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RESEARCH ARTICLE

Molecular Prevalence, Risk Factors Profiling, and Hematological Alterations Associated with *T. equi* Infection in Mules

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ABSTRACT

Equine theileriosis is a widespread tick disease affecting mules, donkeys, and horses. The current study was designed to study a comparison of microscopy and PCR for molecular detection of *Theileria equi* (*T. equi*) along with the assessment of associated risk factors and hematological alterations in mules of Pakistan. In this study, a total of 166 mule blood samples were subjected to microscopy and PCR. The microscopic and PCR-based positive samples (n = 12) and non-infected samples (n = 12) were subjected to hematological analysis. A data capture form was filled out to assess the risk factors associated with *T. equi* infection in mules. The current study showed that a total of 7.23% mules were found to be positive on microscopic examination, while based on PCR, 12.65% (21/166) mules were found positive for *T. equi* in the study districts. Risk factors profiling showed that vector control status, previous tick history, presence of ticks and tick-borne disease, and housing hygiene were found to be potential risk factors ($p<0.05$) associated with *T. equi* infection in mules. Hematological analysis revealed a decrease in RBCs, Hb, PCV, WBCs, and platelets ($p<0.005$) while a significant increase in lymphocytes and granulocytes, in *T. equi*-infected mules as compared to the healthy group, was observed. The study concluded that *T. equi* infection is prevalent in the mule population of Pakistan. The findings of risk factors profiling will help the livestock farmers adopt better management practices, and hematological analysis will aid veterinarians in promptly diagnosing the infection.

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INTRODUCTION

Ticks and tick-borne diseases (TBD) are the most important issue for livestock animals, affecting ruminants and equines in tropical and subtropical areas (Bouchard *et al.*, 2019). *Theileria equi* (*T. equi*), formerly *Babesia equi*, is a necessary hemoprotozoan that ticks spread. It belongs to the phylum Apicomplexa and is the main cause of equine piroplasmosis in tropical and semitropical regions (Ali *et al.*, 2021). The OIE has listed this illness, also known as theileriosis, as one of the major equine diseases, and it causes considerable monetary losses. This disease not only affects horses but also mules and donkeys (Shah *et al.*, 2020).

Ticks responsible for transmitting *T. equi and B. caballi* belong to the genera *Hyalomma*, *Rhipicephalus*, and *Dermacentor* (Uilenberg, 2006). *Boophilus microplus*, in particular, has been proven to be able to survive and maintain infection by transovarial and transstadial transmission for both *B.*

caballi and *T. equi* infection (Battsetseg *et al.,* 2002). Blood transfusions, the use of contaminated surgical instruments, and syringes of diseased animals are ways by which theileriosis can be transmitted to healthy equines (Short *et al.,* 2012). Equine piroplasmosis has several negative economic effects, including the inability to participate in equestrian sports, high treatment costs, diminished performance, lower productivity of equines, mortality, and export restrictions (Aziz and Barwary, 2019). Equine theileriosis is OIE OIE-listed disease and globally, this disease is more prevalent in tropical and subtropical areas, including Asia, the south and central parts of America, Europe, and Africa (Shah *et al.,* 2020).

Red blood cells (RBCs) contain *T. equi* merozoites, which are tiny and just 2-3um in length. They might have an ovoid or a spherical appearance (Sumbria *et al.,* 2014). According to the OIE (2009), *T. equi* takes 12 to 19 days for incubation. Different manifestations of the disease are possible, including acute, chronic, and peracute. Fever, anemia, jaundice, hemoglobinuria, and sometimes mortality are common symptoms (OIE, 2008). A carrier condition may exist in donkeys without any obvious symptoms, and antibodies may last for an entire life period.

T. equi infection typically presents with more severe clinical signs, although the signs and severity of the disease can widely vary depending on the area (Wise *et al.,* 2013). This disease has different forms, such as acute, per-acute, and chronic. In the acute form of theileriosis, pyrexia, apathy, hemolytic anemia, hemoglobinuria, and jaundice are evident, and sometimes, animals may die (Schein, 1998). In chronic form, the signs are not so observable. These animals can become a source of tick infection (Wise *et al.,* 2013; Schnittger *et al.,* 2012; Scoles and Ueti, 2015). In addition to exhibiting symptoms, animals with chronic illnesses represent a source of infection for ticks (Wise *et al.,* 2013; Scoles and Ueti, 2015; Schnittger *et al.,* 2012) . The severity of clinical signs may vary within species of host, e.g., horses are more susceptible to *B. caballi* than donkeys and mules (Acici *et al.,* 2008) .

