B. bigemina* was 8% (n = 8/100) *and B. bigemina/A. marginale* 1% (n = 1/100). The triple infection with *T. annulata/B. bigemina/A. marginale* was noted as 8% (n = 8/100). It is very interesting to note that *T. annulata* was not found as a single infection. A total no of 82 protozoans were found in infected cross-bred cattle (57/100). In our study the month wise incident of three protozoans detected by SP-PCR for April was 11% (11/100), May 9% (9/100), June 10% (10/100), July 20% (20/100), August 22% (22/100) and September 10% (10/100), respectively. As for *T. annulata* the rate of infection was almost constant from April 3% (3/100), May 3% (3/100), June 3% (3/100), July 5% (5/100), August zero (0/16) and September 2% (2/100), respectively. The same pattern was also observed in *A. marginale*, having rate of infection in April 3% (3/100), May 2% (2/100), June 3% (3/100), July 2% (2/100), August 2% (2/100) and September 3% (3/100), respectively. However, in case of *B. bigemina* the wet months of July at 13% (13/100) and August at 20% (20/100) had highest incident percentage. The incident of protozoa for months of April was 5% (5/100), May 4% (4/100) June 4% (4/100) and September 5% (5/100), respectively. There was significant difference statistically in months and incidence of haemoparasites ($p < 0.029$).

3.4. PCR and genetic makeup of animals

It was noted that the cattle having higher concentration of Friesian blood and lower concentration of Sahiwal blood were much more infected and vice versa. The highest number of infected animals were of 7/8 group at 32% (n = 32/100) followed by 15/16 at 30% (n = 30/100), 31/32 at 9% (n = 9/100), 5/8 at 7% (n = 7/100), 3/4 at 3% (n = 3/100) and 1/2 1% (n = 1/100). There was significant difference amongst the various crosses of cattle and overall incident of protozoan ($p < 0.0474$). It was found that *B. bigemina* infection in blood sample (n=100) tested by SP-PCR was higher in crosses of 7/8 (18%), 15/16 (19%) and 31/32 (7%) than in 5/8 (4%), 1/2 (1%) and 3/4 (2%). The same pattern was almost found in *T. annulata* infected cattle i.e. 7/8 (8%), 15/16 (4%) followed by 5/8 (2%), 31/32 (1%) and 3/4 (1%) while in crosses of 1/2 no infection was detected. In case of *A. marginale* the crosses of 1/2 and 3/4 were free of infections and 7/8 (6%) and 15 (7%) had highest followed by 5/8 (1%) and 31/32 (1%) (see Figure 4). However, for individual protozoans no significant difference was found between various blood ratio (Friesian x Sahiwal) and infection of haemoparasites.

3.5. Phylogenetic studies

The level of nucleotide variation and phylogenetic position of partial sequence of *T. annulata* revealed in this study was compared with the available sequences. The phylogenetic tree showed four major clusters. The first cluster consist of our isolated sequences of cytochrome b gene (OL456214 & OL420757) grouped with isolates from Pakistan (MW354913 & MW354915), India (MZ665960 &MN89344) and Turkey (MK032846), with bootstrap value of 44%. The second cluster was formed by Chinese isolates (MG735208 & MG735209) with bootstrap value of 50%. The third and fourth cluster was formed by Iranian isolates (JQ308837) with bootstrap value of 87% and Spanish isolates (DQ287958 & DQ402154) with bootstrap value of 72%, respectively. Our isolated cytochrome b gene partial sequences were closely related to isolates from Iran and Spain and to a lesser extent to isolates from Pakistan, India, Turkey and China (see Figure 5).

The phylogenetic tree analysis for *B. bigemina* showed that our isolated sequence (OL376658) are most homologous (94-100%) to isolates from Cuba. The isolates from Egypt (MH796638, MH796639 & MH796640) have 88% homology with our sequence. Likewise, current isolates from USA (MH047819, MH050356, MH050357, MH050358, & MH050387 are closer (74 to 84% similarity) to our isolates as compared to isolates from South Africa (MH257710 – 23), which has a homology value of 34 to 73%. Hence, the partial isolated sequence (18s rRNA) in this study has higher homology value to isolates from Cuba, followed by isolates from USA and South Africa, respectively (see Figure 6).

