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PCR-BASED DETECTION OF *BABESIA CANIS* IN PAKISTANI CATS USING RIBOSOMAL RNA MARKER

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ABSTRACT

Babesia canis (*B. canis*) is a tick-borne apicomplexan parasite that infects erythrocytes, causing babesiosis in various domestic and wild animals, including cats. The present investigation was designed to report the PCR-based presence of *B. canis* in 159 cat blood samples collected from three pet clinics in Lahore (Pakistan). Data on the epidemiological and clinical characteristics (gender, age, body conditions, body temperature, mucous membrane, deworming, hematuria, vaccination, vomiting, tick infestation, hydration status, and sampling seasons) were collected through a questionnaire at the sampling site. Three out of 159 (1.9%) cats were found *to be B. canis infected as they amplified a 450bp amplicon from the* 18S rRNA gene of this parasite through PCR. All three parasite-positive cats were asymptomatic and apparently healthy. Risk factor analysis indicated that none of the studied parameters was associated with *B. canis* infection (P>0.05) among screened cats. In conclusion, this is the first epidemiologic study of *B. canis* infection in cats from Lahore, Pakistan. Although the prevalence found is low, the infection should be considered as a differential diagnosis in cats with compatible clinical signs.

***Keywords*:** Cat; *Babesia canis*; PCR; 18S rRNA gene; Lahore, Pakistan

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1. INTRODUCTION

Babesia species are intraerythrocytic apicomplexan protozoa transmitted by ticks, and they are of significant veterinary and public health importance (Bajer and Dwużnik-Szarek 2021; Milošević et al. 2024). Over the past five years, meta-analyses have revealed that zoonotic *Babesia* spp. particularly *B. microti*, *B. divergens*, and *B. venatorum* are detected globally at low but notable prevalence rates in ticks, animals, and humans (Ali et al. 2024). A variety of *Babesia* spp. have been molecularly identified in feline hosts, including *B. canis* (Canis) and *B. vogeli*, as demonstrated in Beijing and mainland China (Zhang et al. 2019), *B. gibsoni* in cats across multiple regions of China (Li et al. 2023), and the novel *B. hongkongensis* in community cats from Hong Kong (Almendros et al. 2023). Hard-bodied ticks of the family Ixodidae are the main vectors responsible for transmitting *Babesia* spp. to vertebrate hosts (Zeb et al. 2023; Zhang et al. 2024). Dermacentor reticulatus, commonly known as the ornate dog tick, is currently recognized as the primary vector transmitting *B. canis* to dogs across Europe, with expanding geographic distribution significantly increasing disease risk (Borowski et al. 2024; Springer et al. 2024).

Infection with *Babesia* spp. Produces diverse clinical presentations, with asymptomatic carriers as well as severe cases of disease having been reported. Clinical signs may include fever, lethargy, pallor, splenomegaly, jaundice, weakness, and even collapse due to hemolysis that causes hypoxic insult, thrombocytopenia, systemic inflammation, and pigmenturia (Krause et al. 2021). Severe cases may progress to life-threatening complications such as acute kidney injury, hepatopathy, pulmonary edema, and neurological manifestations (Matijatko et al. 2020). Typical laboratory abnormalities in feline babesiosis include regenerative hemolytic anemia, often characterized as macrocytic and hypochromic, frequently accompanied by thrombocytopenia and hyperbilirubinemia (Almendros et al., 2023; Li et al., 2023). To the very best of our knowledge, no published data is available regarding the prevalence of *B. canis* in cats from Lahore, the capital city of Punjab province, Pakistan. Therefore, the present study was conducted to document the occurrence of *B. canis* infection in the feline population of this region.

