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PCR OUTPERFORMS MICROSCOPY IN DETECTING LOW-GRADE HAEMOPROTOZOAN INFECTIONS: A COMPARATIVE STUDY OF *BABESIA* SPP. AND *CYTAUXZOON* SPP. IN DOMESTIC AND CAPTIVE WILD FELIDS FROM REWA, INDIA

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ABSTRACT

This study investigated the occurrence of *Babesia* spp. and *Cytauxzoon* spp. in domestic and captive wild felids in Rewa, Madhya Pradesh, India, and evaluated the haematobiochemical alterations associated with them. Over a period of six months (July–December 2025), blood samples were collected from 50 felids (32 domestic cats, 18 captive wild felids including tigers, leopards, and lions). Samples were analyzed for hemoprtotozoa using Giemsa-stained blood smear microscopy and PCR targeting the 18S rRNA gene (for *Babesia* spp.) and ITS-2 gene (for *Cytauxzoon* spp.). Haematological and biochemical profiling of all the felids under study was done. Microscopy detected only one cytauxzoon positive case (2.0%) in a white tiger and no babesia infections in any of the felids. In contrast, PCR revealed a significantly higher detection rate (12.0%; n=6), identifying *Babesia* spp. in four felids (one wild felid, three domestic cats) and *Cytauxzoon* spp. in two felids (one white tiger, one domestic cat). PCR demonstrated superior sensitivity (16.67% vs. 100% specificity), with a statistically significant difference for overall detection (p=0.0498), for *Cytauxzoon* spp. (p=0.557) and for *Babesia* spp. (p=0.0413). Clinically, babesia- positive domestic cats showed mild haematological changes, while the wild felids remained largely asymptomatic. Cytauxzoon- positive domestic cat and the white tiger exhibited severe anaemia, thrombocytopenia, leukopenia, elevated liver enzymes, and acute renal failure, with the domestic cat succumbing within 48 hours and the tiger after three months despite rigorous treatment. The study concludes that PCR is indispensable for accurate surveillance of haemoprotozoan infections in felids, and that regular surveillance is needed to prevent mortalities in highly endangered species.

KEYWORDS: Haemoprotozoan infections, *Babesia* spp., *Cytauxzoon* spp., Felids (domestic and wild), PCR diagnosis, Haematobiochemical alterations

INTRODUCTION

Vector-borne haemoprotozoan infections represent a significant and emerging threat to both domestic and captive wild felids worldwide. Among these, apicomplexan parasites belonging to the genera *Babesia* and *Cytauxzoon* (Order Piroplasmida) are of particular clinical and epidemiological importance. These tick-borne pathogens are capable of causing a spectrum of disease manifestations, ranging from subclinical carrier states to severe, often fatal haemolytic anaemia, thrombocytopaenia, multi-organ failure, and death (Baneth *et al.,* 2020; Grillini *et al.,* 2023).

Babesia spp. are strictly intra-erythrocytic parasites that cause babesiosis, a disease characterized by haemolytic anaemia, fever, icterus, and lethargy in susceptible hosts. The first report of the piroplasms in felids date back to the early 20th century, with *Babesia felis* described from a Sudanese wild cat. Subsequently, several other species have been named from various felids, including *Babesia cati* from Indian wild cats, *Babesia pantherae* from leopards, and *Babesia leo* from lions (Dennig & Brocklesby, 1972; López-Rebollar *et al.,* 1999). While babesia infections in domestic cats are recognized as an important clinical disease in regions such as South Africa, the epidemiology and clinical significance of these parasites in other parts of the world, including India, remain inadequately characterized (Jacobson *et al.,* 2000; Penzhorn *et al.,* 2001). Babesiosis has been documented in captive leopards and other large felids in zoological settings in India (Mishra *et al.,* 2008; Rafiqi *et al.,* 2018).

