What's bugging Himalayan big cats: vector-borne pathogens of wild tigers and leopards in Nepal

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Abstract

The survival of endangered wildlife can be threatened by infectious agents including vector-borne pathogens (VBPs), which can compromise their health and reproductive success. Understanding the prevalence of these pathogens in under-studied areas, such as Nepal, is crucial for conservation efforts. In the current study, bacterial and protozoan VBPs in rescued endangered wild felids [Bengal tigers (*Panthera tigris tigris*) (n = 20) and leopards *(Panthera pardus*) (n = 15)] in Nepal, were screened by species- or group-specific molecular assays. A total of 33 (94%) felids were infected with at least one VBP, with 26 (74%) being co-infected with more than one. The overall infection rate of *Theileria bicornis, Cytauxzoon* sp., *Babesia panickeri, Candidatus* Mycoplasma haemominutum, and *Mycoplasma haemofelis* were 57% (n = 20), 31% (n = 11), 9% (n = 3), 60% (n = 21), and 17% (n = 6), respectively. All samples tested negative for *Hepatozoon, Anaplasma, Ehrlichia, Bartonella,* and *Borrelia*. Infections by *T. bicornis* were higher in leopards, while those caused by *Cytauxzoon* sp. and *Mycoplasma* spp. were higher in tigers. The difference in infection rate of *T. bicornis, Cytauxzoon* sp., and *Mycoplasma* spp. among the wild felids species was statistically significant. This high infection rate of VBPs highlights the need for comprehensive surveillance of VBPs to assess interspecies transmission among wild animals sharing common habitat and the potential for these pathogens to spread to domestic animals and humans.

Keywords: Leopard; Tiger; vector-borne pathogens; Nepal

Introduction

Bengal tigers (*Panthera tigris tigris*) and leopards (*Panthera pardus*) are the major wild felids found in Nepal. Tigers are listed as endangered on the IUCN Red List and are distributed in protected areas and associated forests in the southern lowland of the country with an estimated

49 population of ~355 individuals (Jnawali et al. 2011; DNPWC and DFSC 2022). Leopards are
50 listed as vulnerable on the IUCN Red List and have a wide distribution throughout the country
51 up to 4,400 m above sea level. They share a common habitat with tigers in the southern
52 lowlands and sometimes occur in close proximity to human settlements (Lamichhane et al.
53 2021). Despite increases in their numbers owing to effective conservation initiatives by the
54 Nepalese government, including careful habitat management, these species are still threatened
55 by landscape fragmentation, diseases, and other anthropogenic factors like human-carnivore
56 conflict and poaching (Lamichhane et al. 2021).
57 In endemic areas, wildlife often serves as reservoirs of vector-borne pathogens (VBPs). For
58 example, lions (*Panthera leo*) in South Africa are reservoir of *Babesia leo*, while in the United
59 States, bobcats (*Lynx rufus*) serve as a reservoir of *Cytauxzoon felis*, which sometimes spills
60 over into domestic cats (Cerreta et al. 2022). Emergence and re-emergence of vector-borne
61 diseases (VBDs) including piroplasmosis and anaplasmosis in humans and animals has
62 increased due to factors including changes in climate, global travel, urbanization, habitat
63 fragmentation, and urban encroachment into forests resulting in higher contact between
64 humans, livestock, and wildlife (André 2018). While apparently healthy wildlife can harbor
65 VBPs without clinical manifestation, VBDs have the potential to cause severe disease and even
66 death in both humans and domestic animals (Mukhtar et al. 2022). Bacterial and protozoan
67 zoonotic VBPs belonging to the genera *Babesia, Rickettsia, Anaplasma, Ehrlichia*, and
68 *Bartonella* are particularly notable for their global distribution. Wild and domestic carnivores
69 can be infected by these VBPs and could act as sources of infections for humans and livestock
70 (André 2018).
71 Globally VBDs have been reported in different captive and free-ranging wild carnivores.
72 *Babesia* and *Theileria* were reported in wild felids [lion, leopard, and cheetah (*Acinonyx*
73 *jubatus*)] in Kenya (Githaka et al. 2012) and tigers in India (Nagar et al. 1979). Similarly,

74 *Anaplasma* and *Ehrlichia* have also been reported in different classes of carnivores in both
75 captive and free-ranging populations. (Filoni et al. 2006; André et al. 2010, 2012; Kelly et al.
76 2014; André 2018; Cerreta et al. 2022). Beside these, *Candidatus* Mycoplasma haemominutum
77 and *Hepatozoon canis* were reported in captive tiger populations in Italy (Iatta et al. 2020).
78 Fatal cytauxzoonosis was also reported in captive tigers and African lions (Garner et al. 1996;
79 Jakob and Wesemeier 1996; Peixoto et al. 2007). Three species of hemoplasma have been
80 reported in felids; *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum, and
81 *Candidatus* Mycoplasma turicensis (Tasker 2010; Carneiro et al. 2020). Infections by these
82 aforementioned bacteria have been regularly reported in various wild felids around the world
83 (Haefner et al. 2003; Willi et al. 2007; André et al. 2011; Furtado et al. 2018; Carneiro et al.
84 2020). Although most VBP infection are asymptomatic in wild hosts, recrudescence can be
85 triggered by external factors such as stress (Nijhof et al. 2005), immunosuppression caused by
86 viral co-infection (Munson et al. 2008) and reintroduction of *ex situ*-bred immunologically
87 naïve animals to the wild (Adamson 1962).
88 Knowledge on the prevalence and diversity of VBPs in wild carnivores has been growing.
89 However, information from South Asian countries, including Nepal, remains limited.
90 Therefore, this study aimed to carry out the first molecular survey of common VBPs including
91 *Anaplasma, Ehrlichia, Babesia, Hepatozoon, Cytauxzoon, Borrelia, Bartonella*, and
92 *Mycoplasma* in wild felids in Nepal to fill this gap in knowledge of VBPs in this region.
93

94 **Material and methods**

95 **Study area and sample collection**

96 All tigers were rescued from either buffer zones or protected areas of two national parks
97 (Chitwan National Park and Bardiya National Park), while most of the leopards were rescued

from those areas except for one that was rescued at Koshi Tappu Wildlife reserve in the eastern part of the country. The collection sites of tigers are all in the Terai region whereas leopards from both the Terai and Midhills of Nepal were studied.