The phylogenetic analysis of partial sequence of 16s rRNA gene of *A. marginale* isolated in our study revealed three major clusters. The first cluster consists of our sequence (OL407062) grouped with isolates from Pakistan (MK680804, MK680805, MK680806 & MK680807) with a high bootstrap value of 99% homology. The second cluster with high bootstrap value of 98% homology was isolate from USA (DQ000617). The third cluster from China (OM065781), KSA (AB916498) and South Africa (AF414873) showed moderate homology of 62% bootstrap value. All our isolates showed 99 to 98% similarity with isolates from Pakistan and USA and lesser homology to isolates from China, KSA and South Africa (see Figure 7).

4. Discussion

TTBD infect cattle in wide ecological and geographical regions of Pakistan (Karim et al., 2017; Atif et al., 2022). The exotic livestock breed is highly susceptible to tick and tick-borne diseases even kept in very controlled environment (Young et al., 1988). In the present study it was found that ticks infested 3.92% of total cattle herd. Previous studies have reported that about 28.2% (Rasul and Akhtar, 1975) and 47.9% (Ghafar et al., 2020b) cattle can be infested with ticks. The lower tick infestation in present study may be attributed to (i) better management practices, (ii) use of acaricides in both farms and (iii) organized and commercial farming.

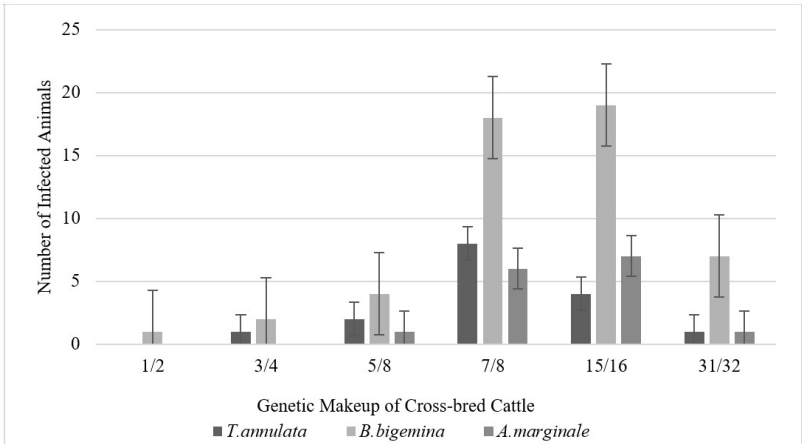

Figure 4. Prevalence of protozoans in various groups of cross-bred (Friesian x Sahiwal) cattle.

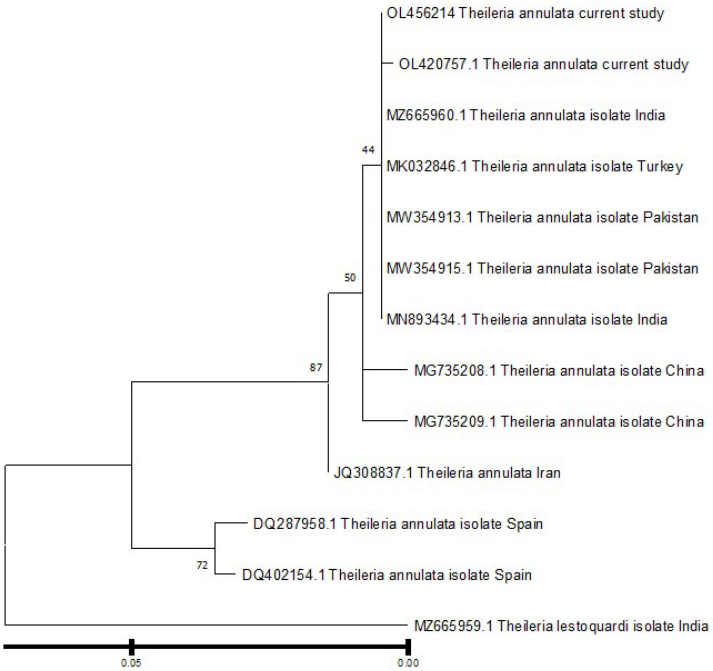

Figure 5. Phylogenetic analysis of *T. annulata* based on Cytochrome b gene.