In contrast, *Cytauxzoon* species (Apicomplexa, Piroplasmida, Theileriidae) have a unique life cycle involving an exoerythrocytic schizogonous stage within macrophages and monocytes, which can lead to vascular obstruction and multi-organ failure. Seven species within the genus cytauxzoon have been identified globally. *Cytauxzoon felis*

is notorious for causing severe and often fatal disease in domestic cats, particularly in the United States, where bobcats (*Lynx rufus*) serve as the natural asymptomatic reservoir (Birkenheuer *et al.,* 2006). In recent years, organisms closely related to *C. felis* have been reported in domestic cats in China, India, and Iran, as well as in wild felids in Brazil, with isolates from Asia and South America belonging to phylogenetically distinct clades (Malangmei *et al.,* 2021; Willi *et al.,* 2022). In Europe, three distinct species—*Cytauxzoon europaeus* (EU1), *Cytauxzoon otrantorum* (EU2), and *Cytauxzoon banethi* (EU3)—have been described, with *C. europaeus* being the most prevalent and generally considered less pathogenic than *C. felis,* although fatal cases are reported (Carli *et al.,* 2012; Grillini *et al.,* 2023).

The transmission of both babesia and cytauxzoon occurs via Ixodid ticks. The vectors for felid *Babesia* species and for *Cytauxzoon* spp. in Europe and Asia remain largely unidentified, though Dermacentor, Ixodes, and Rhipicephalus species are suspected (Penzhorn *et al.,* 2001; Cerreta *et al.,* 2022). Wild felids—including lions, leopards, tigers, bobcats, and lynxes—frequently serve as asymptomatic reservoir hosts, harboring chronic, latent infections that can be transmitted to naïve domestic and captive wild populations (Munson *et al.,* 2008; Andre *et al.,* 2011). Fatal cytauxzoonosis has been reported in managed tigers and lions, with transmission suspected to occur within enclosures via arthropod vectors from endemic reservoirs or asymptomatically infected carnivores (Cerreta *et al.,* 2022).

Conventional diagnosis of haemoprotozoan infections has traditionally relied on microscopic examination of Giemsa- stained peripheral blood smears. However, this method has inherent limitations, including low sensitivity in detecting subclinical, low-parasitaemia, or chronic carrier states, where infected erythrocytes may fall below the 0.5–1% threshold required for reliable visualization (MacNeill *et al.,* 2015). Molecular diagnostic techniques, particularly polymerase chain reaction (PCR) targeting conserved genes such as the 18S rRNA gene for babesia and the ITS-2 region for cytauxzoon, have demonstrated substantially higher sensitivity and specificity. PCR-based studies have consistently identified infections in both domestic and wild felids that were missed by microscopy, establishing molecular methods as the gold standard for surveillance and accurate prevalence estimation (Simking *et al.,* 2010; Chan *et al.,* 2021; Santhosheni *et al.,* 2025).

Despite the growing recognition of haemoprotozoan infections in felids globally, there remains a significant knowledge gap regarding their occurrence, molecular characterization, and clinical impact in many regions of India. Rewa region of Madhya Pradesh, 'The Land Of White Tigers' has significant populations of both domestic cats and captive wild felids (including tigers, leopards, and lions) in facilities such as the Maharaja Martand Singh Judeo White Tiger Safari and Zoo, represents an important but understudied ecological interface. Understanding the prevalence and species diversity of babesia and cytauxzoon in this region, along with the associated haematological and biochemical alterations, is critical for informing diagnostic protocols, treatment strategies, and conservation management. Therefore, the present study was conducted with the objectives of determining the occurrence of *Babesia* spp. and *Cytauxzoon* spp. in domestic and captive wild felids in and around Rewa, Madhya Pradesh, India, using both conventional microscopy and PCR and to compare the diagnostic performance of these two methods. The findings of this investigation are expected to provide evidence-based recommendations for routine molecular screening and improved health management of both domestic and captive wild felid populations.

MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, Rewa, Madhya Pradesh, India, over a period of six months from July to December 2025. The objective of the study was to investigate haemoprotozoan infections, particularly *Babesia* spp. and *Cytauxzoon* spp. in domestic and captive wild felids using both conventional microscopy and PCR and to compare the diagnostic performance of these two methods.