T. equi can be diagnosed by using different methods such as thin blood smear staining examination, serological techniques, i.e., Enzyme-linked immunosorbent assay (ELISA) (Hirata *et al.,* 2002)Indirect fluorescence antibody test (IFAT) (Hirata *et al.,* 2005); (Asgarali *et al.,* 2007), immunochromatographic tests (Huang *et al.,* 2004), complement fixation test (CFT), and by using recombinant antigen, competitive ELISA (cELISA) (Katz *et al.,* 2000) and Polymerase chain reaction (PCR) (Wise *et al.,* 2013). Sensitive PCR-based diagnostics can be used to identify hidden species' genetic differences in addition to addressing the issue of low parasite numbers in blood during concealed infections. 18SrRNA (Lukeˇ *et al.,* 2011; Alhassan *et al.,* 2005; Bashiruddin *et al.,* 1999), β tubulin (Camma and Caccio, 2000), and genes that code for EMA (Battsetseg *et al.,* 2002) have all been used as diagnostic targets for *T. equi*. Compared to serological methods, PCR allows for the most specific and sensitive detection of diverse *Theileria* and *Babesia* species (Sibeko *et al.,* 2008; Jefferies *et al.,* 2007; Buling *et al.,* 2007; Geysen *et al.,* 2003).

To identify and examine the evolutionary relationships of *T. equi*, the 18S rRNA gene is regarded as the most useful genetic marker. This is because of the functions that have been maintained, the slow pace of substitution, and the existence of numerous copies (Hunfeld *et al.,* 2008; Allsopp and Allsopp, 2006). A reverse line blot (RLB) hybridization method has been developed to detect infections caused by *Theileria* and *Babesia*, including equine piroplasmosis, which has higher specificity and sensitivity. The 18SrRNA genes of the parasite are the target of this method. It is based on sequence-specific PCR (Gubbels *et al.,* 1999; Nagore *et al.,* 2004). This assay has been a useful tool for aiding in the detection of mixed infections, as well as novel piroplasm genotypes or species (Nijhof *et al.,* 2005; Nagore *et al.,* 2004; Garcı *et al.,* 2004; Criado-fornelio, 2004 ;Georges *et al.,* 2001).

To date least data is available on molecular and serological diagnosis of *T. equi* in mules in Pakistan. The present study described the prevalence of *T. equi* in the Sahiwal and Mandi Bahauddin districts of Punjab (Pakistan), and the efficiency of various diagnostic assays was compared in this study using microscopic examination and polymerase chain reaction (PCR). This study also evaluated epidemiological risk factors, pathophysiological conditions of the infection, and examined hematological changes. Veterinary clinicians and epidemiologists can benefit from these findings.

MATERIALS AND METHODS

Study Animal: The study was conducted from January to December 2021. The mules (166 animals) were targeted by using the convenient method of sampling, irrespective of sex, color, age, and breed. These mules belonged to various government, private stud farms that were located in the district of Sahiwal and Mandi Bahauddin, Punjab, Pakistan. By looking at the world map, Mandi Bahauudin and Sahiwal districts are situated at 32°34′47″N 73°28′53″E and 30°40′0″N 73°6′0″E respectively. Geographic information system (GIS) mapping was

used to display coordinates of sampling areas for the prevalence of *T. equi* in mules, as shown in Figure 1.

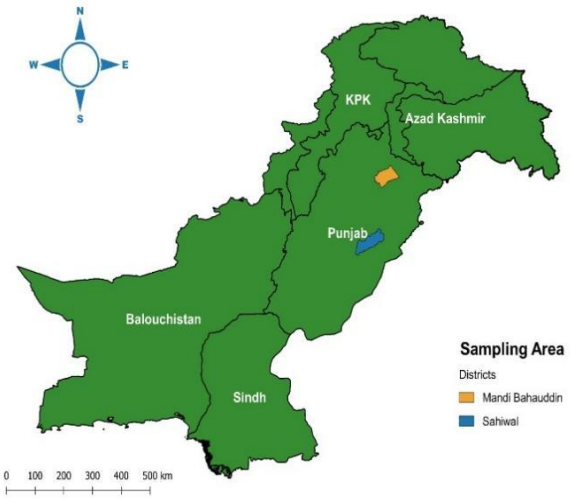

Fig. 1: QGIS map showing the study areas.