Moreover, the prevalence of ticks in various age group of exotic and crossbred cattle is highly debatable and scientists have reported wide range of incidence rates depending on ecological regions, quality of farming practices and control measures. In this study we found 5.3% (13/245) of calves, 3.9% (55/1400) of adults and 3.9% (32/820) of heifers infested with ticks, which is much lower as reported by other researcher (Ahmed et al., 2012) who reported 85% cross-bred calves and 73.2% cross-bred adult infested with *R. microplus*. In another study highest infestation in young calves (47.99%), growing (41.19%), heifer (39.82%) and adult cattle (35.35%) were found (Das and Parasit, 1994). In a recent study it was reported that incident of ticks is higher in calves (i.e.</=1year of age) (55%) than in young animals (i.e. up to 3 years of age) (39%) and adults (48%) (Ghafar et al., 2020b). The reason for low tick infestation in adult animals may be attributed to acquired immunity due to repeated exposure (Tabor et al., 2017) and more attendance to adult cattle as well as in comparison less care to calves (Singh and Rath, 2013; Burrow et al., 2019).

The infestation rate of ticks increases with the progression of summer season. In present study the infestation was highest in wet and hotter, while lower infestation was noted in beginning of summer season and end of summer in September. This is in line with other researchers (Castro and Newman, 2003; Asmaa et al., 2014; Ali et al., 2019) who reported the same pattern of tick infestation.

In our knowledge it is the first study that has established the correlation between tick infestation and various genetic/blood ratio of cross-bred cattle (Friesian x Sahiwal). The cattle having higher concentration of Friesian blood and lower concentration of Sahiwal were among the most infested. Conversely, the animals having lower concentration of Friesian blood and higher concentration of Sahiwal blood were less infested. In a study from Pakistan Rehman et al. (2017) has reported that intensity of tick infestation is significantly lower in indigenous animals compared to exotic and cross-bredt cows. In another study it has been reported that tick infestation is higher in crossbred cattle (72%) as compared to their pure breed (61%) (Ahmed et al., 2012). The cross-bred cattle with higher exotic blood and lower indigenous blood makes the animal prone to tick infestation and vice versa, which is proof of resistance/immunity of indigenous cattle towards tick infestation even in cross-bred animals.

Carrier animals, infected with protozoans, mostly asymptomatic, are an important source of infection in cattle farms. The diagnosis of these animals is very difficult through conventional methods; hence use of more sensitive and specific tests is imperative to avoid spread of disease, control of mortality and minimize economic losses (Altay et al., 2008). It has been reported by Irvin (1987) that in case of mixed infection with *Theileria* and *Babesia*, confusion may arise to differentiate these species solely on the basis of the morphology, developmental stages, hence under field conditions it is imperative to reach on exact diagnosis.

PCR test is used in detection of clinical and subclinical cases of *Theileria*, *Babesia* and *Anaplasma* (El-Ashker et al., 2015). In the study we carried out, the BST revealed lower incidence of haemoparasites. The RLB-PCR detected higher infection rate than BST. The SP-PCR was also more sensitive and specific in detection of haemoparasites. The BST and

