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RESEARCH

The first molecular identification and phylogenetic analysis of tick-borne pathogens in captive wild animals from Lohi Bher zoo, Pakistan

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Abstract
Tick-borne pathogens are causing severe diseases in livestock, wild animals, and humans. Wild animals play a crucial role in tick-borne pathogens' transmission life cycle by serving as reservoir hosts or intermediate hosts, posing a continuous risk for domestic animals and humans. The presence of tick-borne pathogens is often ignored in wild animals kept in zoos, which is a public health concern. In the present study, we investigated these pathogens in tick-infested captive wild animals at the Lohi Bher zoo, Pakistan. Blood samples were collected from 22 animals, which include urials (4) (*Ovis aries vignei*), blackbucks (3) (*Antilope cervicapra*), fallow deer (1) (*Dama dama*), hog deer (6) (*Axis porcinus*), chinkaras (4) (*Gazella bennettii*), white tiger (2) (*Panthera tigris tigris*), a giraffe (*Giraffa camelopardalis*), and African lions (2) (*Panthera leo*). The samples were screened for Piroplasm and *Anaplasma* spp. by polymerase chain reaction targeting different gene loci. We detected three *Theileria* spp. and one *Anaplasma* sp. from the investigated captive wild animals. The *Theileria sp. dama gazelle* was detected from chinkara, *Theileria sp. NG-2012b* from chinkara and giraffe and *T. parva* from African lion, and *Anaplasma bovis* was identified in a giraffe. Moreover, *Theileria* sp. and *Anaplasma* sp. coinfection was detected in one giraffe. Overall, this study shows that *Theileria* spp. and *Anaplasma* spp. are circulating in captive wild animals, which can play an important role in their spread. Further studies are required to monitor tick-borne pathogens in zoo animals and their potential to spread from exotic wild captive animals to local wild and domestic.

Keywords *Anaplasma* · Pakistan · PCR · *Theileria* · Tick-borne diseases · Captive wild animals

Introduction

Recent studies have documented several pathogens as tick-borne, and the infections caused by these pathogens are emerging and reemerging due to their increasing global burden and distribution (Cutler et al. 2021; Parola et al.

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2005). The Piroplasm group and *Anaplasma* spp. are the main cause of most tick-borne diseases (TBDs) (Parola et al. 2005). TBDs not only constrain livestock farming (Namgyal et al. 2021) but also spread infection in humans (Farooq and Moriarty 2021) and wild animals (Parola et al. 2005). In addition, tick-borne pathogens (TBPs) have a diverse and broad host spectrum, which includes canines, cervids, bovines, felines, horses, and humans (Lee et al. 2018).

Wild animals and birds carry numerous tick-borne pathogens in their ecology, which affect human and domestic animals' health (Buczek et al. 2020; Springer et al. 2020). It signifies that for controlling TBDs, it is critical to identify wild animals as hosts and reservoirs of TBPs. Moreover, current climate change and global warming have created an optimum environment for ticks to survive and for an increased incidence of TBDs (Bouchard et al. 2019). These conditions globally intensified the impact of TBDs. The zoonotic potential of TBPs warrants preeminent attention to tick surveillance and tick-borne pathogens in both domestic and wild animals.

Earlier, an epidemiological survey on TBPs in Pakistan was conducted using molecular detection techniques on domestic animals (Hassan et al. 2018; Iqbal et al. 2019). However, limited or no data are available on tick-borne pathogens for wildlife. One reason for this scarcity is the difficulty in obtaining samples from wild animals and the lack of resources in low-income countries that compels to overlook the crucial connection of wildlife in the tick-borne disease transmission. In the present study, tick-borne pathogens, i.e., Piroplasms group and *Anaplasma* spp., were investigated in captive wild animals to explore the role of these animals in the life cycle of tick-borne pathogens. This study investigated the presence and diversity of tick-borne agents in wild animals. It also determines the potential ecological role of exotic wild animals in transmitting TADs.

Materials and methods

Sample collection

Blood samples from 22 tick-infested captive wild animals of Lohi Bher zoo, Punjab, Pakistan, were collected on Whatman FTA™ Classic Card (GE Healthcare, USA) from April 2019 to June 2019 (Table 1). The animals were selected based on a physical examination that showed the presence of tick infestation.