Inclusion Criteria: Those mules showing any of the clinical signs of fever, anorexia, tick infestation, anemia, or pale mucous membrane were included in this study.

Blood Sampling: Blood sampling was done in two steps. Firstly, thin smears of blood (triplets) were prepared by puncture of the ear vein and then air dried on the spot. Secondly, 6ml of blood was taken out from the jugular vein of each animal and then put into gel-coated (3ml) and EDTA (3ml) vacutainers for serological and molecular diagnosis, respectively. Samples of blood were then transported to the Molecular Medicine Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, and stored at -20 ºC.

Risk Factor Analysis: Data regarding the animal, the animal's owner, managemental and environmental factors were recorded for each sample on the questionnaire at the time of sample collection.

Microscopic Examination for *T. equi:* First of all, thin smears of blood were prepared and then fixed with 10% ethanol. These fixed blood smears were stained using Giemsa. After half an hour (30 minutes), the stain was removed and allowed to dry. These stained blood smears were screened and then evaluated for intra-erythrocytic merozoites (piroplasms) by using microscopy under an oil immersion lens (100X).

Molecular Diagnosis: DNA was extracted from 166 uncoagulated samples of blood (n= 166 mules) by using a DNA extraction kit (GeneAll®, Exgene™, 105-101). After this, DNA was quantified by using Nanodrop, and PCR was performed by using specific primers, Forward primer (F=5′-TTCTTAGAGGGACTTTGCGGT-3′) and Reverse primer (R= TGACTTGCGCATACTAGGCA-3′) as used by (Ali *et al.*, 2021) and conditions includes 40 cycles of initial denaturation at the 96°C for 10 minutes and final denaturation at 96°C for 1 minute followed by annealing at temperature of 58°C for 1 minute, extension at temperature of 72°C for 1 minute, and final extension at 72°C for 10 minutes by targeting the 18SrRNA gene with the product size of 220bp.

Hematological Parameters Analysis: A total of 24 mules (12 positive mules and 12 healthy mules) were selected for the hematological analysis. For hematology, a 6ml of blood sample was collected in Gel (3ml) and EDTA (3ml) coated vacutainers. Using the hematological analyzer, various hematological parameters including HGB (Hemoglobin), HCT (hematocrit), RBCs count (Red blood cells), GR (Granulocytes), MCHC (mean corpuscular hemoglobin concentration), PLT (Platelets), WBCs count (White blood cells) and LY (Lymphocytes) were analyzed before treatment and 96 hours after of first treatment and values were compared with reference ranges.

Statistical Design: The prevalence of *T. equi* was calculated by using the formula of Thrusfield *et al. (*2018). Chi-square and multivariable logistic regression were used for the analysis of risk factors. The data regarding hematobiochemical parameters were examined using an independent sample t-test with a 95% confidence interval by using SPSS version 20.00. Potential risk factors linked to disease dynamics were identified as variables with a p-value <0.05 and an odds ratio >1.00. Med-Calc statistical software was used to analyse data about the comparative study of microscopic inspection with a 95% confidence interval.

RESULTS

Prevalence of *T. equi* infection in mules: In this study, a total of 7.23% (12/166) of mules were found to be positive on direct microscopic examination, with a frequency of 6.02% (05/83) in Sahiwal and 8.43% (12/97) in Mandi Bahaudin respectively while based on PCR, 12.65% (21/166) mules were confirmed positive for *T. equi* with a frequency of 09.64% (8/83) in Sahiwal and 15.66% (13/83) in Mandi Bahaudin district (Table 1).

