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Short communication

Molecular epidemiology and phylogenetic analysis of Tams I gene of *Theileria annulata* in Khyber Pakhtunkhwa, Pakistan

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Abstract

Theileriosis is a hemoparasitic disease that affects a wide range of different animal species and is caused by various species of *Theileria*. This study aimed to determine the molecular epidemiology of *Theileria annulata* through microscopy and PCR, in crossbred cattle in some districts of Khyber Pakhtunkhwa, Pakistan. For this study, a total of 384 blood samples were collected from cattle in the Peshawar (n=120), Charsadda (n=94), Nowshera (n=84), and Swabi (n=86) districts. Microscopy and PCR were used to determine the overall prevalence of theileriosis, which was found to be 15.8 and 22.6%, respectively. *Theileria annulata* was detected in blood samples through PCR in the study area, and the target gene i.e., Tams 1, of positive samples was sequenced. The sequences in the current study revealed high sequence homology (ranging from 96 to 100%) with Tams 1 sequences of neighboring countries present in the NCBI database. Season, breed, age, and sex were found to be important risk factors among the several risk factors examined, whereas, among different clinical manifestations, lymphadenopathy showed a strong association with theileriosis. According to Cohen's kappa and ROC analysis, microscopy was proven to be a fair diagnostic test for detecting theileriosis in cattle, and may be used in combination with molecular techniques for screening a large number of animals.

Key words: crossbred cattle, Pakistan, PCR, risk factors, sequencing, theileriosis

Introduction

Theileriosis caused by different species of *Theileria* infects a wide range of livestock species including cattle. Different species of *Theileria* can infect cattle but among them, *Theileria annulata* and *Theileria parva* are highly pathogenic whereas other species of *Theileria* are less pathogenic or avirulent. *Theileria annulata* and *Theileria parva* are the etiological agents for highly pathogenic and economically important diseases of cattle i.e., Tropical Theileriosis and East Coast Fever, respectively (Jabbar et al. 2015). Tropical Theileriosis is widespread in Asia, North Africa, Middle East, Southern Europe, and Asia including Pakistan. Theileriosis causes high morbidity and mortality in exotic and crossbred cattle (Bilgic et al. 2016),

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Table 1. Univariate logistic regression analysis of Theileriosis using PCR in crossbred cattle in Khyber Pakhtunkhwa, Pakistan.

Variables	Categories (n)	Percent positive (%)	Odd ratio	95% CI (Lower bound-upper bound)	P-value
Season	Summer (n=155)	34.8	3.21	1.45-7.1	0.00
	Winter (n=59)	8.4			
	Spring (n=90)	20			
	Autumn (n=80)	12.5			
District	Peshawar (n=120)	22.5	1.13	0.51-2.5	0.88
	Charsadda (n=94)	22.3			
	Nowshera (n=84)	23.8			
	Swabi (n=86)	22.1			
Breed	HF Cross (n=313)	25.9	5.9	2.2-15.2	0.01
	AJC (n=71)	8.4			
Age	Calf (n=60)	45	4.2	2.07-8.51	0.00
	12-24Mo (n=85)	17.6			
	Adult (n=239)	18.8			
Sex	Female (n=300)	25.6	3.6	1.81-8.6	0.01
	Male (n=84)	11.9			

thus inhibiting the introduction of improved cattle into endemic areas. Tropical Theileriosis is characterized by high fever, lymphadenopathy, oculo-nasal discharge, corneal opacity, and anemia, while dyspnea and diarrhea may develop in the later stage of the disease (Al-Emarah et al. 2012).

In Pakistan, there is currently a scarcity of data on the diagnostic accuracy of various diagnostic techniques, the molecular epidemiology of bovine theileriosis, and target gene sequencing. With these limitations in mind, the current study aims to determine the molecular epidemiology of *Theileria annulata* in crossbred cattle through microscopy and PCR, as well as sequencing and phylogenetic analysis of *Theileria annulata* merozoite surface protein (Tams 1).

