Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (1): 41-48, 2023
DOI: 10.9775/kvfd.2022.28455

RESEARCH ARTICLE

Molecular Detection of *Theileria annulata* Infection: An Emerging Disease of Pet Dogs in Pakistan

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Article ID: KVFD-2022-28455 Received: 13.09.2022 Accepted: 06.01.2023 Published Online: 27.01.2023

Abstract: Tick-borne maladies of canines are increasing in sub-tropical and tropical areas around the globe. Theileriosis is an emerging issue in canines, especially dogs but the data regarding the disease prevalence in Asian countries is scarce. Therefore, the current study was designed to check the molecular prevalence of *Theileria* spp. in dogs of Lahore, Pakistan. A total of 102 dog blood samples were initially screened by microscopy and later on by PCR. The results revealed that 10.78% (11/102) and 6.86% (7/102) of pet dogs were found positive by PCR and microscopy, respectively. The phylogenetic analysis showed the sequences resemblance up to 98-99% with *cytb* gene fragments of *Theileria annulata* isolates from Iran, India, Turkey, Sudan, China, and Tunisia. Assumed risk factors revealed a significant (P<0.05) association of house hygiene, tick infestation, and tick prevention applications with the disease dynamics. The infected animals also showed a significant decrease (P<0.05) in values of red blood cells (RBCs), hemoglobin level, PCV, and platelet count. This is the first report regarding the molecular evidence of *T. annulata* infection in dogs of Pakistan. This study will help control the increasing tick-borne maladies of dog population in Pakistan.

Keywords: *Dog, Phylogenetic analysis, Theileria annulata, Risk factors, Hematology*

Theileria annulata Enfeksiyonunun Moleküler Tespiti: Pakistan'da Evcil Köpeklerde Ortaya Çıkan Bir Hastalık

Öz: Köpeklerde kene kaynaklı hastalıklar dünya genelinde sub-tropikal ve tropikal bölgelerde artış göstermektedir. Theileriosis, özellikle köpeklerde ortaya çıkan bir problemdir ancak Asya ülkelerindeki hastalık prevalansına ilişkin veriler yetersizdir. Bu nedenle, bu çalışma Pakistan'ın Lahor kentindeki köpeklerde *Theileria* spp.'nin moleküler prevalansını belirlemek için planlanmıştır. Toplam 102 köpek kan örneği önce mikroskopi ile daha sonra PCR ile analiz edilmiştir. Sonuçlar, evcil köpeklerin sırasıyla %10,78'inin (11/102) PCR ve %6,86'sının (7/102) mikroskopi ile pozitif olduğunu ortaya koymuştur. Filogenetik analiz, sekans sonuçlarının İran, Hindistan, Türkiye, Sudan, Çin ve Tunus'tan izole edilen *Theileria annulata* izolatlarının *cytb* gen fragmanlarıyla %98-99'a varan benzerlik gösterdiğini ortaya koymuştur. Varsayılan risk faktörleri, ev hijyeni, kene istilası ve kene önleme uygulamaları ile hastalık dinamikleri arasında anlamlı bir ilişki olduğunu ortaya koymuştur (P<0.05). Enfekte hayvanlar ayrıca eritrosit (RBC), hemoglobin seviyesi, PCV ve trombosit sayısı değerlerinde önemli bir düşüş göstermiştir (P<0.05). Bu çalışma, Pakistan'daki köpeklerde *T. annulata* enfeksiyonunun moleküler tanısına yönelik ilk rapordur.

