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Original Article

Detection and Molecular Characterisation of Zoonotic Babesia (Babesia microti) in Dogs on Livestock Farms of Dera Ghazi Khan, Pakistan

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Abstract: Objective: This study was conducted to investigate the prevalence and molecular characteristics of Babesia microti in dogs. **Methods:** The study was conducted on livestock farms from June 2022 to October 2022 in Dera Ghazi Khan, Pakistan. Blood samples from 179 apparently healthy dogs were collected and analyzed using polymerase chain reaction (PCR) targeting the 18S rRNA gene of B. microti. **Results:** The results revealed a 1.7% prevalence of B. microti among the sampled dogs, indicating a low but notable presence of this zoonotic parasite in the region. Molecular characterization of the B. microti isolates demonstrated over 90% similarity to previously registered isolates in GenBank, suggesting genetic consistency within this geographic area. The study also noted that all positive dogs exhibited elevated body temperatures, underscoring the clinical relevance of fever in identifying potential cases of babesiosis. **Conclusion:** These findings highlight the zoonotic risk of B. microti on livestock farms and provide insights into its prevalence and genetic diversity, supporting risk evaluation and the development of targeted control and awareness measures for animal and human health.

Keywords: Babesia microti, Zoonotic babesiosis, Dogs, Tick-borne disease, Pakistan

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1. Introduction

Over the past few years, the occurrence and dissemination of tick-borne pathogens (TBPs) have markedly increased, driven by factors such as climate change, globalization, and the movement and growth of human and animal populations. This trend poses a significant threat to both animals and humans, exacerbating the public health burden of tick-borne diseases (Dantas-Torres et al., 2012; Welc-Falęciak et al., 2012). Babesiosis is a tick-borne disease caused by protozoan parasites of the genus *Babesia*, is a prime example of such emerging infectious diseases. The condition affects a variety of hosts, including domestic animals and humans, and is transmitted primarily by ticks of the genus *Ixodes* (Welc-Falęciak et al., 2012).

Among the *Babesia* species, *Babesia microti* is a significant zoonotic pathogen known for causing babesiosis in humans and animals (Gray et al., 2010; Vannier & Krause, 2012). In dogs, babesiosis can be caused by several *Babesia* species, including *B. canis*, *B. vogeli*, *B. microti*, and *B. gibsoni* (Caccio et al., 2002; Solano-Gallego & Baneth, 2011). *Babesia microti* specifically is a protozoan parasite that invades and proliferates within red blood cells, leading to clinical manifestations of babesiosis in infected hosts (Akram et al., 2019). Babesiosis typically results in a mild to moderate illness. However, individuals with compromised immune systems or those taking immunosuppressive medication may experience severe and life-threatening symptoms (Hatcher et al., 2001; Hildebrandt et al., 2021; Menis et al., 2015). Notably, dogs exposed to ticks or infested with them, such as those living outdoors, on farms, or used for hunting, are at a higher risk of contracting a *Babesia spp*. infection (Miró et al., 2015).

Dogs, being one of the most commonly kept pets worldwide (Asher et al., 2011), often coexist in environments such as livestock farms where they interact closely with other animals and humans. This interaction increases the risk of zoonotic transmission of pathogens. The presence of dogs on farms can facilitate the spread of tick-borne diseases, both within the animal population and to humans. This implies that there is increased interaction between dogs and humans. Recent findings have documented the presence of B. microti in cats and dogs in urban areas like

Lahore and Rawalpindi (Akram et al., 2019). However, the prevalence and geographic distribution of this parasite in more rural and less studied areas, such as Dera Ghazi Khan (DG Khan), remain poorly understood.

Climate change and environmental factors are likely influencing the distribution of tick populations and the prevalence of tick-borne pathogens, potentially expanding the range of diseases like babesiosis. Understanding the prevalence of *B. microti* in dogs on livestock farms in DG Khan is crucial, as it could reveal new insights into the epidemiology of this zoonotic parasite and highlight potential risks for both animal and human health. In this context, the present study was conducted to investigate the occurrence of *Babesia microti* in dogs reared on livestock farms in Dera Ghazi Khan, Punjab, Pakistan. Specifically, this study aimed to detect *B. microti* using PCR and to examine its association with selected host-related characteristics, including age, sex, breed, and tick infestation status. Our goal is to better understand the role of healthy dogs as potential carriers of *B. microti*, thereby informing future public health interventions and improving disease management strategies.

