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Research Article

Molecular Analysis and Associated Risk Factors of *Theileria annulata* in Cattle from Various Zones of Balochistan, Pakistan

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Abstract

Theileriosis is a protozoan parasite that is transmitted by ticks and infects a wide range of animals worldwide. This study aims to assess the molecular prevalence and related risk factors of theileriosis in Balochistan, Pakistan. Standard microscopy methods, polymerase chain reaction (PCR), 18S small subunit ribosomal RNA gene sequencing, and phylogenetic analysis were used. For this purpose, a total of 408 blood samples were collected from tick-infested cattle in Zhob, Loralai, and Quetta districts of Balochistan, Pakistan. Microscopy and subsequent PCR analysis confirmed the highest prevalence of *Theileria annulata* in Loralai district (11.76% and 12.75%), followed by Zhob district (11.27% and 12.25%), and Quetta district (8.34% and 9.56%), respectively. Moreover, the prevalence of *T. annulata* was higher in young cattle (85.82%), followed by female cattle (58.87%), and exotic crossbred cattle (33.33%) in the study area. However, various variable such as sex, area, and breeds of the cattle were not significantly correlated ($P>0.05$) with the presence of *T. annulata,* except for the age of animals ($P<0.05$). In addition, sequencing and phylogenetic analyses revealed that the isolated *T. annulata* was closely related to the isolates from Türkiye, Italy, Egypt, Iran, and Pakistan. Hence, these findings will contribute to the development of more effective control strategies for theileriosis in the cattle population of Balochistan, as well as in Pakistan on a broader scale.

Keywords: Balochistan, Cattle, Phylogeny, Prevalence, Risk factors, *Theileria annulata*

Introduction

Agriculture is the backbone of Pakistan's economy, with livestock contributing 14.04% to the country's overall national GDP [1]. In Balochitsan province, 85% of population is directly or indirectly linked to livestock, which contributes over 45% to the province's GDP [2]. The cattle population in Pakistan is around 51.5 million, playing a significant role in animal husbandry for milk, meat, and hides [3]. Livestock is the main source of livelihood for the local people in Balochistan, especially cattle breeding, which serves as a key source of income [4]. A recent study has revealed that Ixodid ticks have a substantial impact on infesting livestock and transmitting a wide range of pathogens in Pakistan. Lack of farmer awareness about the threat of theileriosis and the presence of tick vectors on pasture and animals are key factors leading to severe economic losses in the livestock industry [5].

Ixodidae ticks can transmit parasitic, viral, bacterial, and rickettsial diseases to animals and humans, and affect the host in several ways, including reduced body weight, milk production, reproduction, and reduced quality of hides [6]. Similarly, tropical theileriosis in cattle is caused by a tick-borne haemoprotozoan parasite *Theileria annulata*, which is transmitted by ticks of the genus *Hyalomma*, and the Ixodidae [7]. Tropical theileriosis is frequently reported from North Africa, Europe, Middle East, India, and Central Asia, including Pakistan [8]. In Pakistan, *T. annulata* is transmitted by *Hyalomma anatolicum* to bovine animals [1]. While, *T. annulata* infection is characterized by high fever, anorexia, enlargement of

superficial lymph nodes, anemia, hemoglobinuria, ocular-nasal discharges, cessation of rumination, and diarrhea in cattle [9]. Henceforth, *T. annulata* not only affects the health of animals but also causes serious economic losses in terms of vaccination, treatment, reduction in milk yield and live weight of the animal, while it also increases the inter-calving interval, delay in the age of maturity and causes up to 70% mortality in infected animals [10].

In Balochistan, parasitic infections are the most prevalent among all other animal diseases [11]. This could be attributed to the poor healthcare system, open grazing practices, and inadequate animal management, resulting in significant economic losses [11]. In addition, there is scarce information on the molecular identification of parasitic species in the cattle population of Balochistan. Therefore, this study was designed to investigate and characterize the molecular prevalence of theileriosis and its associated risk factors in different zones of Balochistan, Pakistan. The aim is to develop comprehensive control and therapeutic strategies for theileriosis infection in cattle.