Table 1: Prevalence of *T. equi* infection in mules based on diagnostic tests

Location	Microscopic		PCR	
	No. Positive	(%)	No. Positive	(%)
Sahiwal (n=83)	05/83	6.02	8/83	09.64
Mandi Bahaudin (n=83)	07/83	8.43	13/83	15.66
Total (n=166)	12/166	7.23	21/166	12.65

Comparative evaluation of Microscopy to gold-standard PCR: In this study, a total of 166 samples were inspected by using microscopy and PCR. For comparative analysis, microscopy results were contrasted with a more sensitive and gold standard test, PCR. Out of (n=166) samples, 9 samples exhibited positive results for both microscopy and PCR, while (n=12) microscopy-based negative isolates exhibited bands on PCR, and (n=03) microscopy-based positive samples were found negative on PCR (Table 2). Med Calc statistical software was used to calculate the microscopic examination's sensitivity, specificity, and accuracy as 42.86%, 97.93%, and 90.96%, respectively. Additionally, it was found that the negative and positive likelihood ratios were 0.58 and 20.71, respectively. For microscopic examination, the positive predictive value was 75.00% while the negative predictive value was calculated as 92.21% (Table 3).

Table 2: Data obtained from microscopic examination

Microscopy	*T. equi* PCR		
	Positive	**Negative**	**Total**
Positive	True positive (9)	False-positive (03)	TP+FP (12)
Negative	False-negative (12)	True negative (142)	FN+TN (154)
Total	TP+FN (21)	FP+TN (145)	TP+FN+FP+TN (166)

True positive = Mules with PCR positive and ME positive
True negative = Mules with PCR negative and ME negative
False-positive = Mules with PCR negative and ME positive
False-negative = Mules with PCR positive and ME negative.

Table 3: Comparison of Microscopic examination with PCR for the diagnosis of *T. equi* infection

Statistic parameter	Value	CI 95%
Sensitivity	42.86%	21.82% to 65.98%
Specificity	97.93%	94.07% to 99.57%
Positive Likelihood Ratio	20.71	6.09 to 70.44
Negative Likelihood Ratio	0.58	0.40 to 0.85
T. equi Prevalence	12.65%	8.00% to 18.69%
Positive Predictive Value	75.00%	46.87% to 91.07%
Negative Predictive Value	92.21%	89.09% to 94.49%
Accuracy	90.96%	85.53% to 94.85%

Assessment of epidemiological risk factors: Regression analysis of significant and potentially significant risk factors was performed to evaluate potential risk factors (Table 4). These results exhibited that *T. equi* was shown to be more prevalent in mules having a previous tick history (16/79; 76.2 percent) as compared to no previous tick history (05/87; 23.8 percent). Likewise, *T. equi* was shown to be more prevalent in having tick infestation (18/86; 85.7 percent) as compared to no tick infestation (03/80; 14.3 percent). Moreover, Mules that were reared with no vector control strategy (16/82; 76.2 percent) had a higher prevalence of the disease as compared to mules reared with a vector control strategy (5/84; 23.8 percent). Also, Mules reared in good hygiene conditions (04/73; 19.0 percent) were affected less as compared to poor house hygiene (17/93; 81.0 percent), as given in Table 4.

Risk factor analysis by multiple logistic regression: Potential risk factors (p<0.05) for *T. equi* in mules were further analyzed by a multiple logistic regression model. The most significant possible risk variables for *T. equi* in mules, as determined by the multivariate logistic regression model, were vector control (OR=4.703), previous tick history (OR=3.041), presence of ticks (OR=4.045), and housing hygiene (OR=3.713). The mules with no vector control strategy, previous tick history, presence of ticks, poor housing hygiene, and previous history of TBD were 4.703, 3.041, 4.045, and 3.713 times had higher chances of getting *T. equi* infection in comparison to mules with vector control practices, no previous tick history, no presence of ticks, and good housing hygiene respectively (Table 5).

Table 4: Assessment of epidemiological risk factors

Variable	Variable levels	Study population (n=166)	Positive (%)	*p*-value
Age	≤ 6 years	78	11 (52.4%)	.868
	7-12 year	61	7 (33.3%)	
	>12 year	27	3 (14.3%)	
Previous Tick History	Yes	79	16 (76.2%)	.005
	No	87	5 (23.8%)	*
Presence of ticks	Yes	86	18 (85.7%)	
	No	80	3 (14.3%)	.001 *
Vector Control	Yes	84	5 (23.8%)	.009
	No	82	16 (76.2%)	*
Animal living area	Open	94	12 (57.1%)	.959
	Closed	72	9 (42.9%)	
House Hygiene	Good	73	4 (19.0%)	.014
	Poor	93	17 (81.0%)	*
Body Condition Score	Normal	61	8 (38.1%)	
	Fatty	9	1 (4.8%)	.984
	Emaciated	96	12 (57.1%)	
Cracks	Present	83	13 (61.9%)	.243
	Absent	83	8 (38.1%)	
Management	Grazing	138	18 (85.7%)	.735
	Stall fed	28	3 (14.3)	
Deworming/Vaccination	Yes	63	7 (33.3%)	.641
	No	103	14 (66.7%)	
Grooming	Yes	84	9 (42.9%)	.448
	No	82	12 (57.1%)	
Rearing system	Same Spp.	71	7 (33.3%)	.448
	Different Spp.	95	14 (66.7%)	