The tiger and leopard samples were collected through the human – carnivore conflict mitigation program (Fig. 1). Generally, when there is a human – carnivore conflict scenario, animals are captured from the conflict sites. Rescue team carries out an overall physical examination and decides whether to keep the offending animal in a zoo or release it back to the wild, based on its health status. Tigers and leopards were immobilized jointly by a team from the Department of National Park and Wildlife Conservation and the National Trust for Nature Conservation with a standard protocol based on a combination of Medetomidine[@] (0.07 mg/kg BW) and Ketamine[@] (3 mg/kg BW) by using a dart gun, with the effects later reversed by Atipamezole[@] (0.35 mg/kg BW). Samples were collected from 20 tigers (15 males, 5 females; 18 adults, 2 young) and 15 leopards (8 males, 7 females; 13 adults, 2 young) between 2014 and 2021. The blood samples were collected from cephalic and saphenous veins and stored in the molecular laboratory of the National Trust for Nature Conservation – Biodiversity Conservation Center (NTNC-BCC), Sauraha Chitwan. Ethylenediamine tetraacetic acid (EDTA)-treated vacutainer tubes with blood samples were labeled with age, sex, date, and location of collection and stored at − 20°C. The animal's age was categorized based on the shape and color of canine teeth, as well as the color of their body coat (Jhala and Sadhu 2017).

Conventional PCR

DNA was extracted from 200 µl of whole blood samples using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany, RRID: SCR_008539) following the manufacturer's standard protocol. Extracted DNA was stored at – 20°C before further processing. Almost full length of the 18S ribosomal RNA gene (rDNA) (1,400 – 1,600 bp) of the genera *Babesia, Theileria,* and

123 *Hepatozoon* (BTH) was amplified using a nested polymerase chain reaction (PCR) (Masatani
124 et al. 2017). The Anaplasmataceae including *Anaplasma* and *Ehrlichia* was screened by several
125 sets of nested PCRs targeting heat shock protein (*groEL*) or citrate synthase (*gltA*) genes (Table
126 1). For the detection of *Bartonella* species, *gltA* gene was amplified using PCR. *Mycoplasma*
127 species were firstly screened by PCR amplifying 16S rDNA (Ribeiro et al. 2017) and
128 subsequently *M. haemofelis* was characterized by the primers targeting the partial RNase P
129 gene (Tasker et al. 2003). All primers employed in this study are shown in Table 1.
130 All PCR reactions were conducted in a 25 µl reaction mixture containing 12.5 µl of 2×
131 TaKaRa Ex Premier^{TM} DNA Polymerase (Takara Bio Inc., Shiga, Japan, RRID: SCR_021372),
132 0.5 µl of 200 nM of each primer, 1.0 µl of template DNA, and molecular grade water to adjust
133 volume. The cycling conditions were set with an initial denaturation at 94°C for 1 min,
134 followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at each temperature shown
135 in Table 1 for 15 sec, and extension at 68°C for 20-100 sec (1 min/kb), and final extension at
136 68°C for 5 min. The second PCR products were subjected to 1.5% agarose gel electrophoresis,
137 stained with Gel-Red (Biotium, Hayward, CA, USA), and visualized under UV light.
138
139 **Real-time PCR**

140 The samples were screened by a 16S rDNA real-time PCR for the detection of *Borrelia* as
141 described previously by Parola et al. (2011). The details of the primers and probe are presented
142 in Table 1. The reactions were performed in a 20 µl reaction mixture containing 10 µl of
143 THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan), 300 nM of each primer, 200 nM
144 of probe, 5.0 µl of template DNA, and molecular grade water. The reaction was performed in
145 a C1000 thermal cycler with a CFX96 Real-Time PCR Detection System (BioRad Laboratories,
146 Hercules, CA, USA) at 50°C for 3 min, 95°C for 1 min, and 45 cycles of 95°C for 15 sec and

60°C for 1 min. Each run included a blank, a control, and serially diluted plasmid standards (10^6, 10^4, and 10^2 copies/reaction).

In-Fusion cloning

Samples with double peaks in the electropherograms in the direct sequencing of second BTH-PCR products were selected for In-Fusion cloning to obtain sequences of single colonies. The PCR products amplified with the BTH infusion 2nd F and BTH infusion 2nd R primers were purified and cloned into the pUC19 vector (TaKaRa Bio Inc.). The infusion reaction and conditions were set as previously described (Pandey et al. 2024).

Sanger sequencing

The PCR products were purified using ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) or NucleoSpin Gel and PCR Clean Up Kit (Macherey-Nagel, Düren, Germany). The forward and reverse primers were used in the Sanger sequencing of the purified PCR products. The purified products were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, RRID:SCR_018046). Sequence data editing and trimming of primer annealing sites was done using ATGC software (GENETYX Corporation, Tokyo, Japan, RRID:SCR_002917). The obtained sequences were submitted to the DNA Data Bank of Japan (DDBJ) under the accession numbers: LC863643-LC863662 for 18S rDNA of *T. bicornis,* LC863663-LC863673 for 18S rDNA of *Cytauxzoon* sp., LC863674-LC863676 for 18S rDNA of *B. panickeri,* LC863677-LC863682 for 16S rDNA of *Mycoplasma* sp., LC863683-LC863703 for 16S rDNA of *Candidatus* M. haemominutum, and LC863637-LC863642 for RNase P gene of *M. haemofelis*.

