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RESEARCH ARTICLE

# Identification and Molecular Characterization of *Theileria annulata* with Associated Risk Factors in Naturally Infected Camels from Selected Districts in Punjab, Pakistan

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**ABSTRACT**

Camel production in Pakistan is adversely affected by several pathogenic infections and insufficient veterinary facilities. Haemoparasitic diseases significantly affect health and productivity of camels causing a substantial financial burden to camel breeders and owners. The present study was designed for the identification and molecular detection of haemoparasites particularly piroplasms (Theileria spp. / Babesia spp.) infection in naturally infected local one-humped camels (*Camelus dromedaries*) in Punjab by using parasitological as well as molecular tools like polymerase chain reaction (PCR) followed by phylogenetic analysis. Blood samples (n=400) were collected from camels suspected for piroplasms infections in ten districts of Punjab and processed for blood smears and PCR targeting 18S rRNA gene. The findings revealed that Theileria is the most common parasite in camels of all study areas with overall prevalence of 12% and 13.5% by microscopic examination of GSBS and PCR, respectively. The phylogenetic analyses of the isolates on sequencing revealed that all analyzed isolates were closely related to *Theileria annulata* present in NCBI from several parts of the world. However, all samples tested for presence of Babesia spp. were found negative by microscopy and PCR. Chi square based risk factors analyses exhibited significant ($P<0.05$) association between gender, age, tick infestation, previous tick history and prevalence of *Theileria*. In conclusion, current study on haemoparasites is evident for first ever molecular identification of *Theileria annulata* infection in camels of Pakistan along with assessment of potential risk factors associated with disease. Recent outcomes are ascertaining it as a silent killer with damaging effects on immune system.

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## INTRODUCTION

Camel contributes a key role in pastoral communities (called Rohillas in Cholistan desert) by fulfilling basic necessities of their living such as milk, meat, racing, riding and packing (Khan *et al*., 2016). It is a widely distributed domestic animal in deserts and semi desert areas of different continents. The global camel population is about 35 million (FAOSTAT 2019). Pakistan is ranked at 8$^{th}$ position globally among camel raising countries with 1.1 million heads (GOP, 2019-2020). In Pakistan, camels are mainly kept by desert people and nomadic pastoralists, in order to cater their socio-economic needs (Ahmad *et al*., 2010).

Ticks and tick-borne diseases (TBDs) have negative effects on production and reproduction of camels, leading to anemia, fever, wasting and death in heavy infections (El-Naga and Barghash, 2016).

Among piroplasm infections, Theileriosis and Babesiosis are most significant tick-transmitted haemoparasitic diseases of camels caused by the intracellular blood protozoans (Karim *et al*., 2017). *Theileria* in camels was firstly reported in Russia, named as *Theileria camelensis* (Rutter, 1967) while Babesiosis caused by *Babesia caballi* was diagnosed first time in camels of Sudan (Abd-Elmaleck *et al*., 2014). *Babesia* species parasitize the erythrocytes of domestic animals and humans, causing anemia in the affected host (Swelum

*et al*., 2014). Ticks of the genus *Hyalomma* (H) especially, *H. dromedarii* and *H. annatolicum* are accountable for the transmission of piroplasms. In Pakistan *H. dromedary* is the most prevalent (83%) tick species affecting camels (Gadahi *et al*., 2013).

Diagnosis of haemoparasitic infection in camel through conventional microscopy is a challenging task (Alsaad, 2009). However, PCR being specific and sensitive molecular technique is extensively used for the diagnosis of latent infections (Hussain *et al*., 2016). The DNA sequencing has appeared as impeccable diagnostic tool established on PCR amplification consuming generic primers that amplify exceptionally conserved ribosomal gene sequences (Ullah *et al*., 2022). Haemoparasitic diseases in camels are usually neglected in Pakistan. Most documented studies are on camel trypanosomiasis but there is scarce information on theileriosis in camels of Punjab. Therefore, the current study was planned to determine the prevalence of piroplasm infection along with the sequence analysis to study the phylogenetic relationships of detected isolates of local camels in study areas.