Anahtar sözcükler: *Köpek, Filogenetik analiz, Theileria annulata, Risk faktörleri, Hematoloji*

How to cite this article?
Nawab Y, Ijaz M, Ayyub Rm, Ahmed A, Muzammıl I, Ghumman Nz, Javed Mu, Adıl A: Molecular detection of *theileria annulata* Infection: An emerging disease of pet dogs in Pakistan. *Kafkas Univ Vet Fak Derg,* 29 (1): 41-48, 2023.
DOI: 10.9775/kvfd.2022.28455

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INTRODUCTION

Canine theileriosis is a globally emergent vector-borne malady, transmitted by ticks [1,2]. Various species of *Theileria* (phylum Apicomplexa) have been reported to cause the disease in dogs [3]. In the infective stage of the pathogen, sporozoites infect leukocytes and mature into merozoites which finally infect erythrocytes of vertebrate hosts to form piroplasms [4]. The first reported *Theileria* spp. in dogs was *Theileria annae* [5,6] which was later on reported in various countries including Germany [5], Croatia [7], Spain [8-11], USA [12], Barcelona [13], Sweden [14], Portugal [15], Serbia [16], France [17] and Southern Europe [18]. However, *Theileria annulata* has been identified and reported by Bigdeli et al.[19] and Aktas et al.[20] in Southern Iran and Turkey, respectively. Furthermore, in diseased dogs of Paraguay, France, South Africa, and Nigeria, *Theileria equi* has also been confirmed [3,21-23]. Though, the molecular occurrence of *Theileria* spp. in pet dogs of Pakistan is not yet reported.

Clinically the diseased dogs present a wide range of signs including anorexia, lethargy, anemia, pale mucous membranes, jaundice, tachypnea, splenomegaly, tachycardia, and yellowish-orange to brownish colored urine [24]. Furthermore, the severe manifestation of similar clinical signs in other animals has also been reported [25]. The routine diagnosis of the disease is based on the history of tick infestation, clinical findings, and hematological alterations. Microscopic examination of blood smears can also be used for the detection of the responsible pathogen [26]. The serological methods used for the disease diagnosis include IFAT and ELISA [27] but molecular technique like Polymerase Chain Reaction (PCR) is considered a more sensitive, expedient, and reliable technique for diagnosis [28,29].

Mainly *T. annulata* is considered to be involved in persistent infection of cattle. However, the pathogen is reported to cause severe maladies among dogs across the globe, but the data regarding the molecular detection of *T. annulata* in pet dogs is limited in our country. In Pakistan, theileriosis has been reported in bovines, small ruminants, and equines [30-35], but the presence of *T. annulata* infection in pet animals especially dogs is not reported. Therefore, the current study proves to be the first molecular evidence of *T. annulata* infection in dogs of Pakistan. Assessment of risk factors and alterations in hematology values associated with the *Theileria* infection was also a part of the current study.

MATERIAL AND METHODS

Ethical Statement

The current study was approved by the ethical committee of advanced studies research board vide approval number DAS/8438 in the University of Veterinary and Animal Sciences, Lahore. No animal was harmed during the sampling and all the standard operating procedures were followed throughout lab activities.

Study Design

The study was conducted at different private and government veterinary hospitals of district Lahore, Pakistan from March to September 2020 (*Fig. 1*). A total of 102 pet dogs (n=102 dogs) having tick infestation or