Phylogenetic analysis

The sequences obtained in this study were compared with those available in GenBank database by the BLAST (RRID: SCR_001010) analysis. We aligned the obtained sequences with the related sequences obtained from the database using MAFFT v7.450 software (RRID:SCR_011811) (Katoh and Standley 2013). After visual inspection, we performed the necessary trimming and editing of the sequences using MEGA7 (RRID: SCR_000667) (Kumar et al. 2016). Completely identical sequences were detected and brought together by "pgelimdupseq" command in Phylogears2 v2.0 (Tanabe 2008), followed by the best-fit model estimation based on the Bayesian information criterion by Kakusan4 (Tanabe 2011), choosing the general time-reversible (GTR) gamma invariant model for 18S rDNA and 16S rDNA. The phylogenetic analysis was conducted using MrBayes v3.2.6 (RRID:SCR_012067) (Ronquist et al. 2012) with 5,000,000 generations at a sample frequency of 100,000 discarding the first 10% of trees as a burn-in. The consensus tree of 18S rDNA was rooted using the sequences of *Toxoplasma gondii* as an outgroup and was visualized using FigTree v1.4.4 (RRID:SCR_008515) (Rambaut 2012). The RNase P gene sequences of *M. haemofelis* and *M. haemocanis* were aligned using MUSCLE v3.8.425 (RRID:SCR_011812), implemented via the AliView tool ver1.28 (RRID:SCR_002780). The K2P genetic distance between *M. haemofelis* and *M. haemocanis* was calculated using MEGA v10.

Statistical analysis

To assess the impact of different factors (including age, sex, and species) on the prevalence of VBPs in the examined wild felids, Fisher's exact test was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA, RRID:SCR_002798). In addition,

odds ratios and corresponding 95% confidence intervals were assessed. $p < 0.05$ was designated as statistically significant.

Results

Detection of protozoan VBPs

A total of 35 blood samples of tiger (n = 20) and leopard (n = 15) were obtained during the study period and examined for piroplasm infections. The overall infection rates of *Theileria bicornis, Babesia panickeri*, and *Cytauxzoon* sp. in wild felids were 57% (n = 20), 9% (n = 3), and 31% (n = 11), respectively (Table 2). The infection rate of *T. bicornis* was higher in leopards 87% (n = 13) than in tigers 35% (n = 7). The infection rate of *Cytauxzoon* sp. was higher in tigers 50% (n = 10) than in leopards 7% (n = 1). *Babesia panickeri* infection was only detected in tigers 15% (n = 3). All sample were negative for *Hepatozoon*. The representative gel image of the BTH PCR products is provided in Supplementary Fig. 1.

The 18S rDNA sequences of *T. bicornis* obtained in this study were identical (query cover: 99-100%) to *T. bicornis* reported from rescued rhinos (*Rhinoceros unicornis*) in Nepal (GenBank accession number: LC817751, Pandey et al. 2024). The *B. panickeri* found in the tiger in this study showed 99.30% identity (query cover: 100%) with *B. panickeri* sequence reported from a domestic cat (*Felis catus*) in India (GenBank accession number: MN267560, Panicker et al. 2020). The *Cytauxzoon* sp. detected in this study showed 97.28% identity (query cover: 100%) with *Cytauxzoon* sp. sequence isolated from a common palm civet (*Paradoxurus hermaphroditus*) in Singapore (GenBank accession number: ON606000, Chong et al. 2025).

Phylogenetic analysis based on 18S rDNA sequences showed that the partial sequence of *Babesia* sp. (GenBank accession number: LC863674) obtained in this study clustered with *B. panickeri* from India and another *Babesia* sp. from India. The *Cytauxzoon* sp. (GenBank accession numbers: LC863663 and LC863664) sequences obtained from tigers and leopards

belonged to the same clade with *Cytauxzoon* sp. reported from Singapore. *Theileria bicornis* (GenBank accession numbers: LC863643 and LC863650) sequences obtained from tigers and leopards belonged to the same clade with *T. bicornis* reported from the one-horned rhino in Nepal (Fig. 2).

Detection of bacterial VBPs

A total of 77% (n = 27) of the samples tested positive for *Mycoplasma* infection. Phylogenetic analysis of 16S rDNA sequences showed that the *Mycoplasma* sequences obtained in this study belong to two different clades: one clade comprising the *Candidatus* Mycoplasma haemominutum and the other consisting of *M. haemocanis* and *M. haemofelis* (Fig. 3). The samples positive for *M. haemofelis*/*M. haemocanis* were further subjected to RNase P gene PCR. The representative gel images of 16S rDNA and the RNase P gene PCRs are provided in Supplementary Figs. 2 and 3, respectively. All the sequences obtained for RNase P gene were identical to each other and showed 99% identity with *M. haemofelis* (GenBank accession number: EU078617). Multiple sequence alignment of the partial RNase P gene sequences of *M. haemofelis*, *M. haemocanis*, and *M. haemofelis* obtained from Nepal indicated the presence of nucleotide differences in the positions 41, 82, 109, 110, 112, and 134 (Fig. 4). The K2P distance calculation showed that the Nepalese *M. haemofelis* sequence had an average genetic distance of 0.019 (range: 0.012 to 0.028) with other *M. haemofelis* sequences reported from lion in Tanzania, cats in Israel, Australia and the US, Eurasian lynx in Switzerland, European wildcat in France, Iberian lynx in Spain. The average K2P distance between Nepal *M. haemofelis* and *M. haemocanis* sequences previously reported form dog in Germany and the US was 0.065 (range: 0.050 to 0.084) (Supplementary Table1).