Study population and sample collection

A total of 50 felids were included in the study, comprising 32 domestic cats and 18 captive wild felids irrespective of age, sex, or breed. Blood samples were taken from those domestic cats presented to the Veterinary Clinical Complex (VCC) of the College of Veterinary Sci. & A.H., Rewa, District Veterinary Hospital and private clinics in and around Rewa. Ethical approval was secured from the Institutional Animal Ethics Committee under CPCSEA guidelines. The blood samples from wild felids, including tigers, leopards, and lions, maintained at the Maharaja Martand Singh Judeo White Tiger Safari and Zoo, Mukundpur, Madhya Pradesh, were taken with prior permission from the Principal Chief Conservator of Forest, Wildlife Department.

Approximately 5 mL blood samples per animal were collected aseptically at regular intervals taking care with minimizing stress. In domestic cats, the blood was drawn from cephalic or medial saphenous veins, while in wild felids blood was drawn from the tail vein. Out of the 5 ml blood collected from each animal, 2 mL was placed in EDTA-coated tubes for hematological and molecular analysis, and the remaining blood was taken in plain tubes for serum separation.

Microscopic examination

Thin blood smears were prepared immediately after collection, air-dried, and fixed in absolute methanol for 1–2

minutes. Staining was done with Giemsa (1:10 dilution) for 45 minutes, followed by examination under oil immersion (100× objective). Haemoprotozoan parasites, including *Babesia* spp. and *Cytauxzoon* spp., were identified based on morphological criteria from standard references (Benjamin, 1982; Soulsby, 1982).

DNA extraction and PCR amplification

Genomic DNA was extracted from EDTA blood samples using the phenol–chloroform extraction method following established protocols (Sambrook *et al.,* 1989; John *et al.,* 1991). The quality and purity of extracted DNA were assessed using agarose gel electrophoresis and spectrophotometric analysis, and samples with OD260/280 ratios between 1.7 and 1.9 were used for further analysis.

Molecular detection of haemoprotozoan parasites was carried out using polymerase chain reaction (PCR). For *Babesia* spp., the 18S rRNA gene was targeted using primers described by Brahma *et al.,* 2019. For *Cytauxzoon* spp., the ITS-2 gene region was amplified using primers reported by (Brown *et al.,* 2009; Naidenko *et al.,* 2022), with optimized PCR conditions.

The PCR reaction mixture (25 µL) consisted of PCR master mix, forward and reverse primers, nuclease-free water, and genomic DNA template. The amplification involved initial denaturation, followed by 35 cycles of denaturation, annealing, and extension, with a final extension step. Amplified products were analyzed using 0.8% agarose gel electrophoresis in Tris-borate-EDTA buffer. DNA bands were visualized under a UV transilluminator and documented using a gel documentation system. Infection occurrence was determined by both microscopy and PCR. Data were analyzed using standard procedures (Snedecor and Cochran, 1994), expressed as Mean± Standard Error. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Tick-borne apicomplexan parasites, particularly *Babesia* spp. and *Cytauxzoon* spp., represent significant haemoprotozoan threats to both domestic and captive wild felids worldwide. Whereas *Babesia* spp. are strictly intraerythrocytic haemoprotozoa causing haemolytic anaemia, fever, and icterus in domestic cats, wild felids typically remain asymptomatic reservoir hosts. *Cytauxzoon* spp., by contrast, undergo exoerythrocytic schizogony within macrophages and monocytes, potentially causing vascular obstruction and multi-organ failure in susceptible hosts (Baneth *et al.,* 2020; Grillini *et al.,* 2023). The present study determined the occurrence of *Babesia* spp. and *Cytauxzoon* spp. in 50 felids (32 domestic cats and 18 captive wild felids) in and around the Rewa region, Madhya Pradesh, India, using blood smear examination and molecular diagnostic techniques and compared the diagnostic performance of these two methods.

Occurrence of *Babesia* species and *Cytauxzoon* species-

Detection of *Babesia* species and *Cytauxzoon* species by conventional microscopy:

Microscopic examination of Giemsa-stained peripheral blood smears from all 50 felids did not reveal any intraerythrocytic stages of *Babesia* spp. in either domestic or wild animals. However, one captive white tiger was confirmed positive for *Cytauxzoon* spp., displaying characteristic intraerythrocytic piroplasm-like signet-ring organisms on the blood smear (Fig.01). The overall microscopic occurrence of *Cytauxzoon* spp. was 2.0% (1/50), rising to 5.56% (1/18) among wild felids, while no domestic felid was positive by smear examination.