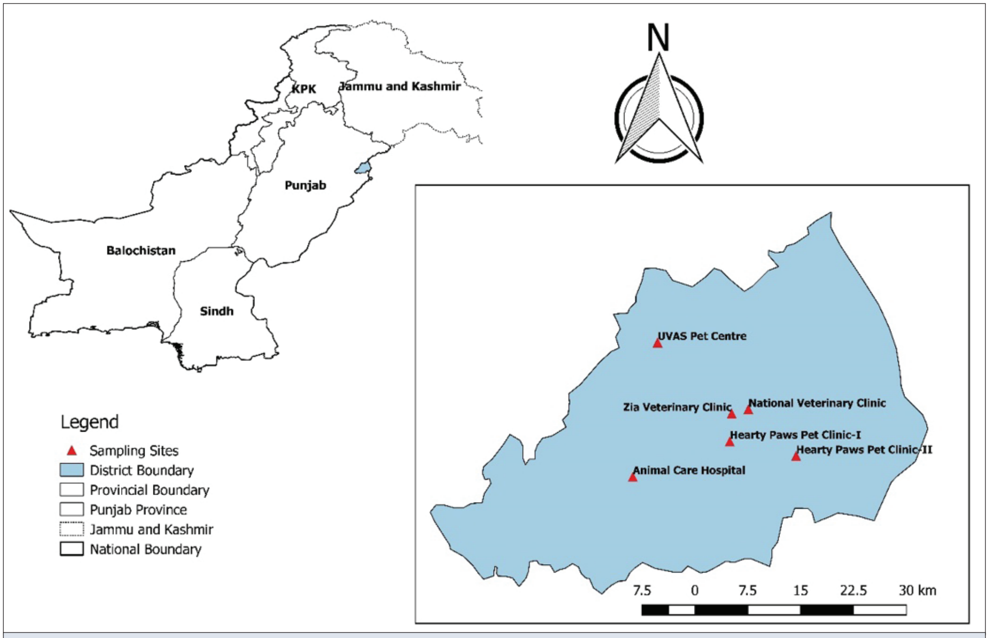

Fig 1. QGIS map showing the sampling sites

showing signs like pyrexia, pale mucous membrane, jaundice, anemia, and anorexia were involved in the study regardless of breed, sex, and age of animals. Consent was obtained from officials before sampling.

Blood sampling was done from study animals in two ways. For microscopic examination, blood smears were made in triplets from ear vein puncture and air-dried on spot. Later on, samples were evaluated through microscopy by performing Giemsa staining of all slides. After that, for molecular analysis, 3 mL of blood was drained aseptically through a cephalic vein from each dog and transferred to Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore. The association of the disease with various risk factors was elaborated by collecting information about various environmental and animal management factors on a predesigned questionnaire.

DNA Extraction

The DNA from each 200 µL blood sample was extracted by using a DNA extraction kit (GeneAll® Exgene™ Blood SV mini 105-101) following the manufacturer's directions. The testing of purity and concentration of all extracted genomic DNA samples was performed using Nano-drop at 260/280 nm. For further processing, DNA samples were kept at -20°C.

PCR Amplification

The amplification of *cytb* gene of *Theileria* species was executed using primers consisting of forward primer as F=GGGAGCTACAGTCATAGGTGGT and reverse primer as R=TCCTGCCATTGCCAAAAGTC and the conditions used for running the PCR reaction were as mentioned by Zaheer et al.[36]. PCR products were visualized under UV light in a 1.5% ethidium bromide-stained agarose gel after gel electrophoresis.

Sequencing

The positive amplified bands were sliced from the gel under ultra-violet light using a sterile blade and subjected to purification by a gel extraction kit (GeneAll® Expin™ Gel SV (102-150). For sequencing, samples of purified genomic DNA were shipped to 1st Base biological technology, Singapore. The sequencing results were evaluated through the NCBI Blast and CLUSTAL W alignment method. A phylogenetic tree was constructed on sequence distance using the Maximum likelihood method of phylogeny testing on Mega X (Molecular Evolutionary Genetics Analysis version 10.0) software.

Hematological and Statistical Analysis

Various hematological parameters including; WBCs count, RBC count, hemoglobin (Hb), and packed cell volume (PCV) were analyzed using a hematology analyzer. The statistical analysis was performed on the data regarding risk factors and the hematological parameters using SPSS version 20.00. The association of risk factors like age, sex, breed, previous tick history, type of acaricide, tick control status, interval of acaricide application, house hygiene, tick infestation, and housing type, was analyzed statistically using logistic regression analysis and Chi-square test. An independent sample t-test was used to evaluate the changes in blood parameters with a confidence interval of 95%. The variables with P-value <0.05 and odd ratio >1.00 were assumed to be significantly associated with disease occurrence.

RESULTS

Epidemiology of *Theileria* spp. in Pet Dogs

The study showed a 10.78% (11/102) molecular-based prevalence of canine theileriosis in dogs from Lahore, Pakistan. The sequencing of samples declared the gene sequence to be of *T. annulata*. However, the microscopic-based examination showed a 6.86% prevalence of *Theileria* spp. infection in studied animals.