243 Overall, the infection rates of *Candidatus* M. haemominutum in tiger and leopard were 85%
244 (n = 17) and 27% (n = 4), respectively. The infection rates of *M. haemofelis* in tiger and leopard
245 were 5% (n = 1) and 33% (n = 5), respectively.
246 None of the samples were positive for *Anaplasma, Ehrlichia, Borrelia*, and *Bartonella*.
247
248 **Detection of co-infection and association with host factors**

249 The overall infection with at least one VBP was 94% (n = 33). Single infection occurred in
250 20% (n = 7) of the wild felids, while co-infection with more than one VBP occurred in 74% (n
251 = 26) (Table 3). Co-infection of *T. bicornis* and *Mycoplasma* spp. (40%, 14/35) was the most
252 common coinfection in wild felids followed by *Cytauxzoon* sp. and *Mycoplasma* spp. (22%,
253 8/35).
254 Our investigation revealed that there was no significant association between the infection
255 rate of each VBP and age or sex of the wild felids. The infection rates of *T. bicornis*,
256 *Cytauxzoon* sp. and *Mycoplasma* spp. were significantly different between felid species (Table
257 4). The infection rate of *T. bicornis* was higher in leopards than in tigers (87% vs 35%), while
258 those of *Cytauxzoon* sp. and *Mycoplasma* spp. were higher in tigers than in leopards (50% vs
259 7%, 90% vs 60%, respectively) (Table 2).
260
261 **Discussion**

262 Globally, VBPs have been reported in wild carnivores, including captive and free-ranging
263 felids. However, there is a lack of information regarding these infections in wild felids in Nepal.
264 In the current study, we report a remarkably high prevalence of VBPs at 91% in two wild felid
265 species, including *T. bicornis, Babesia* sp*.,* and *Cytauxzoon* sp.
266 In this study, *T. bicornis* was the most common piroplasm with a higher prevalence in
267 leopards (87%) than tigers (35%). In previous studies, *Theileria*-like parasites were also

detected in free-ranging cheetahs by microscopic examination of blood smears in the Serengeti National Park and Ngorongoro Crater Conservation Area, Tanzania (Averbeck et al. 1990). Thereafter, Githaka et al., (2012) reported an unknown species of *Theileria* in a cheetah in Kenya. In a captive setup, *Theileria parva* was reported in an African lion in Lohi bher Zoo (Mukhtar et al. 2022). Phylogenetic analysis showed that *T. bicornis* from tigers and leopards belonged to the same clade as *T. bicornis* reported from one-horned rhinos in Nepal, highlighting the possibility of interspecies transmission of *T. bicornis* among the wild animals in the region. This is the first report of *T. bicornis* in wild felids. *Theileria bicornis* was also reported from rhinos and nyalas in South Africa (Nijhof et al. 2003; Pfitzer et al. 2011) and cattle in Uganda (Muhanguzi et al. 2010), suggesting that it has a broad host range. King'ori et al. (2019) reported *T. bicornis* in *Amblyomma tholloni* collected from African elephants in Kenya, suggesting the possibility that *Amblyomma* spp., which are widely distributed in Nepal, may act as vectors of *T. bicornis* (Bohara and Shrestha 2015). Considering the wide host range and a lack of information on the vector of *T. bicornis* in Nepal, there is a need for further investigations identifying *T. bicornis* and its tick vector(s) in livestock populations in the region. Currently the pathogenicity of *T. bicornis* is also unknown; further investigation is required.

Various *Babesia* species have been previously reported from wild felids in Africa such as *Babesia lengua, Babesia felis, B. leo* and *Babesia vogeli.* In this study, *Babesia* infection rate was found in 15% (3/20) of the tested tigers. This rate is higher than that found in free-ranging cheetahs (7.5%, 3/40) in Namibia and lower than in lions (50%, 28/56) in Swaziland, Southern Africa (Bosman et al. 2007). Additionally, *B. lengua* was reported at 28.5% (39/137) in cheetahs in North West Province, South Africa (Bosman et al. 2010). Similarly, *B. leo* and *B. felis* were observed at a prevalence of 25% (6/24) in lions, while *B. lengua* had a prevalence of 32% (6/19) in spotted hyenas in Zambia (Williams et al. 2014). In captive wild felids in Zimbabwe, *B. leo* was detected in lions (59%, 51/86) and cheetahs (100%, 4/4), while *B. vogeli*

was detected in lions (12%, 10/86), servals (100%, 2/2), and Southern African wildcats (*Felis silvestris cafra*) (100%, 6/6) (Kelly et al. 2014). These differences in infection rates could be influenced by a number of factors, including host susceptibility, environmental conditions, vector abundance, and experimental biases such as sample size and detection methods (Alvarado-Rybak et al. 2016; Santoro et al. 2019). Infection with *Babesia* spp. is usually asymptomatic, although pathogenicity may occur due to concurrent immunodeficiency, such as the observed mortality in African lions co-infected with canine distemper virus (Munson et al. 2008). In this study, *Babesia* was reported only in tigers; the variation in results could be due to the difference in sampling location of the two wild felids as most leopards were rescued from the Midhill region while tigers were rescued from the Terai region. Phylogenetic analysis showed that the *B. panickeri* obtained in this study is closely related with *B. panickeri* reported from a cat in India and *Babesia* sp. from a lion in India (Rafiqi et al. 2018; Panicker et al. 2020), suggesting the possibility of interspecies transmission of *B. panickeri* between domestic and wild animals. It is noteworthy that the transmission of pathogens between domestic and wild animals may allow the pathogen to evolve and become more virulent within the new environment (Seal et al. 2021).