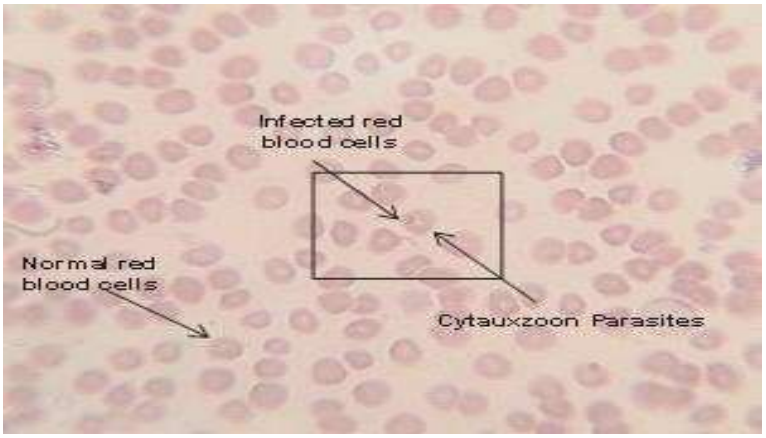

Figure 1. Blood smear showing *Cytauxzoon* spp. merozoite in infected red blood cell

The low detection rate observed in this study is consistent with the recognized limitations of light microscopy in diagnosing subclinical or low-parasitaemia haemoprotozoan infections.

Similar under-detection during chronic and carrier stages has also been reported in earlier studies (Birkenheuer *et al.,* 2006; Steever, 2017). In the Indian context, microscopy has likewise shown limited diagnostic utility, with reports indicating failure to detect cases that were later confirmed through molecular techniques (Malangmei, 2019). This reduced sensitivity is likely attributable to low parasitaemia, subclinical carrier states, or early stages

of infection that fall below the detection threshold—generally requiring approximately 0.5–1% infected erythrocytes for reliable visualization (MacNeill *et al.,* 2015). Collectively, these findings emphasize that reliance on blood smear examination alone is inadequate for the accurate detection of babesia and cytauxzoon infections in felids.

Detection of *Babesia* species and *Cytauxzoon* species by PCR:

Molecular screening using PCR targeting the 18S rRNA gene of *Babesia* spp. and the ITS-2 gene of *Cytauxzoon* spp. revealed a markedly higher detection rate. *Babesia* spp. DNA was identified in four felids (8.0%): one wild felid (5.56%) and three domestic cats (9.38%). *Cytauxzoon* spp. DNA was detected in two felids (4.0%): one wild felid and one domestic cat. The six-fold higher overall detection rate by PCR (12.0%) compared to microscopy (2.0%) clearly demonstrates that molecular methods have substantially higher sensitivity. It was also reported that PCR-based detection has identified *Babesia vogeli* DNA in a significant proportion of anaemic cats, whereas microscopic detection rates remain minimal (Chan *et al.,* 2021). Similarly, the detection of babesiosis in domestic cats aligns with previous studies that reported significantly higher PCR-based positivity compared to conventional blood smear examination, highlighting the superior sensitivity of molecular diagnostics (Simking *et al.,* 2010; Ahmad *et al*., 2011; Panait *et al.,* 2023). Detection of *Babesia* spp. in one captive wild felid (captive leopard) in the present study corroborates earlier reports of babesiosis in large felids in India, where infections have been documented in zoological settings (Mishra *et al.,* 2008; Rafiqi *et al.,* 2018).