Material and Methods

Ethics Statement

This experimental design was duly approved by departmental ethics committee and directorate of advanced studies, University of Veterinary and Animal Sciences (UVAS), Lahore (ID: DAS/2053).

Study Area, Study Animals and Blood Sampling

A total of 408 blood samples were collected aseptically from tick-infested cattle in Balochistan between July 2021 and November 2022. The sampling was conducted randomly, irrespective to the sex, age, and breed of the cattle, from three different zones in Balochistan, Pakistan. Briefly, 136 blood samples were collected from the 1st zone, Suleiman mountainous region "Zhob", while 153 blood samples were collected from the 2nd zone, Northern highlands "Loralai",

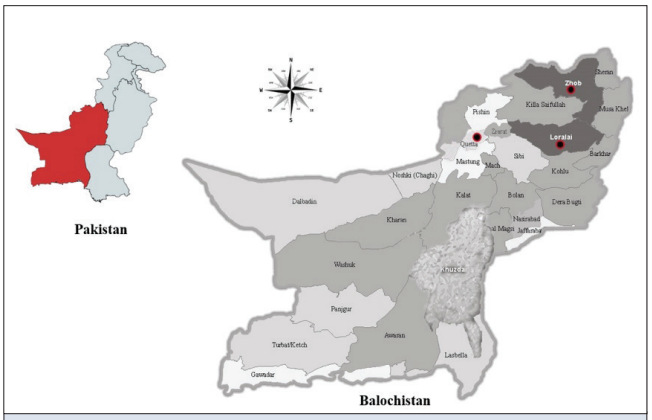

Fig 1. A map created by Mapinfo software, showing the sampling sites; Zhob, Loralai, and Quetta in the Balochistan Province, Pakistan

and 119 blood samples were collected from the 3rd zone, Kachhi basin region "Quetta/Sibi" (Shoaib et al.[12]). The sampling area is depicted in *Fig. 1.* The sample size was determined using the formula provided by Thrusfield;

$N = [Z^2p\ (1 - p)]/\ d^2$

$N= (1.96)^2*0.5(1-0.5)\ /\ (0.05)^2 =384$

Where, N is the desired sample size, P is the predicted prevalence, and d is the 5% precision level.

As mentioned above, the sample size was determined to be 384. However, a total of 408 blood samples were collected in the study area to increase the level of accuracy. A standard performa was used to collect relevant details, such as, age, breed, area, and sex, pertaining to various risk factors [13]. Foe each cattle, five (5) mL of blood was drawn from the jugular vein and placed in vacutainers with EDTA. The blood samples were transported in a cold chain to the Parasitology Laboratory of UVAS, Lahore, and stored at -20°C till further analysis.

Microscopic Analysis

Microscopic analysis was conducted by preparing thin blood smears from the collected samples. The smears were preserved using absolute ethanol and stained with the field's stain A and the field's stain B, as previously described by [14]. Subsequently, the smears were observed under a compound microscope, Olympus CX21 (Olympus, Tokyo, Japan), at a magnification of 100x to detect the presence of *Theileria.*

DNA Extraction and Molecular Analysis

Genomic DNA was extracted from the blood samples using a DNA extraction kit (Thermo Fisher; Cat. No: FABGK001-2), following the manufacturer's instructions. The purity and concentration of extracted DNA were measured using a NanoDrop at 260/280 nm and stored at -20°C for further PCR analysis.

All of the extracted DNA samples were subsequently subjected to PCR, targeting the *T. annulata* cytochrome b gene fragment using specific primers, as previously described by Farooqi et al.[8]. The forward primer used was 5'-ACTTTGGCCGTAATGTTAAAC-3', and the reverse primer was 5'- CTCTGGACCAACTGTTTGG-3', resulting in an amplification size of 450 base pairs.