Assessment of risk factors associated with canine theileriosis revealed tick infestation to be significantly associated (P=0.014) with the disease occurrence. Tick control status was also a significantly proven (P=0.016) risk factor. The other risk factor significant (P=0.036) associated with the disease incidence was house hygiene as dogs raised in good hygiene conditions showed less disease prevalence as compared to dogs raised in poor hygiene conditions. Factors like age, breed, sex, previous tick history, acaricide type, and interval of acaricide application were also statistically analyzed and found non-significant risk factors for the occurrence of the disease. Housing type was also proved a non-significant determinant. However, dogs raised in cages revealed more disease prevalence (14.3%) as compared to the dogs reared in houses made of concrete (8.3%) *(Table 1)*.

Theileria cytb Gene Analysis

This is the first molecular-based evidence for the existence of *Theileria* spp. in dogs of Lahore, Pakistan. The sequencing of PCR products of study samples was done for *cytb* gene of Theileria and was analyzed later on using Bioinformatics software including CLUSTALW and BLAST alignment. Various *cytb* sequences of *Theileria* were obtained from the Genbank database to compare them with study isolates. Phylogenetic analysis was performed using maximum likelihood method *(Fig. 2)*. The study isolates showed a high similarity with the *T. annulata* sequences from the GenBank database. The phylogenetic analysis revealed the present study sequences of *T. annulata* show the highest resemblance with the gene sequence of *T. annulata* from Pakistan which is isolated

Table 1. *Results of Chi-square test on various assumed risk factors associated with theileriosis*

Study Variable	Category	*T. annulata* Positive (%)	*T. annulata* Negative (%)	P Value
Gender	Male	4 (7.7)	48 (92.3)	0.305
	Female	7 (14.0)	43 (86.0)	
Age	≤ 1Years	5 (10.0)	45 (90.0)	0.802
	> 1 Year	6 (11.5)	46 (88.5)	
Breed	German Shepherd	4 (13.3)	26 (86.7)	0.363
	Labrador Retriever	1 (5.9)	16 (94.1)	
	Kohati Gultair	1 (7.7)	12 (92.3)	
	Mix / Non-descript	5 (20.8)	19 (79.2)	
	Pit bull	0 (0.0)	7 (100.0)	
	Pug	0 (0.0)	11 (100.0)	
Tick Infestation	Yes	9 (18.8)	39 (81.2)	0.014*
	No	2 (3.7)	52 (96.3)	
Previous Tick History	Yes	8 (17.0)	39 (83.0)	0.060
	No	3 (5.5)	52 (94.5)	
Tick prevention applications	Yes	1 (2.3)	43 (97.7)	0.016*
	No	10 (17.2)	48 (82.8)	
Route of Acaricide application	Topical	1 (2.9)	33 (97.1)	0.052
	Parenteral	0 (0.0)	10 (100.0)	
	NA	10 (17.2)	48 (82.8)	
Interval of Acaricide application	< 2 Months	1 (2.9)	33 (97.1)	0.052
	> 2 Months	0 (0.0)	10 (100.0)	
	NA	10 (17.2)	48 (82.8)	
Housing Type	Concrete	5 (8.3)	55 (91.7)	0.340
	Iron	6 (14.3)	36 (85.7)	
House Hygiene	Good	3 (5.2)	55 (94.8)	0.036*
	Poor	8 (18.2)	36 (81.8)	

** Indicates risk factors significantly associated with theileriosis in dogs*

from cattle (Accession no. OL420757). Our study isolates showed more resemblance with each other than the others. Furthermore, the current study isolates showed high similarity with the gene sequences from India (Accession no. MN044040, MN044047). However, the gene sequences from other countries like Sudan (LC431534, LC431528), Spain (DQ287958), Turkey (MK693128, MK693130), Iran (MN422305, MT812969), China (KP731977), and Tunisia (KF732026, KF732025) resembled our study isolates of *T. annulata*. Moreover, the isolate of dog from France (Accession no. JX454779) showed a significant variation with our study isolates, hence making an out-group.