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2. MATERIALS AND METHODS

2.1. Subjects, Blood and Data Collection

Cats (n = 159) presented either for routine examination or treatment at the pet clinic of the University of Veterinary and Animal Sciences (UVAS) and two private veterinary clinics in Lahore, Pakistan. None of the sampled cats were clinically diagnosed with babesiosis at the time of collection. To gather epidemiological and clinical information possibly linked with the occurrence of *B. canis*, a structured questionnaire was completed on-site. Parameters recorded included gender, age, body condition, body temperature, mucous membrane status, deworming history, hematuria, vaccination status, vomiting, tick infestation, hydration level, and sampling season. Jugular vein of each cat was aseptically pricked to draw blood (1-2 ml) into EDTA-coated tubes during the period from November 2015 to July 2016. Blood samples were promptly stored at −20°C, and preserved until subjected to DNA extraction and molecular analysis.

2.2. DNA Extraction and PCR

An inorganic manual method was used to extract DNA from the collected whole blood sample following Qamar et al. (2017). PCR protocol of Erdélyi et al. (2014) was replicated for the detection of *B. canis* in cat blood samples by using the oligonucleotide primers targeting 450bp amplicon from the 18S rRNA gene of *B. canis* (Table 1).

Table 1: Pair of primers (Erdélyi et al. 2014) used to amplify 18S rRNA gene sequence of *Babesia canis* in cat blood samples during the present investigation

Primers	Sequence
Piro-A1 (Forward Primer)	5'-AGGGAGCCTGAGAGACGGCTACC-3'
Piro-B (Reverse Primer)	5'-TTAAATACGAATGCCCCCAAC-3'

The PCR reaction mixture consisted of DNA template (50 ng), PCR buffer (1x), $MgCl_2$ (1.5mM), dNTPs (200µM), Taq DNA polymerase (1.5 U, AbClonal, USA), forward and reverse primer (15pmol), balanced with double-distilled water (Erdélyi et al. 2014). For quality assurance, a negative (reaction mixture without DNA) and a positive (*B. canis* DNA donated by Dr. Munir Aktas, University of Firat, Turkey) control were also run in each PCR.

A thermal cycler (Multi GENE OptiMAX, Labnet Inc.) was used for DNA amplification. The thermos cycling profile consists of a single cycle of initial denaturation for 10min at 94°C, followed by 35 cycles of denaturation for 30s at 94°C, annealing for 30s at 60°C, and extension for 30s at 72°C. A final extension step was carried out for 7min at 72°C (Erdélyi et al. 2014). The PCR products were separated through electrophoresis by 2% agarose gel. A 100bp DNA ladder (New England BioLabs Inc.) was used for the size comparison. Gel was observed under a UV trans-illuminator (Biostep, Germany). The 18S rRNA *B. canis* positive amplicons were sliced from the gel, and the NucleoSpin Gel and PCR Clean-up (Macherey Nagel, Germany) kit was used to purify the products, and they were sent to a commercial company (Macrogen, Korea) for DNA sequencing.

2.3. Statistical Analysis

Probability values equal to or less than 0.05 were considered significant. The results were analyzed using the statistical software Minitab (version 17). Chi-square test was applied to compare the parasite prevalence between various body conditions of cats under study. Fisher's exact test was applied to correlate potential risk factors, including gender, age, body condition, body temperature, mucous membrane status, deworming history, haematuria, vaccination, vomiting, tick infestation, hydration status, and season of sampling, with the presence of *B. canis* using.

3. RESULTS

3.1. Prevalence of *Babesia canis*

Analysis of the results showed that PCR had amplified a 450bp amplicon from 18S rRNA gene of *B. canis* in 3 out of 159 feline blood samples (1.9%) examined in the present study (Fig. 1).

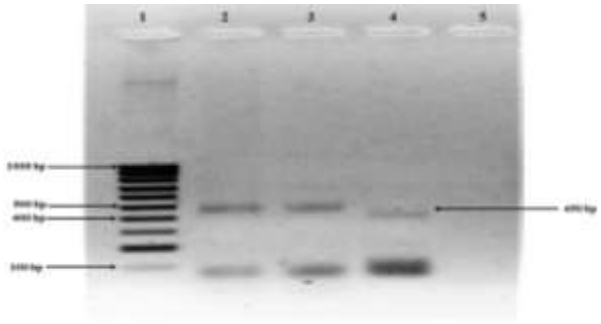

Fig. 1: Amplification of 18S rRNA gene of *Babesia canis* through PCR in cat blood samples collected during present investigation. Lane: 1, 100bp DNA ladder; lane 2, positive control; lanes 3-4, cat blood sample positive and lane 5 negative for the presence of *Babesia canis*.