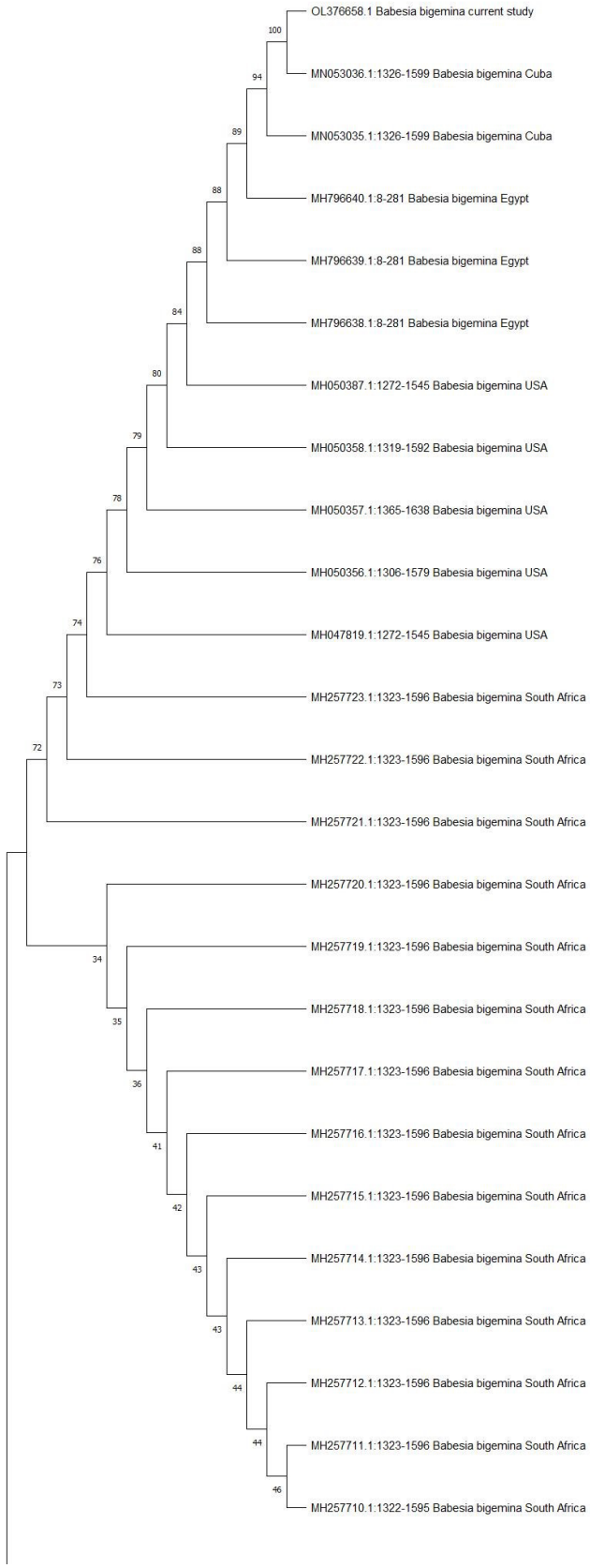

Figure 6. Phylogenetic analysis of *B. bigemina* based on 18s rRNA gene. Scale bar set at 0.05.

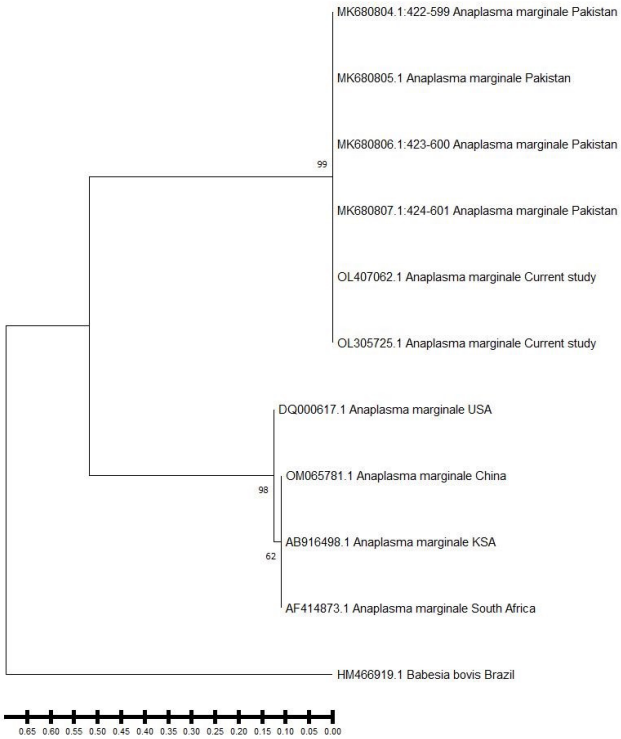

Figure 7. Phylogenetic analysis of *A. marginale* based on 16s rRNA gene.

PCR were used by Durrani and Kamal (2008) in Pakistan for detection of *Theileria* and *Babesia* species in field samples, and concluded that PCR is much more sensitive and specific than BST. The same result has been reported by Nourollahi-Fard et al. (2015), in which BST detected much lower infection of *T. annulata* than PCR.