In this study, we were unable to identify the species of *Cytauxzoon* because the obtained sequences did not cluster with any of the known *Cytauxzoon* species. *Cytauxzoon* species are apicomplexan haemoparasites transmitted by ticks. Both domestic and wild felids are significantly impacted by *Cytauxzoon* infections, which have different clinical impacts. Acute and frequently fatal disease with a 97% mortality rate has been linked to *C. felis* in domestic cats if treatment is not given (Antognoni et al. 2022). This protozoan has been reported in captive as well as free-ranging wild carnivores with fatal incidence observed in captive lions, bobcats, and captive tigers (Garner et al. 1996; Peixoto et al. 2007; Andre et al. 2009). In this study, *Cytauxzoon* sp. was found in 31% (11/35) of rescued wild felids, which is higher than

318 those reported in free-ranging wild carnivores (22%, 6/27) in Mato Grosso State, Brazil (De
319 Barros Silva et al. 2021) and in asymptomatic wild felids (13%, 9/72) in Brazil (André et al.
320 2009). This could be attributed to several factors such as variation in environmental conditions,
321 vector abundance, host susceptibility, and small sample size of this study. Cui et al. (2024)
322 reported *Cytauxzoon manul* in 33% (1/3) of Eurasian lynx (*Lynx lynx*) in northwestern China.
323 Although *Cytauxzoon* species have been described by molecular study in various wild felids
324 from the US, Spain, Brazil, China, and Zimbabwe, this is the first report of its detection from
325 wild felids in Nepal. Phylogenetic analysis based on 18S rDNA sequences showed that
326 *Cytauxzoon* sp. sequences of tigers and leopards belong to the same clade and are closely
327 related to a *Cytauxzoon* sp. from a common palm civet in Singapore. This finding suggests that
328 there is potential regional variation of *Cytauxzoon* sp. Further investigations of *Cytauxzoon*
329 infections in wild and domestic felid populations is critical to understand the clinical
330 significance and transmission dynamics of this uncharacterized *Cytauxzoon* sp.
331 Our data showed a high prevalence of *Mycoplasma* spp. (77%, 27/35) in the tested wild
332 felids. This is consistent with previous findings, where infection rates of 98% in Serengeti lions
333 in Tanzania and 73% in jaguars (*Panthera onca*) in Brazil were reported (Willi et al. 2007;
334 Furtado et al. 2017). In domestic felids, *M. haemofelis* can sometimes cause severe hemolytic
335 anemia, lethargy, pyrexia, splenomegaly, lymphadenopathy, and death in some cases; however
336 clinical outcomes are not well studied in wild felids (Andre et al. 2011; Hicks et al. 2015;
337 Ravagnan et al. 2017). Phylogenetic analysis based on 16S rDNA of *Mycoplasma* showed that
338 the obtained sequences belong to two clades: one containing *Candidatus* M. haemominutum
339 clade and the other consisting of *M. haemocanis* and *M. haemofelis*. *Mycoplasma haemocanis*
340 and *M. haemofelis* cannot be differentiated by 16S rDNA sequences (Birkenheuer et al. 2002;
341 Tasker et al. 2003; Furtado et al. 2018). Transmission of *Mycoplasma* spp. in felids is not well
342 studied but blood sucking arthropods including ticks and fleas have been suggested to be

potential vectors (Shaw et al. 2004; Taroura et al. 2005; Lappin et al. 2006; Barrs et al. 2010; Reagan et al. 2017).

Our investigation revealed that there is no significant association between infection rates of VBPs and the age or sex of wild felids. However, this observation may have been influenced by sampling bias, as most of the felids examined in this study were adults. No significant association between infection rates and the sex of felids may indicate that both sexes have an equal risk of exposure to VBPs and have similar susceptibility to them. Infection with *T. bicornis* is higher in leopards, while *Cytauxzoon* sp. and *Mycoplasma* spp. are higher in tigers. The differences in infection rates of *T. bicornis, Cytauxzoon* sp., and *Mycoplasma* sp. between the wild felids species were statistically significant (Table 4), which could be attributed to difference in susceptibility to pathogens, behavior related to the infestation by vectors, vector preference, and difference in the sampling locations (Alvarado-Rybak et al. 2016; Santoro et al. 2019).

Previous studies detected *Anaplasma bovis* in leopard cats (*Prionailurus bengalensis euptilurus)* in Tsushima island, Japan and in Korea (Tateno et al. 2013; Hwang et al. 2015), *Ehrlichia canis* in captive tigers in Thailand, Iriomote cats (*Prionailurus iriomotensis),* and Tsushima leopard cats in Japan, (Tateno et al. 2013; Changbunjong et al. 2025), *Hepatozoon felis* in leopard cats in Korea, Asiatic lions (*Panthera leo persica*), and tigers in India (Kubo et al. 2010; Pawar et al. 2012), *Bartonella henselae* in Iriomote cats in Japan, African lions in Kruger National Park, and cheetahs in Namibia and South Africa (Molia et al. 2004; Tateno et al. 2013), and *Borrelia burgdorferi* s. l. in various zoo felids in Germany (Stoebel et al. 2003). However, in our study, all samples tested negative for these VBPs. In Nepal, the pathogens in the same genera, including *Anaplasma platys, E. canis*, and *Hepatozoon canis*, have been detected in dogs (Díaz-Regañón et al. 2020; Pandey et al. 2025). This may be attributed to ecological differences, vector abundance, or the availability of reservoir hosts. In addition, the

limited sample size may have reduced the likelihood of detecting these pathogens. Other factors, such as primer mismatches or low pathogen loads in blood, could also have contributed to false negative results. Additionally, seasonal fluctuations in vector activity may play a role, as sampling outside the main transmission periods could lead to undetectable infection. Further studies with larger sample sizes and optimized diagnostic methods are necessary to confirm these results.

In wild felids, VBP infections are typically subclinical (Alvarado-Rybak et al. 2016). But these pathogens can become pathogenic under certain conditions, such as captivity-related stress conditions, immunosuppression, climatic variation, or habitat degradation (Penzhorn 2006). Transmission of VBPs occurred between domestic and wild animals. For example, the primary canine pathogens *E. canis* and *H. canis* were detected in tigers in Thailand and a safari park in Italy, suggesting possible spillover from dogs to wild carnivores (Iatta et al. 2020; Changbunjong et al. 2025; Mongkolphan et al. 2025). The detection of *A. bovis* in domestic cats, dogs, deer, and Tsushima leopard cats in Japan suggests transmission of pathogens between domestic and wild animals (Kawahara et al. 2006; Ooshiro et al. 2008; Sasaki et al. 2012; Tateno et al. 2013; Fukui and Inokuma 2019). Blouin et al. (1984) reported that *C. felis* was experimentally transmitted from infected bobcats (*Felis rufus*) to domestic cats via the tick vector *Dermacentor variabilis. Cytauxzoon felis* spillover to domestic cats often leads to fatal disease (Birkenheuer et al. 2006).