## MATERIALS AND METHODS

**Study area and sample design:** The study was carried out from April 2020 to December 2021. A total of 400 blood samples were collected from suspected camels of different age groups (both sexes) in ten selected districts of province Punjab, Pakistan. The representation includes, three distinct geographical regions i.e., Central Punjab (Faisalabad, Jhang), North Punjab (Mianwali, Khushab, Bhakkar) and South Punjab (Rajanpur, Muzaffar Garh, Bahawalpur, Bahawalnagr and Layyah) (Fig. 1). Areas were selected on the basis of population density of camel (Livestock Census, 2006). Sample size was calculated by using the following equation (Thrusfield 2018).

$$N = \frac{1.96^2 \, P_{exp} \left(1 - P_{exp}\right)}{d^2}$$

Where
N = Number of Samples to be required
$P_{exp}$ = expected prevalence
d = desired precision
Twenty samples (two from each) from apparently healthy animals from all districts were collected as control.

**Sample collection:** Approximately 5 ml blood was drawn from jugular vein by using sterile syringes and transferred into a sterile K3 EDTA tubes (Atlas Medo-O-Vac Francisco$^{R}$) for microscopy and DNA extraction for purpose of amplification by PCR (Modry *et al*., 2017). Blood samples were brought in ice boxes to Molecular Parasitology Laboratory, UVAS, Lahore.

**Parasitological examination:** Thin blood smears of all 400 samples were prepared, air-dried and fixed in absolute methanol following the standard protocol. Initial screening of blood smears was carried out to detect the presence of blood protozoans using light microscopy at 40x and 100x (oil immersion objective) (Coles, 1986).

**DNA extraction and PCR amplification:** After microscopic examination, all blood samples were processed for DNA extraction using WizPrep™ gDNA Mini kit following the manufacturer's instructions.

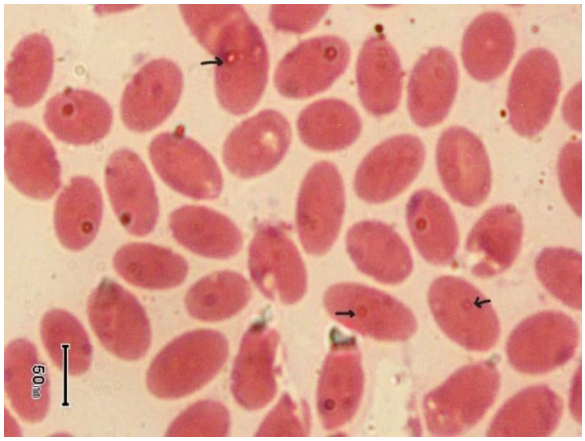

**Fig. 1:** Intra-Erythrocytic Piroplasm *Theileria* Spp. Indicated By Arrow Heads. *at 100x (oil immersion lens).*

Concentrations of DNAs were calculated using Nano Drop (A'aiz *et al*., 2021). For DNA amplification conventional PCR was performed targeting 300 bp region of 18S ribosomal RNA gene with the primers 18SApiF/18SApiR (F: 5′-CGAACGAGACCTTAACC TGCTA-3′, R: 5′-GGATCACTCGATCGGTAGGAG-3′) described previously (Greay *et al*., 2018). PCR was performed with a total volume of 25 µL comprising of 13 µL of commercial ready to use Master Mix (Green Taq Mix Catalog No. P 131, Vazyme Biotech Co. Ltd), 2µL of primers (1µL each forward and reverse); 2µL (50ng/µL) of DNA; and 8µL of double distilled water (Ullah *et al*., 2022). The cycling conditions were followed as initial denaturation at 95℃ for 5 minutes proceeded through 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 seconds and last extension at 72℃ for 5 min (Greay *et al.*, 2018). PCR generated amplicons were fractioned by 1.5% agarose gel electrophoresis at 113 volts, 230MA for 35 minutes to visualize the bands in gel documentation system (Bio Rad Laboratories, USA) with 50 bp standard DNA marker (Thermo Scientific $^{TM}$) (Ullah *et al*., 2022). Positive and negative controls were used for every PCR reaction.