Table 5: Summary of risk factors associated with the occurrence of *T. equi* in mules: variables included in the final logistic regression model.

Variable	Variable Levels	Odds Ratio	CI (95%)	*p*-value
Presence of Ticks	Yes	4.045	15.624-1.047	.001
	No	1		
Previous Tick History	Yes	3.041	9.603-.963	.005
	No	1		
Vector Control	Yes	1	15.034-1.471	.009
	No	4.703		
Housing Hygiene	Good	1	13.085-1.054	.014
	Poor	3.713		

Effect of *T. equi* infection on hematological parameters: For relative assessment, CBC values of *T. equi-infected* and healthy mules were analyzed. In hematology, the infected group of mules exhibited a decrease in RBCs, Hb, PCV, WBCs, and platelets (p=0.000) that were significantly (p=0.000) linked with disease incidence when compared with healthy animals. The results also revealed a substantial rise in lymphocytes (p=0.00) and granulocytes (p=0.000) in theileriosis-infected mules compared to the healthy group (Table 6).

Table 6: Comparative effect of *T. equi* on RBCs (× 10^6 /µL) count between healthy and diseased mules

CBC parameter	Animal category	Mean ± S.D.	*F*-value	CI (95%)	*p*-value
RBCs (×10^6 /µL)	Healthy	9.3667±.57261	.995	-2.84569-(-3.97098)	<0.001
	Diseased	5.9583±.74524			
Hematocrit (%)	Healthy	40.8833±3.75471	2.060	-13.75164-(-19.18169)	<0.001
	Diseased	24.4167±2.54338			
Hemoglobin (g/dL)	Healthy	14.3417±.52477	1.855	-6.07644-(-7.27356)	<0.001
	Diseased	7.6667±.85102			
WBCs (×10^3 /µL)	Healthy	8.2000±.82241	1.964	-2.92583-(-4.14083)	<0.001
	Diseased	4.6667±.59442			
Lymphocytes (%)	Healthy	46.5833±7.65445	2.545	42.07136-25.92864	<0.001
	Diseased	80.5833±11.09839			
Granulocytes (%)	Healthy	49.7583±7.59371	2.829	38.72823-21.25511	<0.001
	Diseased	79.7500±12.46176			
Platelets (×10^3 /µL)	Healthy	242.6667±23.62331	1.093	-74.115377-(-118.88463)	<0.001

DISCUSSION

Worldwide, equine piroplasmosis is a significant economic concern for horses that results in significant financial losses, health issues, and limited equine mobility for equestrian competitions, trade, and export in disease-free nations (Díaz-sánchez *et al.*, 2018; Cabete *et al.*, 2025). Therefore, the objective of this research was to make a comparison of the available diagnostic techniques and assess their applicability for more conclusive *T. equi* infection surveys. This study is the first report designed to compare the different diagnostic tests (microscopic examination and PCR) along with the evaluation of potential risk factors and haematological alterations associated with *T. equi* in the study area.

Microscopic analysis of a thin blood smear stained with Giemsa of the current study revealed 7.23% positive samples. And the results were in agreement with the findings of (Díaz-sánchez *et al.*, 2018; Habibi *et al.*, 2016) who outlined 9%, 9.67%, and 8.9% in Cuba, Kurdistan province of Iran, and Cairo and Giza provinces of Egypt, respectively. While contrary to this study, São Paulo State, Brazil, West Azerbaijan, Turkey, and India reported a lower prevalence of 3.52%, 3.2%, 4.8%, and 4.17%, respectively (Baldani *et al.*, 2010 ; Ebrahimi *et al.*,2018; Guven *et al.*, 2017;Sumbria *et al.*, 2016) and (Mahdy *et al.*, 2016) narrated a high prevalence of 27.4% in Egypt. Due to low levels of parasitemia, microscopic examination is not very sensitive in detecting *T. equi* infection during the carrier and latent stages (Sumbria *et al.*, 2016). However, due to cheap and rapid results, this diagnostic approach is still extensively utilized.