The RLB test, using oligonucleotide probes can be used to detect low level of parasitaemia to simultaneously identify *Theileria* and *Babesia* species, and is very effective and practical tool. In present study, instead of hybridization on probe, the RLB primer was used initially to amplify the DNA fragments in blood samples of infested cattle, and demonstrated on agarose gel, subsequently. The RLB-PCR positive samples can be then subjected to SP-PCR for exact diagnosis of protozoan species. Many researchers around the world have used RLB assay for diagnosis of *Theileria* and *Babesia* species. (Gubbels et al., 1999; Sparagano et al., 2000; García-Sanmartín et al., 2006; Iqbal et al., 2013). It is interesting to note that in present study various positive cases in RLB-PCR were not confirmed by SP-PCR for presence of specific protozoan. Moreover, few negative cases in RLB-PCR showed presence of protozoan in SP-PCR. It can be hypothesized that RLB-PCR may have detected DNA samples of some novel protozoan species, for which the specific primer was not used in SP-PCR in our study. This opens up a new research avenue to optimize the RLB-PCR to detect a larger number of protozoans, simultaneously. The negative samples of RLB-PCR, which were revealed positive in SP-PCR points out for need of further optimization of procedures, chemicals, equipment as well as minimize human errors.

In present study the overall incident of *T. annulata* in tested blood samples by SP-PCR (Cytochrome b gene) was 16%, which was highest in adults 23.6% (13/55), whereas in calves 15.4% (2/13) and heifers 3.1% (1/32), respectively. The infection rate in both farms were almost the same. It is in agreement with the study of Yamchi and Tavassoli (2016) who reported 15.94% tested samples positive for *T. annulata* in cattle. It has also been reported in a study that infection of *B. bigemina* is higher among the age groups of 2-7 years in cross-bred animals (Velusamy et al., 2014). In a study carried out in Khyber-Pakhtunkhwa, Pakistan, Ullah et al. (2021) reported that 23.7% of cattle were found infected with *T. annulata*, however, theileriosis was found to be higher in young animals as compared to adults cross-bred cattle. Another research from Pakistan reported over all *T. annulata* prevalence of 21%, with cross-bred cattle most susceptible (28%) followed by Sahiwal breed (19%) (Parveen et al., 2021). The lower incident rate of infection in our study is in line with Calleja-Bueno et al. (2017) who reported that incident of *T. annulata* was lower in farms, which are being given anti-protozoal drugs at least once a year. However, the reported incident of *T. annulata* in various studies has remarkable differences, which may be as high as 74% (Amiri et al., 2021) or 46.2% (Zeb et al., 2020) or as low as 8% (Rashid et al., 2018). The differences in infection rate may be due to study design, diseased vs healthy vs carrier animals, geographical location, age/numbers of cattle at

farm, management, use of anti-protozoal drugs, season and genetic makeup of cattle. It has been mentioned earlier in our study that only ticks of *R. microplus* was found on infested animals. The absence of *Hyalomma* ticks raises questions about detection of *T. annulata* by BST and SP-PCR in understudied cattle. On scrutiny of farms animal data, it was revealed that these animals were shifted from other farms as well as purchased from local market to diversify the gene pool to increase milk production. It is assumed that these cattle were the carrier of *T. annulata* at time of transfer and/or purchase.

Carrier cattle also infected with *Babesia* are difficult to detect because of the low numbers of parasites that occur in peripheral blood. However, diagnosis of low-level infections with the parasite is important for diagnosis and epidemiological studies (Fahrimal et al., 1992). For PCR the organism-specific primers derived from18S rRNA gene of *B. bigemina* can be used (Laha et al., 2015). In our study the SP-PCR showed overall infection of *B. bigemina* at 51% of tested blood of apparently healthy animals infested with ticks of *R. microplus*. The highest infection was 65.6% in heifers, followed by adults at 45.5% and calves at 38.4%. Our findings are in line with Ganzinelli et al. (2020) who reported high infection rate of 54.7% by *B. bigemina* in purebred cattle using nPCR. These findings are also consistent with Oliveira-Sequeira et al. (2005) who found that *B. bigemina* was present in 92.6% of calves and in 84% of cows. The presence of ticks on calves and cattle is directly correlated with high infection rates of *B. bigemina*, which give substance to our findings. However, in contrast the overall positive rates of *B. bigemina* was also reported as low as 23.6% in cattle (Otgonsuren et al., 2020). Chaudhry et al. (2010) from Pakistan reported 18% positive *B. bigemina* cases in apparently healthy cattle in a farm that were posing threat for the healthy herd population. The difference in infection rate in various studies may be attributed towards different geographical location, animal management and genetic makeup, control measures and use of anti-protozoal drugs.