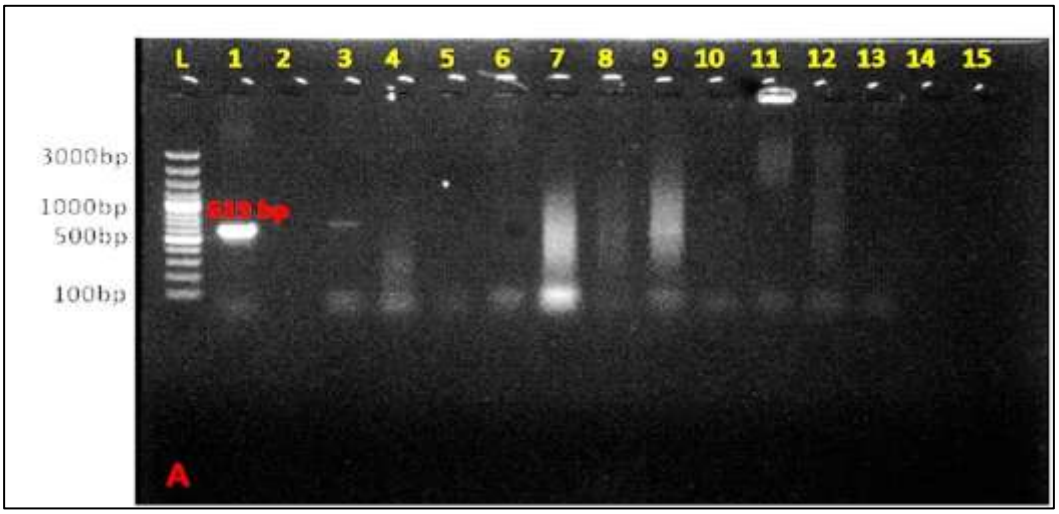

Figure 2. Agarose gel electrophoresis (1.5%) showing PCR amplification products for the detection of *Babesia* genus. Amplification of a 619 bp fragment targeting the *Babesia* genus. Lane L: 100 bp DNA ladder; Lane 1: positive control; Lanes 2–14: clinical samples (lane 3 clinical sample = 619bp); Lane 15: negative control (no- template).

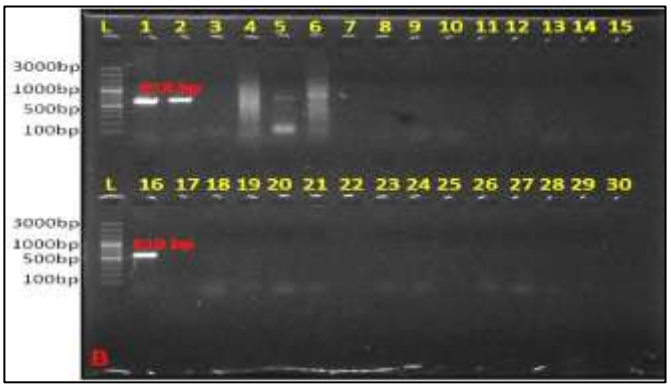

Figure 3. Agarose gel electrophoresis (1.5%) showing PCR amplification products for the detection of *Babesia* genus. Amplification of a 619 bp fragment targeting the *Babesia* genus. Lane L: 100 bp DNA ladder; Lane 1: positive control; Lanes 2–14: clinical samples (lane 2,5,6 lane clinical sample = 619bp); Lane 15: negative control (no-template). Lane L: 100 bp DNA ladder; Lane 16: positive control; Lanes 17–29: clinical samples; Lane 30: negative control (no- template).

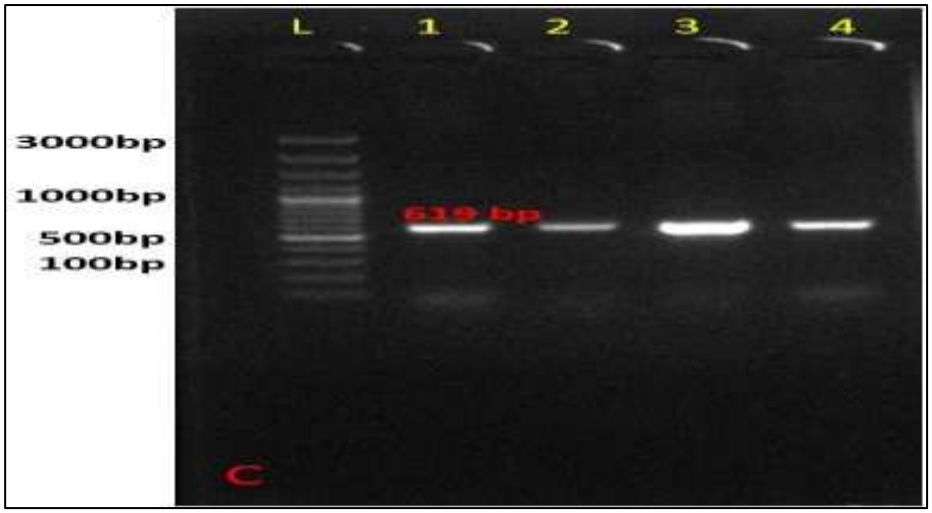

Figure 4. Agarose gel electrophoresis (1.5%) showing PCR amplification products for the detection of *Babesia* genus. Amplification of a 619 bp fragment targeting the *Babesia* genus. Lane L: 100 bp DNA ladder; Lanes 1-4: clinical samples (619bp).