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All three cats that tested positive for *B. canis* were brought to the veterinary clinic for routine examination. Their body temperature, mucous membrane color, hydration status, and overall body condition were within normal limits. In this study, sequencing of *B. canis* DNA was not successful, as the chromatogram peaks obtained from the positive purified PCR products were not reliable, despite being submitted twice for sequencing. Furthermore, subsequent attempts to reconfirm the findings were unsuccessful because the parasite could not be detected in the limited volume of blood samples that remained available.

3.2. Risk Factor Analysis Associated with the Prevalence of *Babesia canis*

Risk factor analysis indicated that *B. canis* infection was not limited to a specific cat breed (Table 2) or their body condition (Table 3). Epidemiological data analysis revealed that none of the studied risk factors was linked with *B. canis* infection (P>0.05) in cats enrolled in the present study (Table 4).

Table 2: Comparison of *Babesia canis* prevalence in various cat breeds

Breed	N	Positive cats	Negative cats
Siamese	04	01 (25)	03 (75)
Stray	36	02 (6)	34 (94)
Cross	49	00 (0)	49 (100)
Pure	22	00 (0)	22 (100)
Persian	04	00 (0)	04 (100)
Local	03	00 (0)	03 (100)
Total	(118)	03 (3)	115 (97)

N represents the total number of cat samples collected from each breed. Values in parentheses are %. P>0.05. Breed data of 41 cats was not available. Hence, it was not included in this table.

Table 3: Association of *Babesia canis* prevalence with the body conditions of cats sampled during the present study from Lahore

Body condition	N	Positive cats	Negative cats	Chi-square test
Average	53	03 (6)	50 (94)	Chi-Sq = 0.047
Poor	16	00 (0)	16 (100)	DF = 2, P-Value = 0.977
Good	43	00 (0)	43 (100)	
Total	112	03 (3)	109 (97)	

N = Total number of samples collected from cats. Values in parentheses are %. P>0.05. Body condition data of 47 cats were not available; hence, they were not included in this table.

Table 4: Association of *Babesia canis* prevalence with the studied parameters describing cat characteristics enrolled during the present study from Lahore

Parameters	Samples collected			*B. canis* +ve cats	*B. canis* -ve cats
	Total	N in each Category			
Animal sex	144	Male	66	01 (2)	65 (98)
		Female	78	02 (3)	76 (97)
Age	146	>1 year	103	01 (01)	102 (99)
		<1 year	43	02 (5)	41 (95)
Body Temperature	159	Normal	129	03 (2)	126 (98)
		Fever	31	00 (0)	31 (100)
Mucous Membrane	159	Normal	146	03 (2)	143 (98)
		Pale	13	00 (0)	13 (100)
Haematuria	159	Present	01	00 (0)	01 (100)
		Absent	158	03 (2)	155 (98)
Vomiting	159	Present	19	00 (0)	19 (100)
		Absent	140	03 (2)	137 (98)
Vaccinated	159	Yes	73	01 (1)	72 (99)
		No	86	02 (2)	84 (98)
Dewormed	159	Yes	67	01 (1)	66 (99)
		No	92	02 (2)	90 (98)
Tick Infestation	159	Present	21	00 (0)	21 (100)
		Absent	138	03 (2)	135 (98)
Season	157	Winter	59	00 (0)	59 (100)
		Summer	98	03 (3)	95 (97)
Hydration status	159	Normal	156	03 (2)	153 (98)
		Dehydrated	03	00 (0)	03 (100)

N = Total number of samples collected from cats. Values in parentheses are %. For some parameters data for all 159 cats was not available. Hence the total number of cats was not same for all parameters.