Materials and Methods

Ethical statement

This study was ethically reviewed and approved by the members of the ethical committee, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar. Animal owners were informed clearly and their consent was taken before the collection of blood samples from their animals.

Study area and Sampling

Samples were collected from suspected cattle populations in different districts i.e., Peshawar, Charsadda, Nowshera, and Swabi. According to the Köppen classification, the climate of the central zone of Khyber Pakhtunkhwa is hot semi-arid, having prolonged hot summers and short, mild to cold winters. A total of 384 blood samples were taken from the jugular veins of suspected cattle in the districts of Peshawar (n=120), Charsadda (n=94), Nowshera (n=84), and Swabi (n=86).

Microscopy

Thin blood smears were prepared from fresh whole blood at the time of sample collection. Blood smears were air-dried and fixed with 100% methanol and stained with 10% Giemsa stain. Smears were washed with running tap water after 15-20 min of staining and air-dried. About 30-50 microscopic fields were examined for the presence of theileria piroplasm through an oil immersion lens i.e., 100x (Ullah et al. 2021).

Extraction of DNA and PCR

For PCR, DNA was extracted from anticoagulant (EDTA) added whole blood samples using an innuPREP DNA Micro kit (Analytik Jena, GmbH, Germany). *Theileria annulata* species-specific primers were used for the amplification of the Tams 1 gene

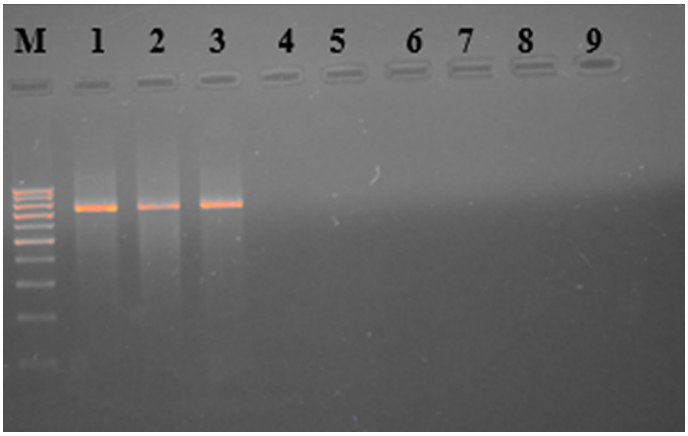

Fig. 1. Gel picture showing 100bp DNA ladder (M) and *Theileria annulata* positive samples having amplicon size of 721bp (1-3) and negative samples (4-9).

(F- GTAACCTTTAAAAACGT, R- GTTACGAACAT GGGTTT), having an amplicon size of 721 bp. PCR reaction cocktail (25µl) was prepared by adding 0.5 µl of each primer (10pmol), 5 µl PCR master mix (Solis biodyne), 5 µl template (30-50 ng/µl) and 11.5 µl PCR water (Ambion Thermo Fisher Scientific). Optimized conditions for PCR reaction were: initial denaturation of 95°C for 3min, followed by 34 cycles of cyclic denaturation at 95°C for 30 sec, annealing temperature of 53°C for 30 sec, cyclic extension at 72°C for 30 sec, and final extension for 5 min at 72°C. Amplification of the target gene was confirmed using gel electrophoresis.

Sequencing and phylogenetic analysis

The PCR product of the Tams 1 gene was sequenced unidirectionally by BGI (Hong Kong). The nucleotide sequences were analyzed initially using BioEdit 7.2.5 and any unnecessary nucleotides were trimmed and were searched for homologous sequences using NCBI BLASTn. All the nucleotide sequences of Tams 1 were aligned using MEGA X (Kumar et al. 2018).

Statistical analysis

Data regarding the sero-molecular prevalence of Theileriosis were analyzed using Binary logistic regression using SPSS version 21 to determine the statistical significance and association between the pathogen prevalence and other independent variables (Ullah et al. 2021), whereas contingency coefficient values were used for determining the association between theileria and signs/symptoms.