Effect of *Theileria* on Hematological Parameters of Dogs

A comparative study on hematological parameters of healthy and diseased dogs was done. The results indicated a non-significant (P=0.475) decrease in the WBCs count of diseased animals as compared to healthy ones. Among WBCs the decreasing pattern in lymphocytes and monocytes was non-significant (P>0.05) while the results of granulocytes were significant (P<0.05). Other hematological parameters like red blood cells (RBCs), hemoglobin level, PCV, and platelet count were found to have a significant (P<0.05) association with the disease incidence. A significant reduction in average values of the parameters was observed in diseased animals *(Table 2)*.

Discussion

The prevalence of tick-borne pathogens in dogs is increasing around the globe and is of significant importance in both veterinary and human health [37,38]. Canine theileriosis is an emerging vector-borne disease

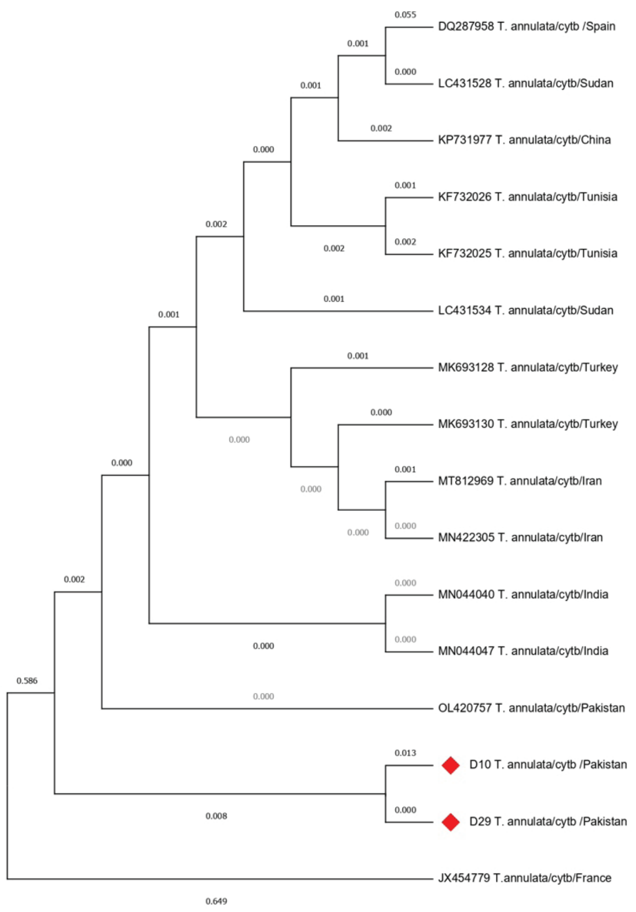

Fig 2. Phylogenetic analysis of *cytb* gene of study isolates with NCBI Genbank reported sequences by using maximum likelihood method and 1000 bootstrap technique

that is distributed and reported globally [1]. The variation in the prevalence of the disease in various countries can be related to alterations in environmental conditions and epidemiological factors like age, sex, breed, habitat of dogs, and management practices [39]. The diagnosis of canine tick-borne pathogens by morphological identification in blood smear through the use of microscopy is not reliable. Therefore, a molecular technique like PCR is required for chronic and subclinical infection in carrier dogs [40]. In Pakistan, vector-borne diseases of dogs like babesiosis [41] and ehrlichiosis [42] have been reported on the molecular level, but till now, no data is available on the molecular characterization of canine theileriosis in dogs. The present study is the first report of canine theileriosis in dogs concerning the molecular diagnosis, phylogenetic analysis of the isolated sequences, and the relationship of presumed risk factors with disease dynamics in pet dogs of Lahore, Pakistan.

In the current study, overall microscopy and PCR-based prevalence of 6.86% (7/102) and 10.78% (11/102) were reported in dogs of district Lahore, respectively. Similar to the finding of the current study, Bigdeli et al.[19] and Aktas et al.[20] have also confirmed and reported the presence of *T. annulata* in southern Iran and Turkey, respectively. The molecular findings of the present study agreed with the outcomes of Cicuttin et al.[43] who described a 10.1% prevalence of theileriosis in dogs from non-investigated areas of Serbia. The findings also matched to results of Niu et al.[44] and Adamu et al.[23] who reported 6.4% and 4% prevalence of *Theileria spp.* in pet dogs of China and Nigeria, respectively. Similarly, a slightly higher prevalence (13%) of *Theileria* spp. was also reported by Gholami et al.[45] in sheepdogs from Iran. However, conflicting with the present study conclusions, Miro et al.[11] reported a higher molecular-based prevalence (62.5%) of *T. annae* in Spain. Also, Matjila et al.[40] described (41.1%) molecular prevalence of *Theileria* spp. from South Africa. The results of the current study were also different from the outcome of Rosa et al.[22] who reported a higher prevalence of *Theileria* spp. (66.6%).