In our study the overall incidence of *A. marginale* on tested blood samples by SP-PCR was 15%. The calves showed higher infection rate of 30.7% followed by adults at 16.4% and heifers 6.2%. It is in agreement with study of Zafar et al. (2022) who reported prevalence of *A. marginale* from 9-17% in tested blood samples from two southern districts (Lodhran and Dera Ghazi Khan) of Punjab, Pakistan. Our findings are also in agreement with results of Atif et al. (2022) who reported the highest prevalence of *A. marginale* in cattle of less than 1 year old (32.84%) while the lowest prevalence (6.45%) was in animals aged between 1 and 2 years of age. In another study from Khyber-Pakhtunkhwa, Pakistan showed that on PCR (16S rRNA gene) *A. marginale* is found to be 16.3% prevalent in cattle of various areas; with breed and acaricidal treatment as significant determinants (Zeb et al., 2020). The sampling sites, vector species, breeds and breeding system as well as the climatic conditions effect the prevalence of *A. marginale* (Bursakov and Kovalchuk, 2019). As reported in various studies, the prevalence of *A. marginale* has shown to be vary from 3-41% in Pakistan (Afridi and Ahmad, 2005; Farooqi et al., 2018; Turi et al., 2018; Ashraf et al., 2021; Atif et al., 2022). *A. marginale* has also been reported from various countries of the world including India (18.48-45.2%) (Singh et al., 2012; Kumar et al., 2019), Egypt (20%), (Selim et al., 2021), Sri Lanka (32.7%) (Zhyldyz et al., 2019), Iran (38.6%) (Noaman and Shayan, 2010), Algeria (39.4%) (Rjeibi et al., 2018), Brazil (57.5%) (Garcia et al., 2022) and USA (82%) (Hairgrove et al., 2015).

In our study single, double and triple protozoan infection were found in tick infested cattle. The co-infection of protozoans in cattle is not very uncommon, and has been reported in Pakistan as well as by researchers all around the globe. In a study carried out by Atif et al. (2022), based on duplex PCR, the overall prevalence of the two concurrent tick-borne pathogens *T. annulata* and *A. marginale* was found to be 19.79%. In another study in Peshawar, Pakistan, out of 68 positive cases, 12 samples (4.21%) were harbouring single infection. Remaining 26 blood samples showed mixed infection of *A. marginale* with *A. centrale* 4.21%, *B.bovis* with *A.centrale* 3.50%, *T. annulata* with *A. marginale* 0.70% and *T. parva* with *A. marginale* was recorded in 0.70% cases, respectively (Afridi and Ahmad, 2005). A researcher from Kenya reported that more than 50% of the positive samples were infected with at least two haemoparasites, which generally belonged to different genus, and 29 different types of mixed infections were noted and some cattle concurrently harbouring up to five pathogens (Adjou Moumouni et al., 2015). The researchers from all around the globe reported co-infection with two/three protozoans ranging from 10.46% (Ganguly et al., 2020), 15.1% (Zhou et al., 2016), 17.9% (Jirapattharasate et al., 2017), 19% (Bursakov and Kovalchuk, 2019) and 20.0% (El-Dakhly et al., 2020) infection percentage.