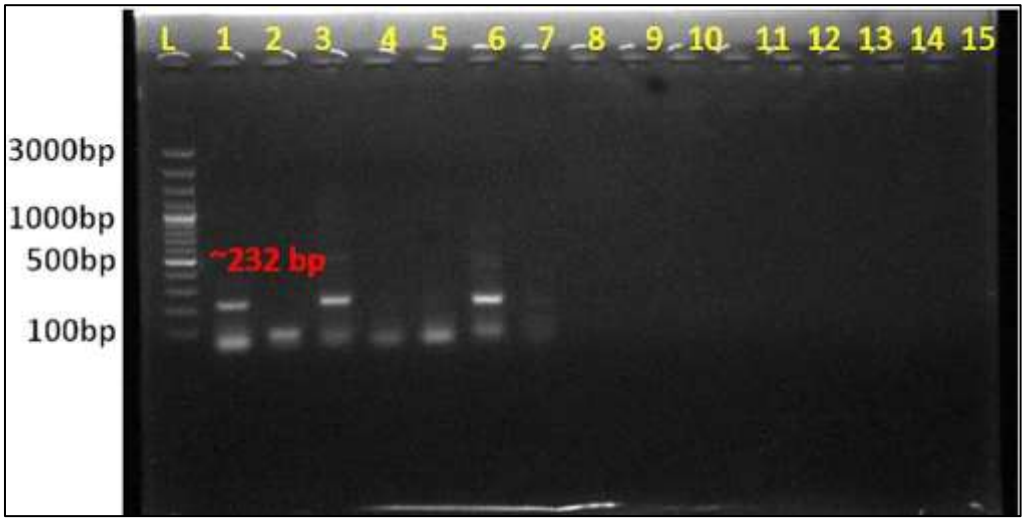

Figure 5. Agarose gel electrophoresis (1.5%) showing PCR amplification products for the detection of *Cytauxzoon* spp. Amplification of a ~ 232 bp fragment targeting the *Cytauxzoon* spp. Lane L: 100 bp DNA ladder; Lane 1: positive control; Lanes 2 (negative control): all clinical sample (lane 3-6 clinical sample).

The identification of *Cytauxzoon* spp. in both wild and domestic felids further supports the hypothesis of reservoir hosts, facilitating spillover transmission between wild and domestic felids (Carli *et al.,* 2012; Grillini *et al.,* 2023; Willi *et al.,* 2022). Within the Indian context, difference in prevalence rates indicate substantial endemicity in certain regions (Malangmei *et al.,* 2021) and may be attributed to geographical differences in vector distribution, climatic influences, and variations in host exposure patterns (Diaz-Regañón *et al.,* 2017; Spada *et al*., 2014; Morganti *et al.,* 2019).

Comparative Diagnostic Performance of Microscopy and PCR:

A marked difference in diagnostic sensitivity was observed between microscopy and PCR. Among 50 samples, PCR detected overall infection in six animals i.e. 12.0% (Table 01)), while microscopy detected only one (2.0%), a statistically significant difference (χ^2 = 3.842, p = 0.0498). Overall, microscopy demonstrated very low sensitivity (16.67%, 95% CI: 0.45–48.3%) with perfect specificity (100%, 95% CI: 92.4–100%). In domestic felids (n = 32), microscopy failed to detect any of the four PCR-positive animals, yielding 0% sensitivity (95% CI: 0–60.2%), a statistically significant finding (χ^2 = 4.267, p = 0.0388). In wild felids (n = 18), microscopy sensitivity was 50.0% (95% CI: 6.8–93.2%), though the small sample size precluded statistical significance (χ^2 = 0.364, p = 0.546). For *Babesia* spp., 18S rRNA-based PCR identified all four positive animals i.e. 8.0% (Table 02) missed entirely by microscopy, with 0% microscopy sensitivity and 100% specificity, representing a statistically significant difference (χ^2= 4.166, p = 0.0413). For *Cytauxzoon* spp., ITS-2 PCR detected two positives i.e. 4.0% (Table 03) versus one microscopically (2.0%); the difference was not statistically significant given the very low case numbers (χ^2 = 0.344, p= 0.557). Piroplasms are generally visible microscopically only in acute or high-parasitaemia cases (MacNeill *et al.,* 2015; Grillini *et al.,* 2023), whereas PCR detects minute quantities of