For each PCR sample, a 20 µL reaction mixture was prepared, consisting of 2 µL of extracted DNA, 25 pmol of each primer, 200 mM of each dNTP, 5 µL of 10X PCR buffer, and 1.5 mM MgCl2 (Promega, Madison, WI, USA), and 1.5 U of Taq DNA polymerase. The first phase of the PCR reaction cycle invovled an initial denaturation step at 94°C for 5 min, followed by thermal degradation for 40 cycles at 94°C for 35 sec, annealing at 51°C for 35

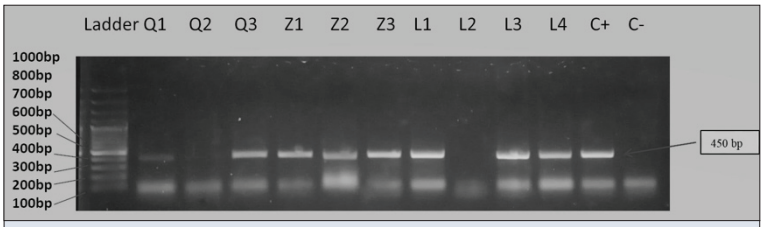

Fig 2. PCR positive products were examined using a gel electrophoresis system to identify *Theileria* species in cattle specimens from three different zones of Balochistan, Pakistan (n=408). A molecular weight marker of 1000 base pairs is visible on the lane ladder. Lane C+ve displays the positive control *(Theileria. annulata* clinical isolate), while Lane C-ve displays the negative control. The other lanes represent the results of the positive and negative samples

sec, and extension at 72°C for 35 sec. The final elongation was performed for 10 min at 72°C, followed by a hold step at 4°C. Each PCR run included a positive control (lab-isolated clinical isolate) and a negative control (sterile water). Positive bands were confirmed using gel electrophoresis and then trimmed for sequencing analysis.

Sequencing Analysis

The PCR bands testing positive for haemoparasites (constituting 50% of the bands positive for *T. annulata*-specific primers) were excised from a 1.5% agarose gel and purified using a gel extraction kit (WizPrepTM; Ref. W70150-300), following the manufacturer's instructions. The purified DNA samples were sent to CELEMICS Product & Services in South Korea for sequencing. A phylogenetic tree was constructed using Mega 11 software with a maximum likelihood algorithm and bootstrapping at 1000 replications.

The PCR bands testing positive for haemoparasites (constituting 50% of the bands positive for *T. annulata*-specific primers) were excised from a 1.5% agarose gel and purified using a gel extraction kit (WizPrepTM; Ref. W70150-300), following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted using SPSS version 20.00. The prevalence of theileriosis was compared to independent variables such as sex, age, area, and breed using the chi-square (χ^2) test. A P-value <0.05 was considered statistically significant, and a 95% confidence interval was established.

Results

Molecular Epidemiology of Theileriosis in cattle

The results of this study showed an overall 34.56% (141/408) PCR-based prevalence of theileriosis in cattle from 3 different zones of Balochitsan, Pakistan i.e. Loralai having a prevalence of 12.75%, Zhob with 12.25% and Quetta with 9.56% *(Fig. 2)*. However, microscopic examination indicated a prevalence of 31.37% (128/408),

Table 1. *Results of the microscopic identification of Theileria species in cattle from three different zones of Balochistan, Pakistan (n=408)*

Sample No.	Districts	Positive n (%)	Negative n (%)	Total n (%)
1	Quetta	34 (8.34)	85 (20.83)	119 (29.17)
2	Zhob	46 (11.27)	90 (22.06)	136 (33.33)
3	Loralai	48 (11.76)	105 (25.74)	153 (37.50)
Total		128 (31.37)	280 (68.63)	408 (100)

Table 2. *PCR based identification of the Theileria species in cattle from 3 different zones of Balochistan, Pakistan (n=408)*