Results and Discussion

All the blood samples were screened initially through microscopy where bovine theileriosis was recorded as 15.8, 12.7, 14.2, and 20.1% in Peshawar, Charsadda, Nowshera, and Swabi, respectively, whereas the molecular prevalence of bovine theileriosis in Peshawar, Charsadda, Nowshera, and Swabi districts was 22.5, 22.3, 23.8, and 22.1%, respectively (Table 1). Microscopy revealed piroplasms in theileria-infected cattle erythrocytes, whereas PCR positive samples had an amplicon size of 721bp for *Theileria annulata* (Tams 1 gene) (Fig. 1). *Theileria annulata* merozoite surface protein (Tams 1) was used for the detection of *Theileria annulata* in blood samples in the present study, which has been used by various researchers around the world and was found to be a suitable candidate for the molecular detection of *Theileria annulata* (Farooqi et al. 2017). Sequences obtained for the PCR product were submitted to the Genebank database (Accession numbers: ON023258-61) and a partial sequence of *Theileria parva* 32kDa surface antigen (Accession number: XM761484) was included as an outgroup species in the phylogenetic analysis (Fig. 2). There was no geographical specificity in the Tams 1 gene sequence types, and nearly identical sequences were found in different geographical areas, implying a panmictic population structure (Habibi 2013).

When comparing the diagnostic accuracy of various tests, microscopy had a sensitivity and specificity of 60.92 and 97.97 %, respectively, whilst PCR was used as the gold standard test for detecting bovine theileriosis. Microscopy had AUC values of 0.791, revealing that microscopy is a fair diagnostic tool for bovine theileriosis. Microscopy can be used only in the acute stages when parasitemia is high, but is almost worthless in asymptomatic animals or when parasitemia is low (Rajendran and Ray 2014).

Theileriosis is a tick-borne disease, and because ticks are more active in the summer, the prevalence of theileriosis was also higher in this season, an observation supported by other researchers worldwide (Ullah et al. 2021). Age-related prevalence of theileriosis was

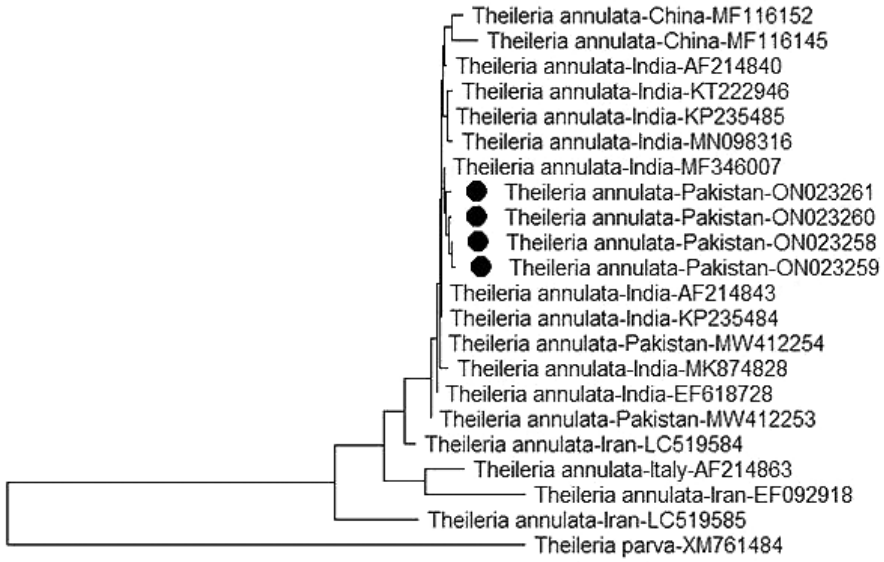

Fig. 2. Neighbor-joining phylogenetic analysis inferred from *Theileria annulata* Tams 1 gene (merozoite piroplasm surface antigens) sequences. Species name, followed by country and accession numbers. Samples sequenced in the current study are marked with circles.

higher in calves (23.8%), followed by adult (19.4%), corroborated by Atif et al. (2012). The prevalence of theileriosis was high in HF cross (25.9%) but was lower in AJC (8.4%). The susceptible breed (exotic and their crosses) produces high levels of macrophage pro-inflammatory cytokine-dependent acute phase proteins, whereas the tolerant breed i.e., Bos indicus produces significantly lower levels and can manage the cytokines storm (Ferrolho et al. 2016).