**DNA Sequencing and phylogenetic analysis:** Ten positive PCR products were selected, purified by GeneJET Gel Extraction Kit (Catalog. No; 00520774) and sent to Lab Genetix (Pakistan) for sequence analysis. DNA sequences retrieved were blasted to the databases of the GenBank to find the homologous sequences in accession nos. ON045834, ON045835, ON045836, ON045837, ON045838, ON045839 and phylogenetic tree was constructed. The GenBank databases BLAST (Basic Local Alignment Search Tool) and databanks of the NCBI (Bethesda, MD, USA) (www.blast.ncbi.nlm.nih.gov/ Blast.cgi) were employed for analysis (Alanazi *et al*., 2020). Phylogenetic analysis was performed using MEGA 7 software through neighbor joining method with 1000 bootstrap. The phylogenetic tree was assembled using the Maximum Likelihood method applying Neighbor-Join and BioNJ algorithms (Lan *et al*., 2021).

**Risk factors assessment:** Detailed information about each herd (age, sex, location, body condition, tick infestation, previous ailment, vaccination, deworming

status and herd size) was collected on a predesigned questionnaire during sample collection for analysis as well as evaluation of risk factors related with *Theileria annulata* infection in camels.

**Statistical analyses:** Statistical analyses were executed in R statistical language (R version 4.1.3). The association between various risk factors and *T. annulata* infection was determined by univariable analysis i.e., chi-square test. All the risk factors with p-value less than 0.2 were analyzed at multivariable level using binary logistic regression. Odds ratio (OR) and CIs for each significant variable were identified using a multivariable logistic regression model. All statistics were considered significant at P<0.05. The map was constructed on GIS.

## RESULTS

**Parasitological identification:** Examination of Giemsa's stained blood smears demonstrated the presence of piroplasms (Theileria/ Babesia) within the erythrocytes in two forms i.e., coma shape and ring shape (Fig. 1). Out of the 400 camels examined, 48 were harboring piroplasms with an overall prevalence of 12%. Highest prevalence was recorded in camels of district Bahawalpur (35.7%) (Table 1).

**PCR detection and molecular characterization:** PCR results revealed that 13.5% (54/400) blood samples were found infected with *Theileria*. piroplasms. Approximately 300 bp of 18S rRNA was amplified (Fig. 2). District wise the highest prevalence (35.7%) of piroplasms was recorded in district Bahawalpur by both microscopy and PCR with no significant variation (P=0.087, 0.106) between the districts (Table 1). Among different regions, the highest prevalence was recorded in districts of south Punjab (17.14 %) followed by north (12.86%) and central (3.70%) region with significant (P<0.05) difference (Table 2). None of the samples tested positive by microscopy was found negative by PCR. However, two unidentified samples by microscopic examination got amplified by PCR. None of the samples were found to be co-infected. The phylogenetic analyses of present study isolates revealed the existence of *Theileria annulata* in camels of Punjab. The sequences deposited revealed homology on BLAST with reported isolates and grouped within a clade of *Theileria annulata* isolated from Pakistan and south Asian countries like China, West Bengal, Uterpardesh, Maharashter etc. (Fig. 3). None of the sequences revealed DNA related to Babesia spp.

**Risk factors associated with *T. annulata* in camels:** The univariate analysis revealed that the prevalence of theileriosis was higher in females (17.13%) than males (7.38%) with a significant difference (P=0.012). Overall Age was found significant factor (P= 0.018) associated with occurrence of disease. The highest prevalence (17.45%) was observed in middle aged animals (2-5 years), followed by (14.63%) in older animals (>5 y) and (4.60%) in young ones (<2 y). Tick-infested camels showed the highest prevalence (20.67%) compared with non-infested animals (5.73%) and exhibited significant (P<0.001) association with *T. annulata* infection in camels. Previous tick history was found significantly (P=0.023) associated with theileriosis in camels. For significant variables values of odds ratio (OR) and 95 % confidence interval were determined by using Multivariate logistic regression Model (Fig. 4).

Other species in surroundings, body conditions, feeding and watering style, deworming status, vaccination status, housing system, flies prevalence, flies control status, purpose of rearing and herd size exhibited statistically non-significant association with *Theileria annulata* infection in camel (Table 3).