The detection of the same species/genotypes of VBPs in both tigers and leopards indicates that wild felids living in the same region are threatened by common VBPs. However, the interspecies transmission between wild and domestic animals is yet to be evaluated. The habitat overlaps between wild felids and domestic animals suggest potential VBP transmission. As shown in Fig. 1, most wild felids habitats are within or near human settlements, which suggests a risk of VBP spillover to domestic animals and potentially to humans. This study provides

data on subclinical VBPs infection in tigers and leopards in Nepal. Such data are essential for conservation planning and long-term health monitoring. Comprehensive surveillance of VBPs and their vectors is crucial for understanding the epidemiology of these infections, including their transmission routes and species involved in spreading the pathogens. This information will be vital for developing effective control measures against these infections. The main limitations of this study are the small sample size and the fact that all samples were collected during rescue operations. These factors may limit the generalization of the results to the wider wild felid population in Nepal. Future studies should focus on comprehensive longitudinal survillance, vector ecology, and clinical correlation to better understand the epidemiology of VBPs in wild felids.

Supplementary Information

The online version contains supplementary material available at [DOI].

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Data availability

The sequences obtained in this study are available are available under the accession numbers provided in methodology section. Further information related to this study is available upon request.

Declarations

Ethical approval

The study was based on sample collected during human – carnivore conflict mitigation program. Legal permission for wild felids captures and handling was granted by the chief of National Park Office and samples were collected by a registered veterinarian. Ethical approval for the study was obtained from the Ethical Clearance Committee of the Nepal Veterinary Council (Ref No: 56/2079.80).

Consent for publication

All authors have read and approved the manuscript.

Competing interest

None

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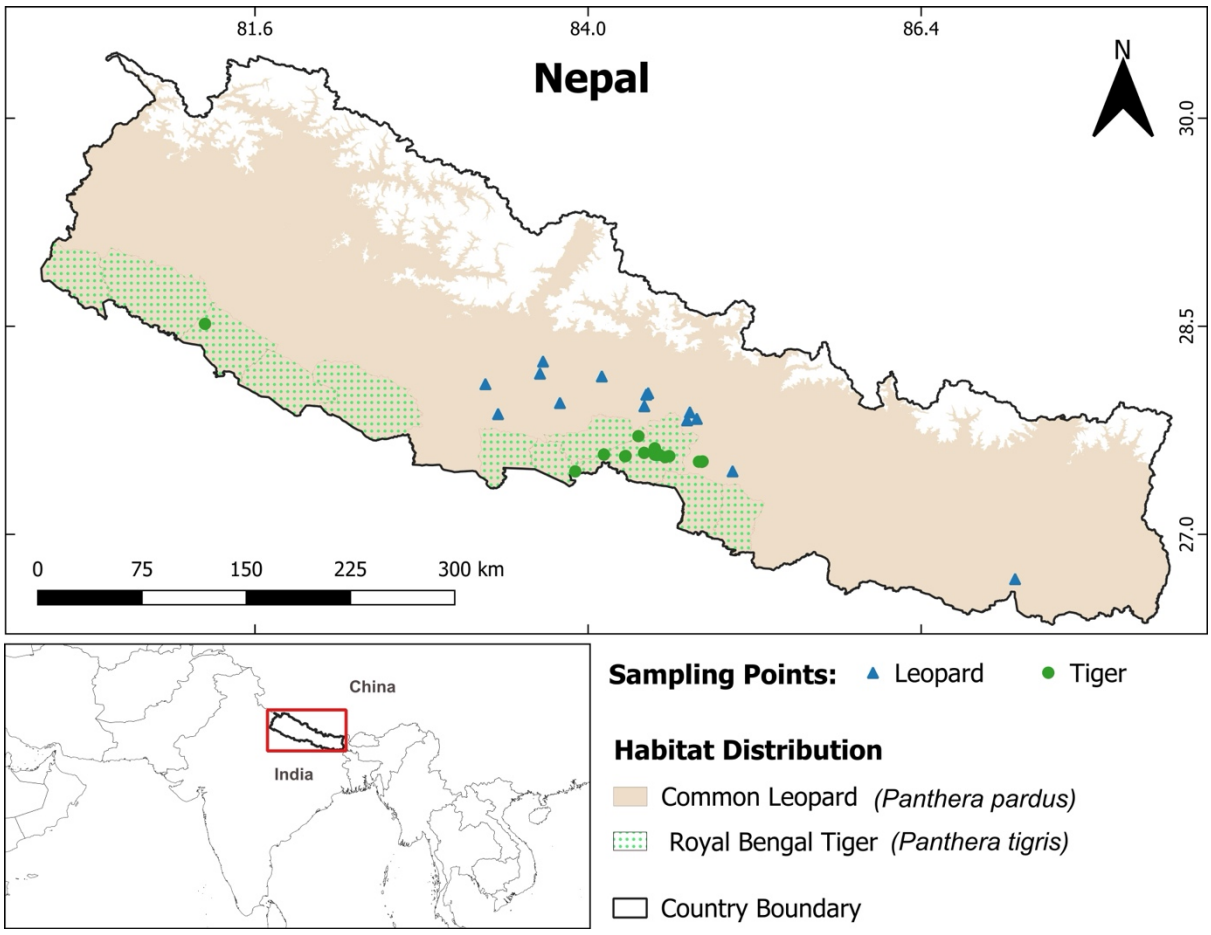

Fig. 1 Map of Nepal showing the sampling locations where vector-borne pathogens in wild felids were screened. The habitat of each animal was given by Jnawali et al. (2011).