The current study report was also contrary to the observations of [9,12,13] who reported 1.9%, 0.7%, and 0.6%

Table 2. *Effect of canine theileriosis on various hematological parameters of dogs based on independent sample t-test*

Parameter	**Unit**	***Theileria* Negative**	***Theileria* Positive**	**F Value**	**Mean Difference**	**Confidence Interval**	**P Value**
		Mean ± SD	**Mean ± SD**				
WBCs	x 10^3/µL	8.74±1.64^a	7.4±3.38^a	1.044	1.26	-5.139-2.619	0.475
Granulocytes	%	77.32±6.21^a	57.44±15.69^b	1.915	19.88	-2.469-37.290	0.03*
Monocytes	%	5.68 ±1.97^a	4.52±1.92^a	0.013	1.16	1.681-4.001	0.374
Lymphocytes	%	20.98±4.25^a	15.60±9.03^a	1.057	5.38	4.919-15.679	0.263
RBCs	x 10^6/µL	6.62±0.952^a	3.61±0.974^b	0.003	3.01	1.596-4.407	0.001*
Hemoglobin	g/dL	14.72±2.09^a	7.72±2.70^b	0.097	7.00	3.475-10.524	0.002*
PCV	%	41.64±3.69^a	24.64±5.32^b	1.164	17.00	10.312- 23.687	<0.001*
Platelets	x 10^3/µL	239.40±48.41^a	71.60±26.16^b	5.068	167.80	111.04-224.55	<0.001*

*^{aa} indicates a non-significant difference between healthy and diseased animals; ^{ab} indicates a significant difference between healthy and diseased animals; * indicates the significant association of blood parameters with the disease*

molecular prevalence of *T. annae* in Spain, Mississippi (USA), and Barcelona, respectively. While, Inacio et al.[3] reported a lower prevalence (0.3%) of *T. equi* in dogs from Paraguay, and Xu et al.[46] also reported the lowest prevalence (0.1%) of *T. orientalis* in dogs from China. The difference between *Theileria* distributions may be due to different geographical regions and variable numbers of prevailed vectors in each region, however, management practices and hygienic conditions also confer the difference in the prevalence of *Theileria* in every region.

The current report indicated a higher prevalence of the disease in female dogs (14.0%) compared to male dogs (7.7%) which was as per the outcomes of Miro et al.[11] who recognized a higher infection rate in females dogs (67.2%) comparative to male dogs (65.5%) of Spain. The results were also supported by the study of Aktas et al.[20] who reported a slightly higher infection rate in female dogs (5.5%) as compared to male dogs (5.3%) from Turkey. In the present study, the higher disease incidence in female dogs could be attributed to the fact that female dogs suffer extra stress during pregnancy and lactation making them more prone to infections. The results revealed a higher prevalence (11.5%) in adult dogs (>1 year), compared to younger ones (≤1 year) (10.0%) which was in agreement with the outcomes of Aktas et al.[20]. However, current study findings were contrary to the observations of Miro et al.[11] who found higher disease prevalence in younger dogs (≤3 years) (81.54%) as compared to older ones (>3 years) (46.81%). The higher prevalence of the disease among old animals might be due to an increased opportunity for exposure to ticks over time as described by Hegab et al.[47].