Sample No.	Districts	Positive n (%)	Negative n (%)	Total n (%)
1	Quetta	39 (9.56)	80 (19.61)	119 (29.17)
2	Zhob	50 (12.25)	86 (21.08)	136 (33.33)
3	Loralai	52 (12.75)	101 (24.75)	153 (37.50)
Total		141 (34.56)	267 (65.44)	408 (100)

suggesting that the PCR method is more sensitive compared to microscopic identification *(Table 1, Table 2)*. In addition, the presence of intraerythrocytic inclusion

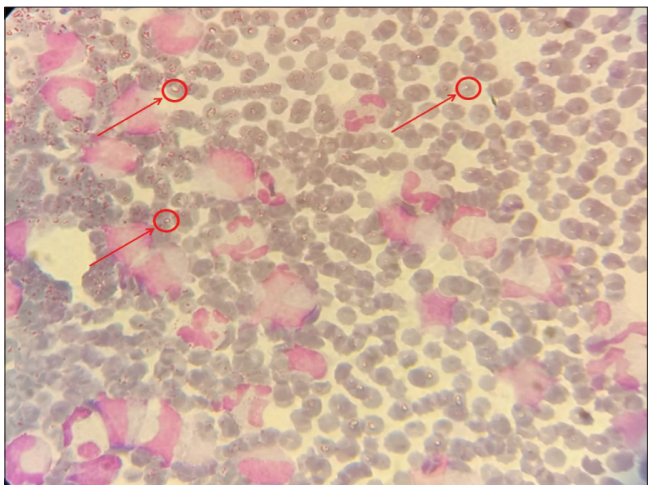

Fig 3. Thin blood smear of cattle samples collected from 3 different livestock zones of Balochistan, Pakistan (n=408), showing typical *Theileria* piroplasms, intraerythrocytic bodies, under oil immersion lens after field staining

Table 3. *Results of chi-square test on various risk factors associated with theileriosis in cattle from three different zones of Balochistan, Pakistan (n=408)*

Parameters		Quetta, (n=39) % Positive	Zhob, (n=50) % Positive	Loralai, (n=52) % Positive	Total (n=408)		Chi-square Value	P-value
					%Positive (n=141)	%Negative (n=267)		
Breed	Sahiwal	20.51	16.00	13.46	16.31	18.73	6.085	0.808
	Friesian	33.34	30.00	23.08	28.38	22.85		
	Red Sindhi	7.69	6.00	9.62	7.80	8.99		
	Cholistani	7.69	4.00	7.69	6.38	11.98		
	Cross Bred	20.51	36.00	40.38	33.33	26.22		
	Jersey	10.26	8.00	5.77	7.80	11.23		
Age	Young (1-2 year)	97.44	82	80.77	85.82	28.84	6.012	0.049*
	Adult (>2 year)	2.56	18	19.23	14.18	71.16		
Sex	Male	56.41	36	34.62	41.13	37.08	5.215	0.074
	Female	43.59	64	65.38	58.87	62.92		
Area	+Ve	9.56	12.25	12.75	34.56	-	0.483	0.786
	-Ve	19.61	21.08	24.75	-	65.44		

** P-value <0.05 was considered significant*

bodies resembling *Theileria* was also confirmed under a light microscope using thin blood smears *(Fig. 3)*.

As shown in *Table 3*, the prevalence of *T. annulata* was higher in young cattle (85.82%), followed by female cattle (58.87%), and exotic crossbred cattle (33.33%) in the studied area. However, various variable such as sex, area, and breeds of the cattle were not significantly correlated (P>0.05) with the presence of *T. annulata,* except for the age of the animals (P<0.05). Exotic cattle breeds, such as Friesian and crossbred, had a higher prevalence of theileriosis compared to local breeds like Sahiwal, Red Sindhi, or Cholistani. This may be attributed to the local breeds being more acclimatized to the conditions in the area. In addition, young animals (0-2 years) were found to be more susceptible to theileriosis compared to adult animals (P<0.05). Female cattle also exhibited a higher prevalence of theileriosis compared to males (P>0.05). These findings suggest that young animals are at a higher risk of theileriosis, compared to adult animals.