Fig. 2 Bayesian phylogenetic tree constructed using the 18S rRNA gene sequences of *Theileria, Cytauxzoon*, and *Babesia.* The tree was rooted with *Toxoplasma gondii* and black dots indicate nodes with posterior probability of > 0.75

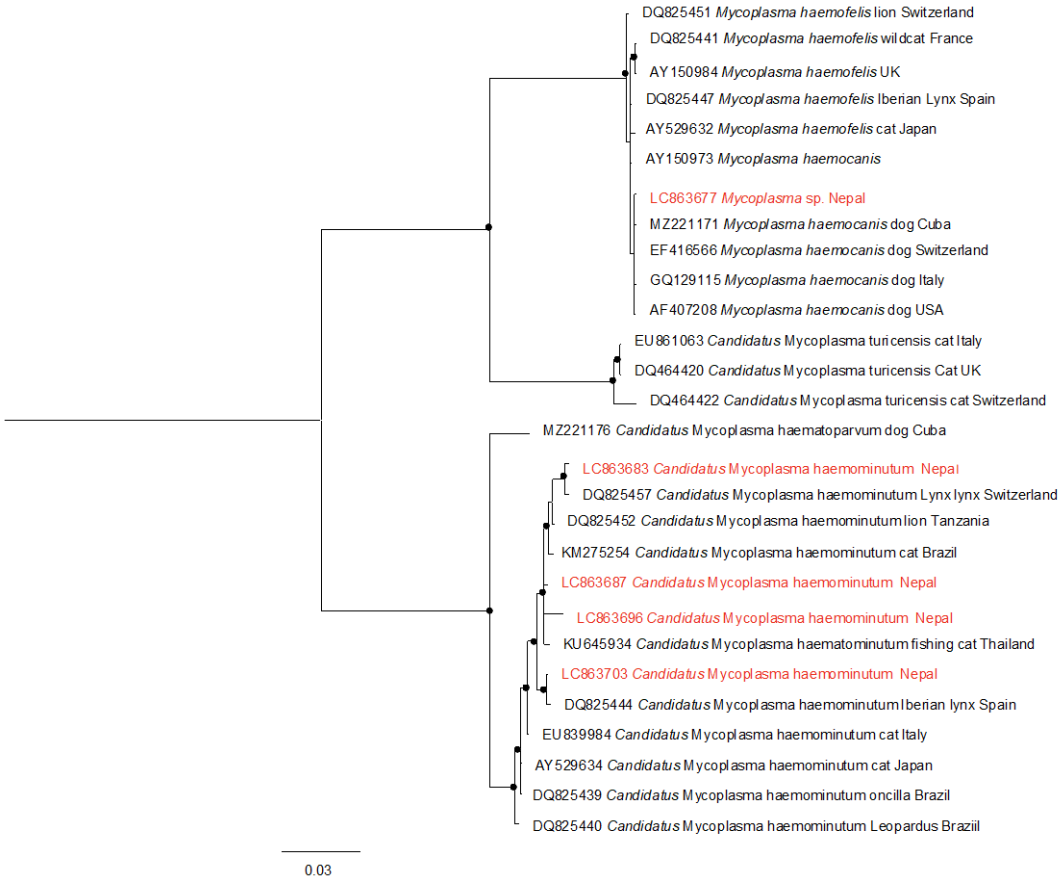

Fig. 3 Bayesian phylogenetic tree constructed using the 16S rRNA gene sequences of *Mycoplasma haemofelis, Candidatus* Mycoplasma haemominutum, and related *Mycoplasma* species. Black dots indicate nodes with posterior probability of > 0.75

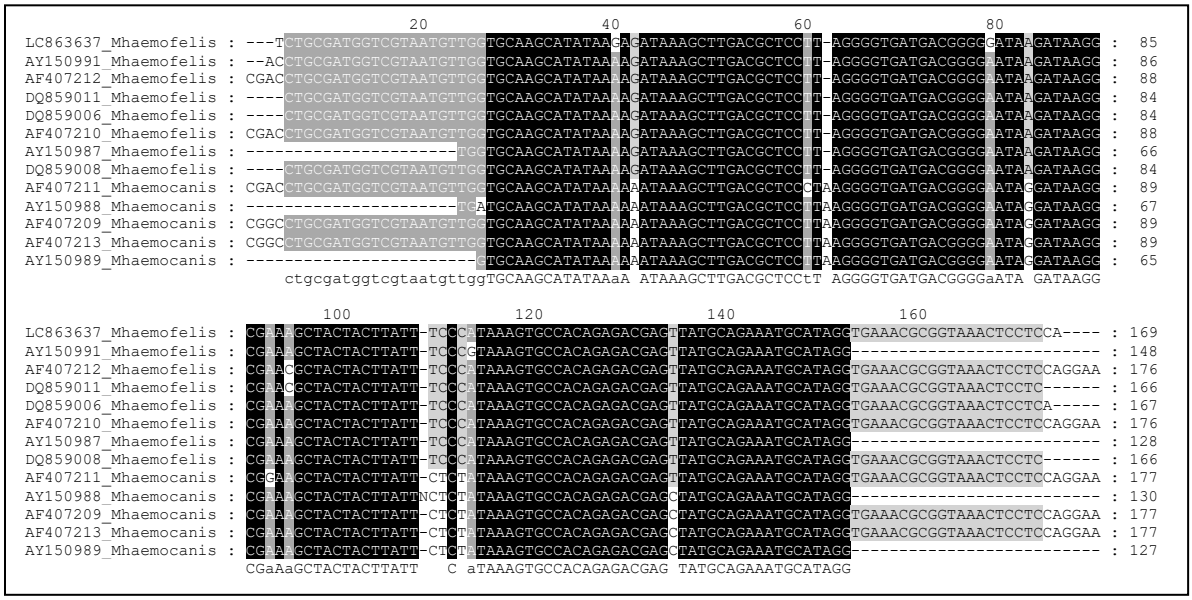

Fig. 4. Multiple sequence alignment of the partial RNase P gene sequences of *Mycoplasma haemofelis* and *Mycoplasma haemocanis*. The *M. haemofelis* (LC863637) sequence obtained from this study is shown at the top of the alignment. Fully conserved sites are shown in black blocks, while partially conserved sites are in grey blocks. Sites at positions 41, 82, 109, 110, 112, and 134 show nucleotide differences between *M. haemofelis* and *M. haemocanis*