2. Materials and Methods

Sample collection

A cross-sectional, convenience-based sampling strategy was adopted, in which dogs present on accessible livestock farms during the study period were enrolled with owner consent. Informed consent was obtained in writing from all dog owners prior to sample collection, after explaining the study objectives and procedures. Owing to the lack of baseline prevalence data for *Babesia microti* in dogs from the study area, a priori sample size calculation was not feasible; therefore, the sample size was determined by field accessibility and logistical considerations. The study was conducted in 155 livestock farms from June 2022 to October 2022 in DG Khan, Punjab, Pakistan. Blood samples were collected from (n=179) healthy dogs on these livestock farms. The general information regarding the dog's age, breed, gender etc., was collected using a pre-designed close-ended questionnaire. The data was collected after explaining the objectives and purpose of the study to the dog's owner. The age of the dogs was categorised into five sets: puppies (< 1 year), young (1 to 3 years), adult (4 to 6 years), old (7 to 10 years), and very old (more than 10 years). A professional veterinarian did the handling of dogs and collection of samples and data. Whole blood from the jugular vein was collected from selected dogs using blood collection tubes with EDTA-anticoagulant. The body of each dog was thoroughly examined for the presence of ticks, specifically ears. The Ethics Committee of the Livestock and Dairy Development Department, vide letter No. 511-2022/L7DD, approved all animal-related procedures.

Inclusion and exclusion criteria

Dogs of any breed and sex were enrolled if they were healthy and did not show signs of acute canine piroplasmosis. Such signs indicating exclusion were severely pale mucous membranes, apathy, loss of appetite, fever, yellowing of membranes indicating jaundice, dark brown urine due to haemoglobin, or dark/bloody faeces.

DNA Extraction

To extract DNA, we modified the previous method by (Saeed et al., 2015). The obtained samples were mixed with 500 µL of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 30 mM DTT, 0.5% SDS) and 0.4 mg/mL proteinase K (Fermentas, USA). The samples were left in a heating block at 55ºC overnight. Once the samples were lysed, they were heated at 95ºC for 10 minutes. Next, a mixture of phenol, chloroform, and isoamyl alcohol (in a 25:24:1 v/v/v ratio) was added, and the samples were vortexed for 40 seconds and centrifuged at 13000×g for 10 minutes. The aqueous phase was transferred to a clean tube, and an equal amount of ice-cold isopropanol was added. The DNA was collected by centrifugation at 13000×g for 14 minutes, washed with 70% ethanol, and dried at 65ºC for 7 minutes. Finally, the DNA was resuspended in 40 µL of sterile distilled water and stored at -20ºC for future use.

Detection of Babesia microti and PCR amplification

To amplify the 18S rRNA gene, a pair of primers, namely forward Bab1: 5´-CTTAGTATAAGCTTTTATACAGC-3' and reverse Bab4: 5´-ATAGGTCAGAAACTTGAATGATACA-3', were utilised. This amplification resulted in a 238 bp fragment, already documented by (Persing et al., 1992). A final reaction volume of 50 ul was created to prepare for DNA amplification. This volume contained 200 mM deoxynucleotide triphosphate (dNTPs), 50 mM Tris (pH 8.3), 50 pmol of each primer, 1.5 mM of divalent cation MgCl2, 5µl of DNA template, and 0.25 U of the Taq DNA polymerase enzyme (Vivantis, UK). Negative and positive controls were also included during PCR amplification to ensure accuracy. The DNA amplification process was carried out using a DNA thermal cycler (MultiGene OptiMax PCR system). In the current study, we utilised the same thermal profile as by (Persing et al., 1992). To amplify *Babesia microti*, we subjected the sample to an initial denaturation for 5 minutes at 94°C, followed by 32 cycles of denaturation for 1 minute at

94°C, annealing for 1 minute at 55°C, and extension for 2 minutes at 72°C. Finally, we achieved an overall extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis on a 2% agarose gel and visualised using a UV Trans illuminator (Biostep, Germany).