**Table 1:** Comparison of region wise prevalence of *Theileria annulata* through microscopic vs PCR in camels

| District | Animals Tested (N) | Microscopy $^{+ve}$ (%) | P-value | PCR$^{+ve}$ (%) | P-value |
|---|---|---|---|---|---|
| Jhang | 39 | 02 (05.10) | 0.087* | 02 (05.10) | 0.106* |
| Faisalabad | 15 | 00 (00.00) | | 00 (00.00) | |
| Mianwali | 41 | 05 (12.20) | | 04 (09.80) | |
| Khushab | 40 | 04 (10.00) | | 05 (12.50) | |
| Bhakkar | 90 | 10 (11.10) | | 13(14.40) | |
| Rajan Pur | 86 | 11(12.80) | | 11 (12.80) | |
| Muzaffar Garh | 38 | 04(10.50) | | 06 (15.80) | |
| Bahawalpur | 14 | 05 (35.70) | | 05 (35.70) | |
| Bahawalnagar | 22 | 06 (27.30) | | 06 (27.30) | |
| Leyyah | 15 | 01 (06.70) | | 02 (13.30) | |
| Total | 400 | 48 (12.00) | | 54 (13.50) | |

*The results are not significant at P >0.05

**Table 2**: Region wise prevalence of Theileria in camels

| Region | Positive | Negative | Prevalence % | P value |
|---|---|---|---|---|
| Central | 2 | 52 | 3.70 | 0.04 |
| North | 22 | 149 | 12.86 | |
| South | 30 | 142 | 17.14 | |

Central: Faisalabad, Jhang: Northern: Mianwali, Bhakkar, Khushab: Southern: Bahawalnagar, Layyah, Muzaffar Garh, Bahawalpur, Rajan Pur

**Table 3:** Univariate analysis of risk factors associated with theileriosis in camels of Punjab

| Variable | Variable levels | Positive | Negative | Prevalence (%) | P-value |
|---|---|---|---|---|---|
| Gender | Female | 43 | 208 | 17.13 | 0.012 |
| | Male | 11 | 138 | 07.38 | * |
| Age | <2Y | 04 | 083 | 04.60 | 0.018 |
| | >5Y | 24 | 140 | 14.63 | * |
| | 2-5Y | 26 | 123 | 17.45 | |
| Tick infestation | Yes | 43 | 165 | 20.67 | 0.000 |
| | No | 11 | 181 | 05.73 | * |
| Previous tick history | Yes | 37 | 173 | 17.62 | 0.023 |
| | No | 17 | 173 | 08.95 | * |
| Other species in surrounding | Yes | 41 | 225 | 15.41 | 0.101 |
| | No | 13 | 121 | 09.70 | |
| Body condition | Normal | 09 | 091 | 09.00 | 0.446 |
| | Emaciated | 45 | 255 | 15.00 | |
| Deworming status | Yes | 02 | 014 | 12.50 | 1.000 |
| | No | 52 | 332 | 13.54 | |
| Vaccination status | Yes | 01 | 004 | 20.00 | 0.511 |
| | No | 53 | 342 | 13.42 | |
| Housing system | Concrete | 01 | 012 | 07.69 | 0.424 |
| | Desert | 34 | 180 | 15.89 | |
| | Soil | 19 | 154 | 10.98 | |
| Flies prevalence | Intensive | 28 | 189 | 12.90 | 0.900 |
| | S. intensive | 26 | 157 | 14.21 | |
| Flies control status | Yes | 15 | 089 | 14.42 | 0.809 |
| | No | 39 | 257 | 13.18 | |
| Type of feeding/ watering | Indoor | 13 | 112 | 10.40 | 0.330 |
| | Outdoor | 41 | 234 | 14.91 | |
| Purpose of rearing | Drought | 30 | 179 | 14.35 | 0.660 |
| | Exhibition | 00 | 002 | 00.00 | |
| | Milk/Meat | 24 | 165 | 12.70 | |
| Herd size | >10 | 06 | 055 | 09.84 | 0.687 |
| | 1-3 | 23 | 133 | 14.74 | |
| | 4-6 | 14 | 85 | 14.14 | |
| | 7-10 | 11 | 73 | 13.10 | |

* (P<0.05) significant difference.