Table 1. The list of primers and probe used in this study

Name	Sequence (5'-3')	Target organism(s)	Target gene	Purpose	Product size (bp)	Reference
BTH 1st F	TGAAACTGCGAATGGCTCATTAC	*Babesia, Theileria*, and *Hepatozoon*	18S rRNA	1st PCR	1,400 ─ 1,600	(Masatani et al. 2017)
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	*Babesia, Theileria*, and *Hepatozoon*	18S rRNA	2nd PCR	1,400 ─ 1,600	(Masatani et al. 2017)
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
BTH18SinterF	ATTTTCCGACTCCTTCAGCA	*Babesia, Theileria*, and *Hepatozoon*	18S rRNA	Sequencing		(Ogata et al. 2021)
BTH18SinterR	AACTAAGAACGGCCATGCAC					
GroEL-643s	ACTGATGGTATGCARTTTGAYCG	Anaplasmataceae	*groEL*	PCR	630	(Barber et al. 2010)
GroEL-1236as	TCTTTRCGTTCYTTMACYTCAACTTC					
F4b	CCAGGCTTTATGTCAACTGC	Anaplasmataceae	*gltA*	1st PCR	950	(Inokuma et al. 2001)
HG1085R	ACTATACCKGAGTAAAAGTC					
F1b	GATCATGARCARAATGCTTC	*Anaplasma* and *Ehrlichia*	*gltA*	2nd PCR	430	(Inokuma et al. 2001)
HG1085R	ACTATACCKGAGTAAAAGTC					
BhCS781.p	GGGGACCAGCTCATGGTGG	*Bartonella*	*gltA*	PCR	380	(Norman et al. 1995)
BhCS1137.n	AATGCAAAAAGAACAGTAAACA					
Bor16S3F	AGCCTTTAAAGCTTCGCTTGTAG	*Borrelia*	16S rRNA	qPCR	148	(Parola et al. 2011)
Bor16S3R	GCCTCCCGTAGGAGT CTGG					
Bor16S3P	FAM-CCGGCCTGAGAGGGTGAACGG-TAMRA					
16S_HAEMOforw	GGCCCATATTCCTRCGGGAAG	*Mycoplasma*	16S rRNA	PCR	950	(Ribeiro et al. 2017)
16S_HAEMOrev	ACRGGATTACTAGTGATTCCA					
RNasePFor1	CTGC GATGGTCGTAATGTTG	*Mycoplasma haemofelis*	RNase P	PCR	160 ─ 210	(Tasker et al. 2003)
RnasePRev1	GAGGAGTTTACCGCGTTTCA					
InFusin_BTH 2ndF	AAAACGACGGCCAGTGGCTCATTACAACAGTTATAGTTT	*Babesia, Theileria*, and *Hepatozoon*	18S rRNA	Cloning	1,400 ─ 1,600	(Pandey et al. 2024)
InFusin_BTH_2ndR	GACCATGATTACGCCCGGTCCGAATAATTCACCGG					

Table 2. Vector-borne pathogens detected in the wild felids in Nepal

Vector-borne pathogen	Overall (n = 35) (%)	Tiger (n = 20) (%)	Leopard (n = 15) (%)
Theileria bicornis	20 (57)	7 (35)	13 (87)
Cytauxzoon sp.	11 (31)	10 (50)	1 (7)
Babesia panickeri	3 (9)	3 (15)	0
Candidatus Mycoplasma haemominutum	21 (60)	17 (85)	4 (27)
Mycoplasma haemofelis	6 (17)	1 (5)	5 (33)
Anaplasma/Ehrlichia	0	0	0
Bartonella	0	0	0
Borrelia	0	0	0

Table 3. Single and co-infection of vector-borne pathogens in wild felids in Nepal

	Tiger	**Leopard**
Single infection		
Theileria bicornis	0	5
Mycoplasma spp.	0	1
Cytauxzoon sp.	1	0
Co-infection		
Theileria bicornis + *Mycoplasma* spp.	7	7
Cytauxzoon sp. + *Mycoplasma* spp.	8	0
Babesia panickeri + *Mycoplasma* spp.	2	0
Theileria bicornis + *Mycoplasma* spp. + *Cytauxzoon* sp.	0	1
Babesia panickeri + *Mycoplasma* spp. + *Cytauxzoon* sp.	1	0
Total	19	14

Table 4. The association between different variables (age, sex, and species) and the infection with vector-borne pathogen

Variable	Category	No. tested	*Theileria bicornis*			*Cytauxzoon* sp.			*Mycoplasma* spp.		
			No. positives	OR (95 % CI)	p value	No. positives	OR (95 % CI)	p value	No. positives	OR (95 % CI)	p value
Age	Young (≤ 1 yr)	4	2	0.72 (0.09-5.82)	1	0	0.20 (0.01-4.02)	0.285	3	0.88 (0.08-9.79)	1
	Adult (≥ 1 yr)	31	18	1.39 (0.17-11.15)		11	5.05 (0.25-102.50)		24	1.14 (0.10-12.79)	
Sex	Male	23	11	0.31 (0.07-1.43)	0.162	9	3.21 (0.57-18.21)	0.259	19	2.38 (0.47-11.93)	0.402
	Female	12	9	3.27 (0.70-15.30)		2	0.31 (0.05-1.76)		8	0.42 (0.08-2.11)	
Species	Tiger	20	7	0.08 (0.01-0.48)	0.004	10	14.00 (1.54-127.70)	0.009	18	6.00 (1.00-35.92)	0.051
	Leopard	15	13	12.07 (2.10-69.46)		1	0.07 (0.01-0.65)		9	0.17 (0.03-0.99)	

OR odds ratio; CI, confidence interval