parasite DNA, establishing its primacy for surveillance in low-occurrence regions (Mishra *et al.,* 2008; Rafiqi *et al*., 2018; Santhosheni *et al.,* 2025). The consistent superiority of PCR supports its adoption as the preferred diagnostic tool for haemoprotozoan detection in felid populations.

Table 01. Comparison of microscopy and PCR detection of haemoprotozoan infections in felid populations1

Population	Total animal (n)	Microscopy+(%)	PCR+ (%)	Sensitivity (95% CI)	Specificity (95% CI)	χ^2	p-value
Overall	50	2.0 (1/50)	12.0 (6/50)	16.67 (0.45-48.3)	100 (92.4-100)	3.842	0.0498*
Wild felids	18	5.56 (1/18)	11.11 (2/18)	50.0 (6.8-93.2)	100 (81.5-100)	0.364	0.546
Domestic felids	32	0 (0/32)	12.50 (4/32)	0 (0-60.2)	100 (89.1-100)	4.267	0.0388*

Note: Data presented as percentage positive. Sensitivity and specificity are shown with 95% confidence intervals, calculated by the Wilson score method using PCR as the reference standard. Chi-square (χ^2) test was used to compare detection by microscopy and PCR; * $p < 0.05$ was considered statistically significant.

Table 02. Performance of 18S rRNA gene PCR (*Babesia* spp.) versus microscopy2

Population	Total animal	Microscopy+	PCR+	Sensitivity (95%	Specificity	χ^2	p-value
Overall	50	0 (0/50)	8.0 (4/50)	0.0 (0–60.2)	100.0 (92.1–	4.166	0.0413*
Wild felids	18	0 (0/18)	5.56	0.0 (0–97.5)	100.0 (82.4–	1.000	0.317
Domestic	32	0 (0/32)	9.38	0.0 (0–70.8)	100.0 (89.1–	3.125	0.0775

*Statistically significant ($p<0.05$). Microscopy results for *Babesia* spp. evaluated using 18S rRNA gene-based PCR as the reference standard.

Table 03. Performance of ITS-2 gene PCR (*Cytauxzoon* spp.) versus microscopy3

Population	Total animal (n)	Microscopy+ (%)	PCR+ (%)	Sensitivity (95% CI)	Specificity (95% CI)	χ^2	p-value
Overall	50	2.0 (1/50)	4.0 (2/50)	50.0 (6.8–	100.0 (89.5–99.9)	0.344	0.557
Wild felids	18	5.56 (1/18)	5.56 (1/18)	100.0 (2.5–	100.0 (80.5–100)	0.000	1.000
Domestic felids	32	0 (0/32)	3.13 (1/32)	0.0 (0–97.5)	100.0 (89.1–100)	1.033	0.309

Microscopy results for *Cytauxzoon* spp. evaluated using ITS-2 gene-based PCR as the reference standard.

CONCLUSIONS

This study demonstrates that babesia and cytauxzoon infections circulate in domestic and captive wild felids in Rewa, with PCR proving statistically superior to microscopy for detection, especially for *Babesia* spp. in domestic cats warranting its routine adoption for surveillance. Wild felids exhibited subclinical infections, consistent with reservoir host status, while domestic cats and captive tigers developed severe, often fatal cytauxzoonosis. These divergent clinical outcomes highlight complex host–parasite interactions influencing pathogenesis. Routine molecular screening of both captive wild and domestic felid populations is recommended to accurately estimate prevalence, facilitate early therapy, and strengthen health strategies.

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Ethics approval Not applicable. **Conflict of interest**

All authors declare that they have no conflict of interest.

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