In our study the wet months of summer showed highest percentage of protozoan infection. Our observations are supported by Atif et al. (2022) who reported that incidence of all ticks and tick-borne pathogens are higher in summer, followed by spring, autumn, and winter. The Okafor et al. (2018) summed up that summer/hotter months has higher incident of *A. marginale* than spring/winter/colder months of the year. In a study Ashraf et al. (2021) reported highest prevalence of *A. marginale* was observed during autumn (18.3%) followed by summer (9.7%) and winter season (7.1%). Our findings are also in agreement with Jaimes-Duenez et al. (2018) who reported higher values of infection of Babesiosis during the wet season and late wet season. Siddique et al. (2020) found high incidence in summer (23.41%) followed by autumn (20.47%), spring (17.77%) and winter (7.29%), respectively.

To best of our knowledge, this research is the only study which reported that cross-bred cattle (Friesian x Sahiwal) having various genetic/blood concentration, contributed from sire (Friesian) and dam (Sahiwal), has different level of vulnerability to heamoparasites. It was found that cross-bred cattle having higher concentration of Friesian blood and lower concentration of Sahiwal blood are generally more susceptible to infections of haemoparasites and vice versa. It is very interesting finding and in line with many researcher who have reported that imported breed and their cross-breds are much more vulnerable to protozoan infections than local/indigenous breeds (Fivaz et al., 1992; Ashraf et al., 2021; Atif et al., 2022). In Pakistan influx of

imported animals has increased drastically in last few decades, and these are bred with local cattle to get their cross-bred offspring for better genetic potential and disease resistance. However, the challenges of adaptability and disease proneness of these imported animals and their cross-bred offspring were always a point of concern in farming sector. There are studies which generally suggest that indigenous/cross-bred are better to purebred cattle in resistance to tick/protozoan infection (Asmaa et al., 2014; Rehman et al., 2017) and protozoan infection (Siddique et al., 2020). With confidence it can be said that no study has been carried out in Pakistan on suitability of various genetic makeup of cross-bred (Friesian x Sahiwal) cattle in local environment. Our study presents the first evidence/glimpse that cross-bred animals of various genetic/blood makeup vary in their immunity level towards TTBD. On basis of this research the farmers may be suggested to preserve the blood level of cross-bred cattle (Friesian x Sahiwal) between ratio of 1/2 (50:50) to 3/4 (75:25) to get maximum milk production capacity of exotic genes as well as benefit from better potential/ resistance against TTBD of indigenous blood. Further research, basing on wide geographical region and data, is required to be carried out to reach on conclusive outcome basing on concrete scientific evidence.

To confirm the results of PCR, sequencing of isolates of *T. annulate, B. bigemina and A. marginale* was performed. The level of nucleotide variation and phylogenetic position of partial sequence of *T. annulata* revealed that our isolated sequences (cytochrome b gene) are closely related to isolates from Iran and Spain and to a lesser extent to isolates from China and least similar to Pakistan, India and Turkey. In case of *B. bigemina* our partial isolated sequence (16s RNA gene) in this study has higher homology to isolates from Cuba followed by USA and lesser homology to South Africa, respectively. The phylogenetic analysis of partial sequence (16s rRNA gene) of *A. marginale* isolated in current study showed higher similarity with isolates from Pakistan and USA and lesser homology to isolates from China, KSA and South Africa, respectively. The phylogenetic studies by various scientists from Pakistan has reported various homology level of their isolates with partial sequences of other isolates all around the world (Ghafar et al., 2020a; Atif et al., 2022). Further studies are required to map out the complete epidemiological profiles of these haemoparasites in local population of cross-bred cattle in various demographical regions of Pakistan.

5. Conclusion

Ticks of genus *R. microplus* infect the cross-bred cattle (Friesian x Sahiwal) of all ages, and most active during the hotter and humid season of the year. The crosses of cattle possessing higher genetic level of foreign breed are most susceptible to ticks infestation and vice versa. The PCR test was highly sensitive and specific as compared to conventional blood smear test for diagnosis of haemoparasites. RLB-PCR is a reliable and rapid test for screening of cattle suffering from blood parasites. Amongst the major haemoparasites transmitted by ticks the *B. bigemina* was most prevalent followed by *T. annulata* and *A. marginale*. The haemoparasites infected all animal age groups, prevalent in hot and humid weather and diagnosed mostly in crosses of cross-bred cattle with higher level of foreign blood. Single and double and triple infection of haemoparasites were also noted in animals.

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