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4. DISCUSSION

Cats have coexisted with humans for centuries and, as companion animals, they contribute significantly to the psychological well-being of people living in today's urbanized societies (Chomel 2014). However, they are also frequently exposed to vector-borne pathogens and can act as important reservoirs and hosts in the transmission cycles of these agents (Malheiros et al. 2016; Bilal et al. 2025). Geographically, Lahore District extends from 31°15′ to 31°45′ N and 74°01′ to 74°39′ E. Sheikhupura lies to its north, India is present in the east while River Ravi marks its north-western edge. Temperatures fluctuate between 18°C and 38.8°C on average, with mean annual rainfall of 629 mm. (Akram et al. 2019). To date, there is no published data on the prevalence of tick-borne infections in the local feline population. Therefore, this study was aimed to report the occurrence of *B. canis* in blood samples collected from cats in Lahore, Pakistan.

In the current investigation, PCR was employed to generate a 450 base pair amplicon of the 18S rRNA gene of *B. canis* from feline blood samples. The results demonstrated that 1.9% (3/159) of the cats screened were positive for *B. canis*. Reports from different regions of the world have also described the occurrence of *Babesia* spp. in cats. For instance, Vilhena et al. (2013) examined 320 feline blood samples obtained from northern (n = 140) and central (n = 180) Portugal, and identified four samples positive for *B. canis*, yielding an overall PCR-based prevalence of 1.3%. Likewise, Simking et al. (2010) tested 1490 blood samples from cats in Thailand using nested PCR targeting the 18S rDNA gene and detected *Babesia* DNA in 21 samples (1.4%). Malheiros et al. (2016) explored vector-borne pathogens in cats from Brazil, where PCR testing of 30 samples revealed *B. vogeli* in two animals (7%). Georges et al. (2008) carried out a similar study in Trinidad, analyzing 15 feline blood samples, of which one (6.7%) was positive for *B. canis vogeli*. André et al. (2015) investigated 151 cats (54 males, 95 females, and two with unrecorded sex) from Campo Grande, Mato Grosso do Sul, Brazil, and identified *B. vogeli* in 8 cases (5.3%). In another study, Maia et al. (2014) collected blood from 649 cats, including 320 domestic and 329 stray animals, from veterinary clinics and shelters in southern Portugal. PCR analysis revealed that 43 (6.6%) were infected with *Babesia* spp. Variations in prevalence rates reported among these studies may be attributed to differences in climatic and geographic conditions, vector abundance, and seasonal timing of sampling (Razzaq et al. 2015).

In this investigation, possible risk factors associated with babesiosis were also analyzed. The analysis of the collected data demonstrated that none of the investigated parameters showed any significant association with the prevalence of *B. canis* (Table 4). These outcomes are comparable with the observations of Simking et al. (2010), who carried out a related investigation in cats from Thailand and documented that the occurrence of *B. canis* was not significantly influenced by age, general health status, environmental conditions, or the presence of ectoparasites. Similarly, Vilhena et al. (2013) explored the prevalence of *B. canis* in Portugal using PCR and reported that factors such as gender, type of housing, and clinical condition of the animals had no association with *B. canis* infection.

5. CONCLUSION

In conclusion, we have utilized PCR to amplify the 18S rRNA gene of *Babesia canis* to report its presence in feline blood samples collected from Lahore, Pakistan. To the best of our knowledge, this is the pioneer evidence of *B. canis* infection in cats within this country. We suggest that this diagnostic approach be considered by veterinarians, cat owners, and public health authorities for the identification of *Babesia* spp. infections in cats showing clinical signs consistent with babesiosis.

DECLARATIONS

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Conflict of Interest: The authors declare that they have no competing interests.

Data Availability: Data will be available upon request from the corresponding author.

Ethics Statement: All the experimental protocols and animal handling procedures were approved by the ethical review board of the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, via application number IPAB/Ethics/14-2015. The experimentation was conducted in accordance with applicable laws, and ARRIVE guidelines.

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Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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