Sequencing Analysis of *Theileria* 18S rRNA Gene in Cattle

PCR positive samples were sequenced to analyze the 18S rRNA gene fragments of *Theileria*. The sequences were subjected to analysis using tools such as BLAST & CLUSTAL W alignments. Nucleotide sequences of the *Theileria* 18S rRNA gene were obtained from the NCBI GenBank for comparison. Phylogenetic analysis and BLAST queries revealed a 99% sequence similarity

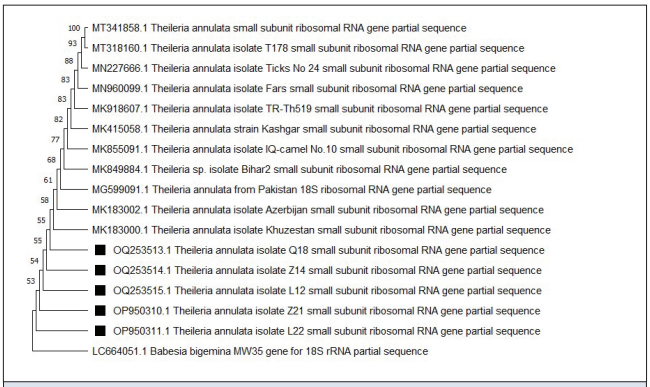

Fig 4. Phylogenetic tree illustrating sequence similarity among samples OQ253513.1, OQ253514.1, OQ253515.1, OP950310.1, OP950311.1, and previously reported sequences of the 18S small subunit rRNA gene of *Theileria annulata*. The partial 18S rRNA gene sequence of *Babesia bigemina* was included as an outgroup

to *T. annulata* 18S rRNA gene from various countries including Pakistan (MT318159), Iran (MN960099), Italy (MT341858), Türkiye (MK918607), and Egypt (MN227666). Notably, some of the sequences obtained in this study have been submitted to the NCBI GenBank *(Fig. 4)*.

Discussion

Tropical theileriosis is a well-known hindrance to the growth of the dairy industry worldwide and causes significant economic losses to the livestock industry [15,16]. However, there is a lack of sufficient and up-to-date molecular epidemiological studies on tick-borne

infections, particularly theileriosis, which hinders the development of preventive and control strategies [16]. In Balochistan, where a majority of the people rely on livestock for their livelihood, tick-borne diseases pose serious risks to the livestock industry [17]. Therefore, this study was designed to investigate the molecular prevalence and relevant risk factors of theileriosis in cattle from three different zones of Balochistan: Zhob, Loralai, and Quetta. Initial examination of thin blood smears from the sampled animals showed intra-erythrocytic bodies of theileriosis under a light microscope, consistent with previous findings Ekoka Mbassi et al.[14], Adehan et al.[18] and Chauhan et al.[19], which also observed similar intra-erythrocytic bodies in the blood smears of the *Theileria* positive animals.

In this study, a PCR-based prevalence of theileriosis was observed in cattle from 3 different zones of Balochitsan, Pakistan i.e. Loralai (12.75%), Zhob (12.25%) and Quetta (9.56%), accounting for a total of 34.56%. These results were in line with a study conducted by Durrani et al.[20], which reported a PCR based prevalence of *Theileria* in cattle from China to be 32.4%. Similarly, the prevalence of *T. annulata* in cattle from North-Western Pakistan was found to be 29.9% [21]. However, microscopic examination revealed a lower prevalence of theileriosis, specifically 31.37% (128/408), compared to PCR based prevalence. This findings aligns with previous reports by Chen et al.[22], Zeb et al.[23], and Zaman et al.[24], suggesting that microscopic identification is less precise and sensitive compared to PCR analysis. Therefore, PCR is considered the preferred tool for identifying blood-born parasites [25-,27].