DNA sequencing

To verify the PCR results, the DNA fragments obtained were purified using a PCR purification kit (GF-1 96-well PCR Clean-up kit, Vivantis, UK) and then sent to a commercial laboratory (Macrogen, Korea) for sequencing.

Phylogenetic analysis

The nucleotide sequences obtained in this study were compared with those already published in GenBank. A BLASTn search was carried out, and a phylogenetic tree was created using the Neighbor-Joining (NJ) method with the Kimura two-parameter model. To ensure accuracy, we replicated the bootstrap test 1000 times (Tamura et al., 2007).

Nucleotide sequence accession numbers

The obtained sequences have been deposited in the GenBank database with assigned accession numbers: OQ120558, OQ120559, and OQ120560. These correspond to the 18S rRNA gene sequences of *Babesia microti*.

3. Results

The study examined 179 blood samples from dogs for tick-borne pathogens. Of these, 3 (1.7%) were found to be positive for *B. microti*. Two of the three positive dogs were male, as detailed in Table 1. Additionally, all three positive dogs were categorised as young, between 1 and 3 years of age.

Table 1: Prevalence of Babesia microti in dogs on livestock farms of Dera Ghazi Khan, Pakistan

Parameter	Category	N (Number) =179	*B. microti* positive	Prevalence
Sex	Male	96	2	2.08%
	Female	83	1	1.20%
Age	> 1 year	62	3	4.83%
	< 1 year	117	0	0.00%
Body Temperature	Normal	133	0	0.00%
	Fever	46	3	6.52%
Tick Infestation	Present	13	1	7.69%
	Absent	166	2	1.20%
Total			3	1.68%

Sequencing of the obtained amplicons and subsequent BLAST analysis confirmed that these sequences were B. microti. The PCR amplification yielded a 238 base pair amplicon specific to the 18S rRNA gene of B. microti, demonstrating over 90% similarity with the registered B. microti isolates in GenBank, as shown in Figure 1.

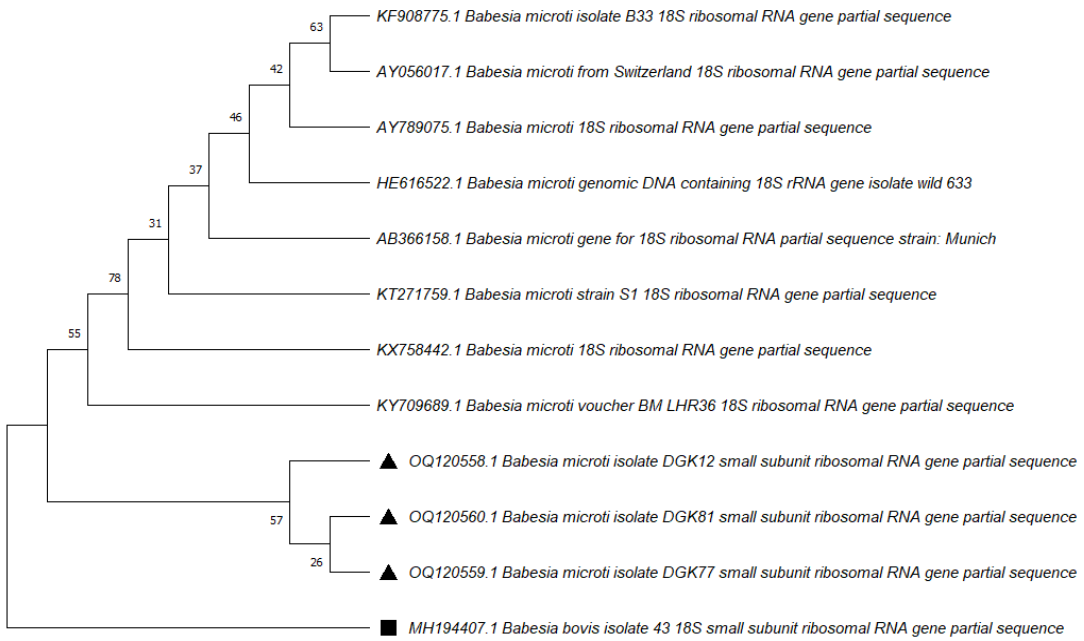

Figure 1: *Phylogenetic analysis of the sequences of the Babesia microti based on 18S rRNA gene using the neighbor-joining method. The optimal tree with the sum of branch length = 1.19793554 is shown. The evolutionary distances were computed using the Kimura 2-parameter method. The pathogens identified in the present study are marked in bold triangle, where bold rectangle indicate outgroup.*