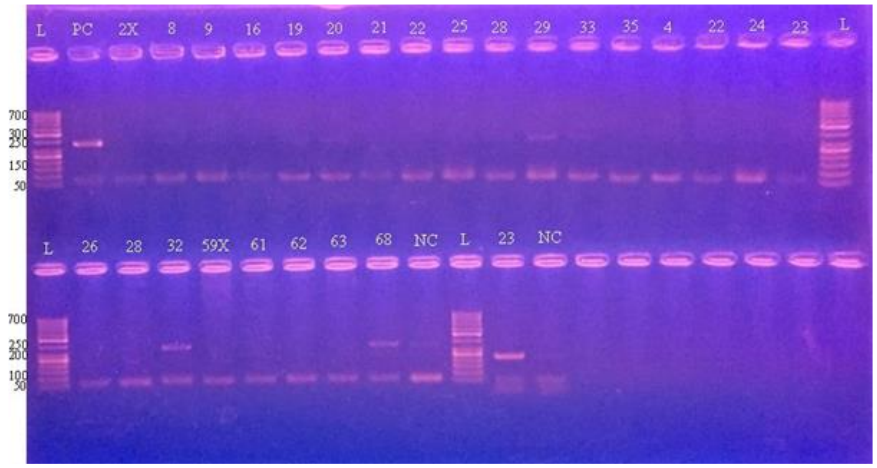


**Fig. 2**: Agarose gel (1.5%) electrophoretogram stained with ethidium bromide. PCR analysis of the 18s RNA gene revealed a 300 bp band derived from *Theileria annulata* isolates from camel blood samples (29,32, 68). L DNA, 50 base pair ladder, PC (Positive control), Sample Ids (2X. 68), NC (Negative control)