Moreover, 12.65% (21/166) prevalence was confirmed by PCR, which is consistent with the results (Mshelia *et al.*, 2020; Slivinska *et al.*, 2016) who reported 13% and 13.95% *T. equi* prevalence in Nigeria, Ukraine, Poland, and Slovakia horses on the molecular basis. While lower than Central Balkan (22.5%), Venezuelan (61.8%), Southwest Mongolia (19.1%), and Xinjiang province (40.8%) prevalence, respectively (Davitkov *et al.*, 2016; Rosales *et al.*, 2013; Rüegg *et al.*, 2007; Zhang *et al.*, 2017) and higher than Turkey (8.8%), and Erzurum, Turkey (2.96%) (Guven *et al.*, 2017; Kizilarslan *et al.*, 2015).

Tick control status, previous tick history, and tick infestation in mules were the utmost important possible risk factors found to have a direct relationship with the rate of *T. equi* infection. This is in line with the results of previous studies (Sumbria *et al.*, 2016; Abbas *et al.*, 2017; Shah *et al.*, 2020; Ali *et al.*, 2021; Javed *et al.*, 2021), which declared that infection rates were higher in equines raised with inadequate tick control measures, prior tick history, and tick infestations than in equines raised without these risk factors.

Horses in Pakistan are frequently immunized against tetanus and equine influenza, but not so for EP (no vaccine available). Other infections, when combined with poor house hygiene, can have an immunosuppressive impact on unvaccinated animals and increase the risk of infection, which is in concordance with results of (García-bocanegra *et al.*, 2013; Sumbria *et al.*, 2016). As a result, mules without an appropriate deworming/vaccination program had a considerably greater prevalence of *T. equi*. This infection was also associated with cohabitation with other livestock animals, as

formerly outlined by Guidi *et al.* (2015). The explanation is that under mixed farming, ticks' capacity to spread is limited, and their survival is reliant on the presence of compatible hosts. Other research studies also found that larger numbers of Ixodes ticks were gathered in mixed farming (Camacho *et al.,* 2005). There were no significant variations in the prevalence of *T. equi* infections across the different groups (age, sex, housing type, anemia, grazing type, BCS, and cracks).

According to this study, the infected group's hematological profile revealed a notable drop in RBCs, Hb, PCV, and WBCs, suggesting that hemolytic anemia was a nearly constant feature of this illness. And similar results were also claimed by (Ali *et al.,* 2021; Mahmoud *et al.,* 2016). The pathophysiology of anemia in canine babesiosis is well established, it is a complex process in horse piroplasmosis. There are three known mechanisms of hemolysis: immune-mediated hemolysis brought on by autoantibodies against the membrane components of infected and uninfected erythrocytes, toxic hemolysis brought on by the parasite's hemolytic factor, and mechanical hemolysis brought on by trophozoite intra-erythrocyte binary fission. It was also believed that similar pathways contributed to EP-induced haemolysis (Ali *et al.,* 2021; Sumbria *et al.,* 2016). Other repeated results among the infected animals studied were Lymphocytosis, granulocytosis, thrombocytopenia, and leukopenia. Higher lymphocytes, granulocytes, and lower platelets, WBCs were also documented by (Ali *et al.,* 2021; Camacho *et al.,* 2005) in *T. equi* infected horses.

Conclusion

In Pakistan, this study is the first report for the relative assessment of *T. equi* infection by using two different diagnostic techniques, including microscopy and PCR. Microscopy has low sensitivity, is relatively cheap, and easy to perform as compared with the gold standard PCR. Therefore, for epidemiological studies, accurate diagnosis of equine piroplasmosis must be done by using conventional and molecular techniques. Although this is a comprehensive approach for the identification of *T. equi* infection, associated potential risk factors, and hemato-biochemical alterations. So that it will be helpful to plan strategic preventative and control measures against this vector-borne illness to avoid economic losses to the equine industry.

Conflict of Interest: The authors have no competing interests.

Authors' Contribution: PB conceived the idea and supervised the research. MJS performed the experiments. PB and MJS wrote the manuscript.

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