In this study, higher disease prevalence was observed in mixed/non-descriptive breeds (20.8%) as compared to pure breeds such as German shepherd (13.3%), Labrador retriever (5.9%), and Kohati Gultair (7.7%). Similar findings were also documented by Miro et al.[11] who described a high prevalence of the disease in Cross-breed/Mixed (c/m) (66.6%) as compared to pure breeds (61.6%). The lower disease incidence in pure breeds was probably due to the reason they spend more time in the gardens and indoor. Additionally, inspection and cleaning practices like bathing and brushing are regularly followed for pure-breed dogs making these dogs less prone to tick-borne infections.

Tick infestation and previous tick history were found to be significant risk factors for disease incidence. In the current study, it is observed that dogs having tick infestation or previous tick history were at greater risk of developing the disease compared to dogs that neither have previous tick history nor current tick infestation. These findings were compiled with the observation of Miro et al.[11] and Ahmed et al.[48] who reported the association of tick infestation with tick-borne maladies in pet animals.

To control vector-borne diseases like babesiosis and theileriosis in dogs, the control of ticks by acaricides or pesticides is considered an effective way. In this report, the dogs without ticks prevention applications revealed a higher prevalence of disease (17.2%). This factor was statistically analyzed and its P-value was noted (0.016) which is a significant ($P<0.05$) contributing factor towards disease dynamics and that could be clarified by the fact that ticks act as a biological vector to spread the *Theileria* infection from diseased dogs to healthy ones. So, the dogs that have a higher exposure to ticks or inadequate tick control approaches will have more chances of disease occurrence.

This study provides the first insight into the genetic characterization of *Theileria* spp. targeting the *cytb* gene in dogs from district Lahore, Pakistan. The local study isolates showed similarity with the *T. annulata* isolates from India, Iran, China, Turkey, Sudan, and Tunisia isolates. The transboundary movement of animals through shared borders with Iran, India, and China and import of various breeds of pet dogs from these countries could be a reason for the similarity of the current study isolates with *T. annulata* isolates of Iran, Turkey, India, and China.

In the current study, the values of RBCs, PCV, Hb, and thrombocytes (platelets) were found significantly decreased in infected dogs. WBCs showed a non-significant decrease in infected animals. Decreased RBCs, PCV, and Hb are considered important indicators of anemia. The current study outcomes were supported by Rosa et al.[22] who reported anemia with thrombocytopenia in 5 out of 6 dogs from South Africa. Thrombocytopenia is the most frequent and typical finding of canine theileriosis and was described by Falkenö et al.[14]. Additionally, Garcia [10] also described anemia along with thrombocytopenia as a common manifestation and leukocytosis as an uncommon finding in diseased dogs of northern Spain. In the present study, the differential WBCs counts indicated a general tendency towards neutrophilia and eosinopenia which was in agreement with the outcomes of Garcia et al.[10]. Additional to these reports, the findings of several other studies including Simes et al.[15] and Keefe et al.[39] were also following the outcomes of the current study. Rosa et al.[22] documented that the thrombocytopenia was probable to be secondary to immune-mediated devastation, platelet consumption, and splenic sequestration in *Theileria*-affected dogs from South Africa. Leukocytosis in addition to thrombocytopenia was also observed in diseased dogs in several other reports [10,14,15,22,40].

This study provides molecular-based evidence of *Theileria* spp. in dogs of Lahore, Pakistan. The amplification of the *cytb* gene fragment by PCR revealed an overall *Theileria* prevalence of 10.78%. Assessment of risk factors revealed that house hygiene, tick control status, and tick infestation were proven significant determinants for disease dynamics. Moreover, hematological parameters of infected animals showed a significant reduction in the RBCs, PCV, Hb, and platelet count. The current study will be helpful to plan control measures for the *T. annulata* infection in pet dogs of Pakistan.

Availability of Data and Materials

The information regarding the datasets analyzed during the present study is available from the corresponding author (M. Ijaz) upon request.

Funding

No source of funding is provided.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.N., M.I.: Conceptualization, methodology, data curation, investigation, resources, project administration, funding acquisition, writing, review, and editing; N.Z.G., A.A., R.M.A.: Methodology, data curation, investigation, software, visualization, validation; M.U.J., A.A.: Writing of original draft; I.M.: Writing, review, and editing. All authors read and approved the final manuscript.

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