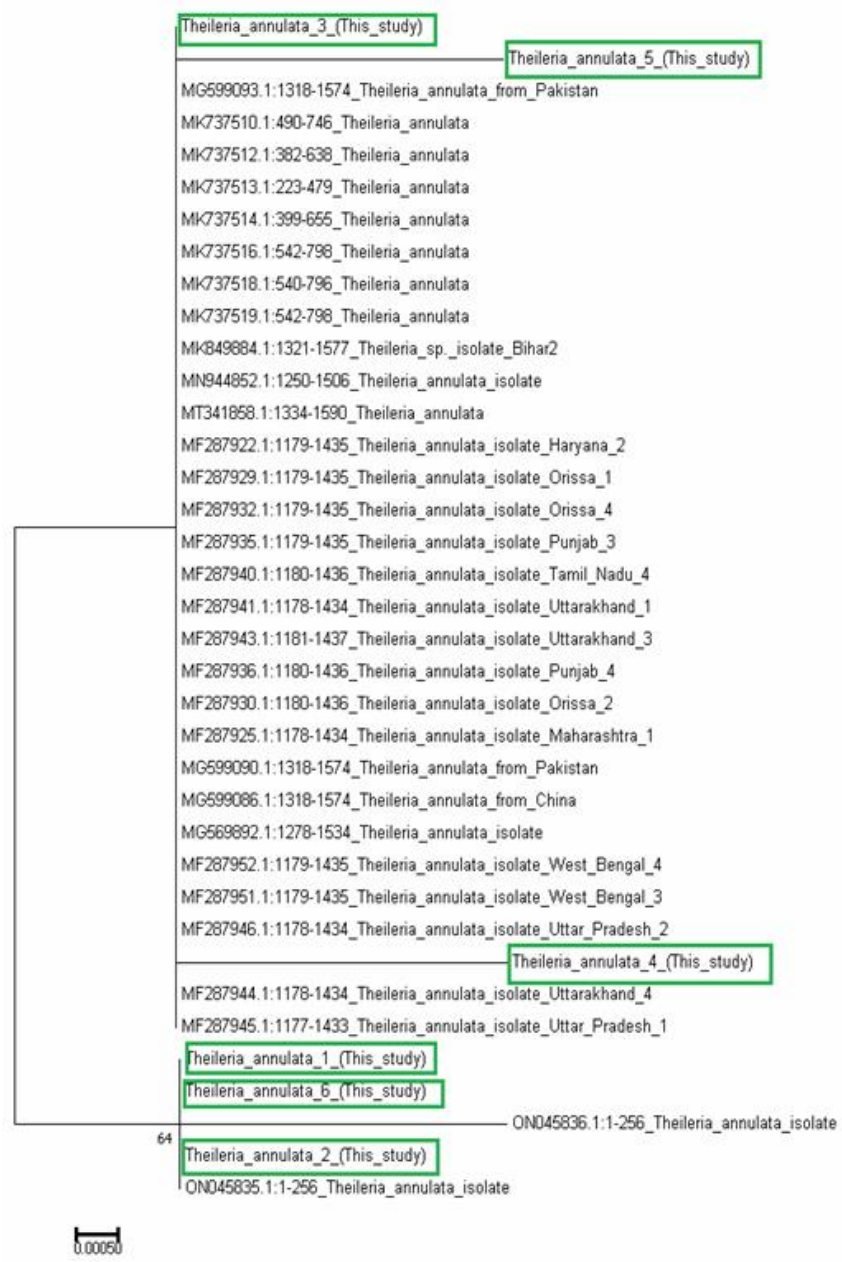


**Fig. 3:** A phylogenetic tree based on 18SApi rRNA, constructed with maximum-likelihood (ML) & neighbor joining (NJ) methods, representing phylogenetic relationships between samples in the current study and other reference sequences retrieved from GenBank

## DISCUSSION

Theileriosis is declared to be the second most significant hemoprotozoal disease after Trypanosomiasis affecting camels. No data pertaining to epidemiology and molecular detection of camel theileriosis is yet available in Pakistan. The recent investigation delivers a comprehensive discernment into the epidemiology, etiology, genetic diversity as well as risk factors related to camel theileriosis.

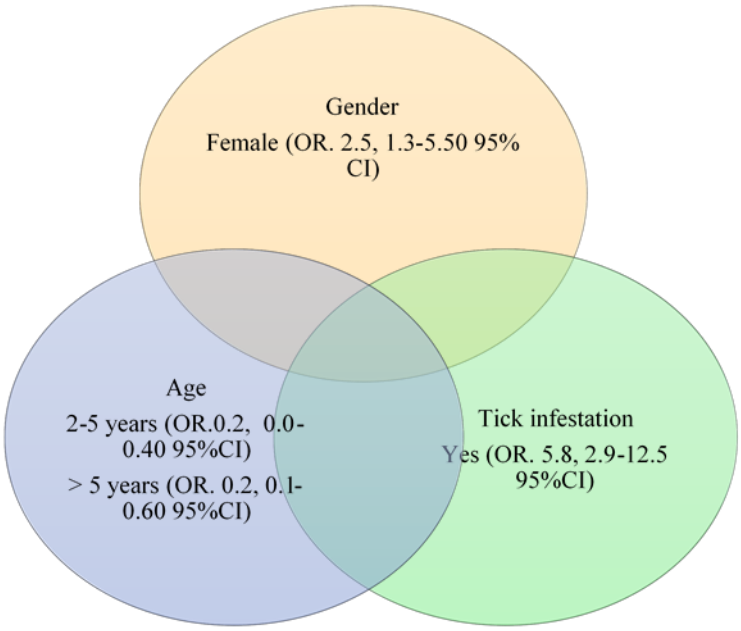


**Fig. 4**: OR and CIs for significant variables (p<0.05) using a multivariate logistic regression Model. (95% CI=95% confidence interval, OR=Odds ratio).

The preliminary screening of camel blood samples was carried out by microscopic examination with Giemsa-stained blood smears. Out of 400 examined samples, 48 (12%) were positive for erythrocytic forms of piroplasms appeared within the RBCs in rod and coma shape.

These finding are nearly similar with studies in Jordan (10%) by Oncel *et al*., (2007), lower than 28.88% and 24.5% recorded by Alsaadi and Faraj, (2020) and Ullah *et al*., (2022) respectively while higher than 6.75% reported in Upper Egypt (Hamed *et al*., 2011). These variations in prevalence may be attributed to different geographical and climatic conditions of the areas, variable sample size, vector prevalence, animal health status and technical skills of diagnostician (Alsaadi and Faraj, 2020). Piroplasms intermittently identified in blood of carrier animals and can't be detected by direct examination in many cases (Faraj *et al*., 2019). Molecular detection by PCR assays permitted detection of parasites with far better precision than conventional microscopy (El-Naga and Barghash, 2016) by identifying coinfections, species differentiation, detection at low parasitic levels and chronic infections (Mirahmadi *et al*., 2022). PCR based findings of this study endorsed that the rate of infection of piroplasms was higher (13.5%) then microscopy which necessitates the substantial role of PCR for piroplasms detection. These findings are in accordance with Ullah *et al*. (2022) and El-Naga and Barghash (2016). Higher and lower prevalence of *Theileria* infection in camels than present study ranged from 4.97% to 75% (Ullah *et al*., 2022; A'aiz *et al*., 2021). DNA sequencing and BLAST analysis of the 18S rRNA sequences demonstrated that all positive samples with piroplasmosis were infected with *T. annulata*. These findings are in accordance with Omer *et al*., (2021) who identified *T. annulata* species in camels with no evidence of *T. camelensis*, *T. dromedrii* and *T. equi* as reported earlier. The majority of reports on occurrence of *T. camelensis* (Ismael *et al*., 2014) and *T. dromedrii* (Mishra *et al*., 1987) in camels are based on microscopic detection except Moezi *et al*., 2016) who amplified sequence of 18S ribosomal DNA fragment to detect *T. camelensis* and *T. dromedrii* in tick infested camels. The results revealed that none of the samples found positive for *Theileria camelensis* and *Theileria dromedarii*. Since this is the first molecular report regarding the occurrence of *Theileria* infection in camels in Punjab, that's why unable to compare results with any other study from Pakistan.