In the present study, age of the animals was statistically significant risk factor for the occurrence of theileriosis in cattle of Balochitsan ($P<0.05$). However, other risk factors such as sex, area, and breeds of animals were statistically non-significant ($P>0.05$). These results were consistent with the reports of Mohammed-Ahmed et al.[28], Farhan et al.[29], and Kawan [30], suggesting that the occurrence of theileriosis in different zones may be linked with various known or unknown variables. Furthermore, the prevalence of theileriosis was observed to be higher in crossbred and Friesian cattle compared to Sahiwal, Red Sindhi, and, Cholistani, breeds in Balochitsan. These findings were similar to the results reported by Ghafar et al.[31] and Rana et al.[32], which indicated a higher prevalence of *T. annulata* in crossbred and exotic breeds of cattle compared to local breeds. Collectively, these findings indicate that exotic breeds and their crossbreeds face an increased susceptibility to tick-borne diseases [33,34]. This vulnerability may be attributed to inherent resistance, grooming behavior, or other biological factors influencing the interaction between native breeds and ticks. Furthermore, it is crucial to explore environmental conditions, climatic factors, and other ecological parameters that could contribute to a more stable or unstable enzootic state in various areas of Balochistan [33,34].

In addition, this study showed that female and young cattle (0-2 years) appeared to have a higher prevalence of *Theileria* than male and adult cattle in Balochistan. These findings were consistent with the results reported by Aslam et al.[35] and Farooq et al.[36], who also observed a higher prevalence of *T. annulata* in younger animals. However, Valente et al.[37] found no significant correlation between sex and the presence of theileriosis in cattle. On the other hand, Marwaha et al.[38] observed that the prevalence of theileriosis was higher in adults compared to young animals, possibly due to the higher immunity level in young animals caused by fetal hemoglobin in their circulatory system [39]. Moreover, the increased occurrence of theileriosis in younger animals, as observed in this study and others, might be linked to the larger body surface area of adult cattle, their outdoor management, prolonged movement over long distances in search of feed and water. In addition, the hormonal issues in female animals could potentially contribute to the higher prevalence of haemoparasitic infections in female cattle [40].

Finally, BLAST and phylogenetic analysis of the study isolates revealed a 99% sequence similarity with the *Theileria* isolates from Italy, Pakistan, Egypt, Iran, and Türkiye. The 18S ribosomal RNA gene sequence of studied isolates was aligned with similar reference sequences, showing significant variation in the genotype of *T. annulata* from 3 different zones of Balochistan, Pakistan. Similar studies on the sequencing and phylogenetic characterization of *T. annulata* have been conducted by Sisson et al.[41], Gargano et al.[42], and Solomon and Tanga [43], which are closely related to this study. Hence, it is important to highlight that the sequence similarities among globally discovered haemoparasites are primarily due to the export and import of animals [44].

The present study concludes that *T. annulata* is the main species causing theileriosis in the cattle populations of Balochistan, Pakistan. Several risk factors such as sex, area, and breeds of the animals, were not significantly associated with the disease dynamics, except for the age of the animals. Consequently, young animals are more susceptible to suffering from theileriosis. Overall, these results will be beneficial in designing more effective control strategies for theileriosis and provide a baseline for further research to eradicate or decrease haemoparasites in the future.

Availability of Data and Materials

The datasets generated during and analyzed during the current study are available from the corresponding author (K. Ashraf & M.U. Rehman) on reasonable request.

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Funding Support

N/A

Conflicts of interest

All other authors declare no conflicts of interest

Ethical Statement

This experimental design was duly approved by departmental ethics committee and directorate of advanced studies, University of Veterinary and Animal Sciences (UVAS), Lahore (ID: DAS/2053).

Author Contributions

KA and MUR contributed to the conceptualization, design, funding and supervision of the study. FK conducted all experiments and wrote the first draft of the manuscript. HA and MA collected, analyzed and interpreted the data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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