The analysis revealed that all B. microti-positive dogs were mixed breeds. Furthermore, a notable association was observed between B. microti infection and elevated body temperature; all infected dogs exhibited fever. An examination of Ixodid ticks showed that 13 out of 179 dogs (7.6%) were infested, and these ticks were subsequently removed.

4. Discussion

Historically, babesiosis in dogs was primarily attributed to B. canis and B. gibsoni (Cacciò et al., 2002). However, recent studies have increasingly recognized B. microti as a pathogen of canine babesiosis (Baneth et al., 2012; Camacho et al., 2002). This study pioneers the documentation of the detection and molecular characterization of zoonotic Babesia species in dogs on livestock farms in Dera Ghazi Khan, Pakistan. The findings offer new insights into the prevalence of B. microti in this region and underscore the potential for zoonotic transmission.

The low prevalence of B. microti observed in this study contrasts with higher rates reported in other regions (Akram et al., 2019; Gabrielli et al., 2015; Stensvold et al., 2015). This discrepancy may be attributed to regional variations in tick populations and environmental factors affecting tick-borne pathogen distribution. While this study identified fever as a common symptom among positive dogs, other clinical signs, such as pale mucous membranes, were not observed, which may reflect local variations or differences in disease manifestation.

The presence of B. microti in dogs highlights the potential risk of zoonotic transmission, especially given the close contact between dogs and humans on livestock farms. Recent evidence suggests that such interactions could facilitate the transfer of infected ticks from dogs to humans (Spada et al., 2014). Although this study provides valuable data, it is limited by the small sample size and the lack of comprehensive tick and human infection assessments. Future research should include more extensive and diverse sample sizes, encompassing different farms and regions, to better understand the prevalence and distribution of B. microti. Additionally, examining tick populations on the farms and conducting studies on human cases of babesiosis in the same area would offer a more complete picture of the potential zoonotic transmission and associated risks.

From a public health perspective, these findings highlight the need for routine molecular surveillance of tick-borne pathogens in dogs on livestock farms, which may serve as sentinels for zoonotic risks. Integrating regular tick control measures for farm dogs, along with awareness

programs for farmers regarding tick management and personal protective practices, could help reduce the circulation of Babesia microti at the animal–human interface. Such targeted interventions may support early risk identification and strengthen local tick control strategies.

Recent studies have highlighted the importance of this research in light of changing climate conditions and the expansion of tick habitats (Lempereur et al., 2011). As climate change continues to influence tick distribution and the epidemiology of tick-borne diseases, ongoing surveillance and research are crucial for effective disease management and public health protection.

5. Conclusion

This study successfully detected and characterized Babesia microti in dogs on livestock farms in Dera Ghazi Khan, Pakistan, marking a significant contribution to understanding zoonotic Babesia infections in this region. The detection of *B. microti* in the examined dogs highlights the presence of this pathogen in local canine populations, raising concerns about its zoonotic potential and the associated risks to human health. The findings underscore the critical need for integrated surveillance and control strategies to manage tick-borne diseases effectively and mitigate the potential for zoonotic transmission. The results of this study also highlight the need for proactive measures to control tick populations and reduce the risk of babesiosis in both animals and humans. As climate change continues to influence the distribution of ticks and tick-borne pathogens, ongoing research and adaptive management strategies will be essential to address emerging public health challenges associated with tick-borne diseases. Future studies involving larger sample sizes, broader geographic coverage, and integrated assessments of ticks and human exposure are needed to overcome the limitations of the present study and to better define the epidemiology and zoonotic potential of *Babesia microti* in Pakistan.

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Ethical approval: Animal handling and sample preparation complied with the Animal Ethics Procedures and Guidelines and were approved by the Animal Ethics Committee of L&DD, Punjab.

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Conflicts of Interest: The authors declare no conflict of interest.

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