In phylogenetic tree, the 18S rRNA sequence *of T. annulata* grouped with sequences from south Asian Countries. Owing to existence of hypervariable regions, the 18S rRNA sequences of *T. annulata* have impact in unfolding genetic diversity and crucial for determining evolutionary patterns (Sivakumar *et al*., 2014).

*T. annulata* identified in present study is the principal cause of tropical theileriosis in cattle (Mohsin *et al*., 2022). The occurrence of this pathogen in camels may be owing to the co-inhabitance of these animals in desert areas (Alanazi *et al*., 2020). This study has provided evidence that piroplasms possess low host specificity and camels can play role in the epidemiology of theileriosis by presenting another host specific piroplasms (Youssef *et al*., 2015). The current work directed no pathognomic clinical sign in the positive cases. This may be ascribed toward the subclinical and chronic nature of *Theileria* infection in camels.

The nonexistence of Babesial DNA is in resemblance with the Alanazi *et al*., (2020). The inability to identify Babesia DNA in the existing study may be explicated by the non-availability of the suitable vector or the host specificity of the vector. However, based on findings of the study seems impossible to predict that camels being not infected with Babesia spp. with available information indicating that DNA of *B. caballi* (Mirahmadi *et al*., 2022), *B. bigemina, B. bovis* (El-Naga and Barghash, 2016*)* exist in camel blood from different parts of the globe.

Current research revealed the *Theileria* infection in male and female camels with a significant difference of prevalence between the genders. The infection in females was 2.5 times higher than males. These outcomes are in agreement with Selim *et al*., (2021) while in contrast with Ullah *et al*. (2018). The higher rate of infection in female may be due to immunosuppression related to pregnancy, parturition and lactation rendering them more vulnerable to the disease (Amira *et al*., 2018).

All age groups were found infected with *T. annulata* with variable rates. Middle aged group (2-5y) displayed the higher rate of infection compare to older (>5y) and young ones (<2y) are in agreement with the previous findings (Alsaadi and Faraj, 2020) while in disagreement to (Mohsin *et al*., 2022) recorded high infection rate in young animals. Maternal antibodies boost immunity in young calves and protect them from various pathogens (Ullah *et al*., 2022). The study indicated tick infestation had a significant association with *Theileria* infection in camels. The rate of infection in ticks infested camels was 5.8 times higher (as compare to non-infested. Similar findings were reported by (Ullah *et al*., 2018). Ticks being the sole vector of *Theileria* are predominantly associated with theileriosis in camels. Higher rate of tick infestation increases the likelihood of infection with *T. annulata* in camels (Selim *et al*., 2021). Likewise, previous tick history was also found significantly associated (with 2.7 times higher rate of *Theileria* infection in camels. Despite the non-significant effect of other species surrounding on *Theileria* infection in camels, the high rate of infection (in camels surrounded by other species supports the findings of the present study.

**Conclusions:** Based on the findings we may conclude that *Theileria annulata* is the only prevailing specie identified in the camels of targeted areas. Further investigations on vector role of tick and tick control programs is suggested to decrease the infection rate in camels

**Authors contribution:** Conceived and designed the experiments: FA, MUR, M.A. Hafeez. Performed the experiments: FA, MUR, GS & KA: Analyzed the data: KA, MUR & MS: Contributed reagents/materials/ analysis tools: Wrote the paper: FA, MAH & MS.

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