The overproduction of proinflammatory cytokines, particularly TNF-α, IL-1, and IL-6, is thought to be responsible for diverse clinical signs of theileriosis in cattle, i.e. fever and lymphadenopathy (Glass et al. 2005). The corneal opacity in cattle infected with theileriosis may be due to leucocytic infiltration of the cornea and lens. Diarrhea was found in 73.5 % of infected cattle and may be attributed to an inflammatory response and ulceration of the abomasal and gastrointestinal tract (Al-Emarah et al. 2012). The accumulation of edematous fluid inside the lungs and thoracic cavity may lead to respiratory distress and dyspnea in cattle infected with theileriosis.

Conclusion

Theileria annulata is responsible for causing theileriosis in cattle in the study area, according to the findings of the current study. According to the study finding, microscopy is a fair diagnostic technique and can be used in tandem with PCR to detect bovine theileriosis. Lymphadenopathy and high temperature were strongly associated with the disease among the clinical manifestations studied.

References

Al-Emarah GY, Khudor MH, Daham HR (**2012**) Clinical, haematological and biochemical study to cattle naturally infected with *Theileria annulata* in north of Basrah province. Al-Qadisiya J Vet Med Sci 11: 54-62.

Atif FA, Khan MS, Iqbal HJ, Arshad GM, Ashraf E, Ullah S (**2012**) Prevalence of *Anaplasma marginale, Babesia bigemina* and *Theileria annulata* infections among cattle in Sargodha District, Pakistan. Afr J Agric Res 7: 3302-3307.

Bilgic HB, Karagenc T, Bakırcı S, Shiels B, Tait A, Kinnaird J, Eren H, Weir W (**2016**) Identification and analysis of immunodominant antigens for ELISA-based detection of *Theileria annulata*. PLoS One 11: e0156645.

Farooqi SH, Ijaz M, Saleem MH, Rashid MI, Oneeb M, Khan A, Aqib AI, Mahmood S (**2017**) Distribution of ixodid tick species and associated risk factors in temporal zones of Khyber Pakhtunkhwa Province, Pakistan. Pakistan J Zool 49: 2011-2017.

Ferrolho J, Domingos A, Campino L (**2016**) Cattle specific immune mechanisms used against the protozoan *Theileria annulata*. Int Trends Immun 4: 45-49.

Glass EJ, Preston PM, Springbett A, Craigmile S, Kirvar E, Wilkie G, Brown CGD (**2005**) *Bos taurus* and *Bos indicus* (Sahiwal) calves respond differently to infection with *Theileria annulata* and produce markedly different levels of acute phase proteins. Int J Parasitol 35: 337-347.

Habibi G (**2013**) A comparative phylogenetic analysis of Theileria spp. by using two "18S ribosomal RNA" and "*Theileria annulata* merozoite surface antigen" gene sequences. Arch Razi Inst 68: 47-52.

Jabbar A, Abbas T, Saddiqi HA, Qamar MF, Gasser RB (**2015**) Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. Parasit Vectors 8: 283.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (**2018**) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Bio Evol 35: 1547-1549

Rajendran C, Ray DD (**2014**) Diagnosis of tropical bovine theileriosis by ELISA with recombinant merozoite surface protein of *Theileria annulata* (Tams1). J Parasit Dis 38: 41-45.

Ullah R, Shams S, Khan MA, Ayaz S, Akbar NU, Din QU, Khan A, Leon R, Zeb J (**2021**) Epidemiology and molecular characterization of Theileria annulata in cattle from central Khyber Pakhtunkhwa, Pakistan. PLoS One 16: e0249417.