Sample collection and animal treatments complied with the approval of the Animal Ethics Procedures and Guidelines Committee of Lohi Bher zoo. Briefly, for lion and white tiger, the animals were immobilized by dart injection of 0.03–0.05 mg/kg medetomidine 20 mg/mL (Kyron Laboratories, Johannesburg, South Africa) and 0.5–1.0 mg/kg Zoletil 100 mg/mL (tiletamine-zolazepam; Virbac, Centurion, South Africa). They were reversed by intramuscular administration of 0.2 mg/kg atipamezole 5 mg/ml (Pfizer, Sandton, South Africa). Blood samples were collected by femoral or cephalic venipuncture, and 125 µl of blood was loaded on the FTA card. Small ruminants were immobilized by zoo technicians manually by using the confinement method. Trapped large ruminants were anesthetized using a portable inhalational anesthetic machine with a precision vaporizer. The whole blood from each animal was collected via jugular vein and loaded onto the FTA card as above. All sample collections were done in the daytime. The dried blood was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) protocol to isolate genomic DNA from the Dried Blood Spots according to the manufacturer's instructions.

Detection of tick-borne pathogens and PCR amplification

The extracted DNA was screened for piroplasms and *Anaplasma* by PCR. The PCR primers, amplicon sizes,

Table 1 The piroplasm and *Anaplasma* spp. identified in captive wildlife from Lohi Bher Zoo, Punjab, Pakistan

Animal species	Scientific name	Total no. of animals	Total no. of animals positive for Piroplasm	Total no. of animals positive for *A. bovis*
Urial	*Ovis aries vignei*	4	0	0
Black buck	*Antilope cervicapra*	3	0	0
Fallow deer	*Dama dama*	1	0	0
Hog deer	*Axis porcinus*	6	0	0
Chinkara	*Gazella bennettii*	4	**3**	0
White Tiger	*Panthera tigris tigris*	1	0	0
Giraffe	*Giraffa camelopardalis*	1	**1**	**1**
African Lion	*Panthera leo*	2	**1**	0
Total		22	**05**	**01**

The bold entries indicate the sampled animals that are detected as positive

and cycling conditions applied in this study are listed in Table 2. Briefly, nested PCR was conducted to detect piroplasm with the primers based on 18S rRNA (Olmeda et al. 1997; Yang et al. 2014), while for *A. phagocytophilum* and *A. bovis*, primers based on 16S rRNA (Barlough et al. 1996; Kawahara et al. 2006) were used. *Anaplasma ovis* and *A. marginale* were tested by conventional PCR employing primers based on the *msp4* gene (de la Fuente et al. 2005; Torina et al. 2012). Reactions were performed in an automatic thermocycler (Bio-Rad, Hercules, USA) with a total volume of 25 μl as previously described by Yang et al. 2017. The DNAs extracted from the whole blood of animals infected with *T. annulata*, *A. phagocytophilum*, *A. bovis*, *A. marginale*, and *A. ovis*, which have been verified by sequencing, were selected as the positive control. Sterile water was used as the negative control for each run. Amplified fragments were electrophoresed on a 1.0% agarose gel containing 10 μl of Goldview (SolarBio, China) and visualized under UV transillumination.

DNA sequencing and phylogenetic analysis

The DNA fragments were purified with the AxyPrepTM DNA Gel Extraction Kit (Axygen, Union City, CA, USA). The purified fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed for sequencing using BigDye Terminator Mix (GenScript, Nanjing, China). The nucleotide sequences obtained in this study were compared with previously published sequences deposited in GenBank by a BLASTn search or by using the ClustalW multiple alignment algorithms in the MegAlign program of the Lasergene software package (Madison, WI, USA). The phylogenetic trees were inferred using the Neighbor-Joining (NJ) method with the Kimura two-parameter model, and the bootstrap test was replicated 1000 times (Tamura et al. 2007).

Table 2 Primers and PCR amplification conditions

Pathogen	Target gene	Primer name	Primer sequence (5'-3')	Annealing temp (°C)	Amplicon size (bp)	Reference
Piroplasm	18S rRNA	Piro1-S	CTTGACGGTAGGGTATTGGC	55	~1410	(Yang et al. 2014
		Piro3-AS	CCTTCCTTTAAGTGATAAGGTTCAC			
		PIRO-A1	CGCAAATTACCCAATCCTGACA	55	~430	Olmeda et al. 1997
		PIRO-B	TTAAATACGAATGCCCCCAAC			
A. phagocytophilum	16S rRNA	EE1	CCTGGCTCAGAACGAACGCTGGCGGC	55	~1430	Barlough et al. 1996
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		SSAP2f	GCTGAATGTGGGGATAATTTAT	60	641	Kawahara et al. 2006
		SSAP2r	ATGGCTGCTTCCTTTCGGTTA			
A. bovis	16S rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCGGC	55	~1430	Barlough et al. 1996
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		AB1f	CTCGTAGCTTGCTATGAGAAC	60	551	Kawahara et al. 2006
		AB1r	TCTCCCGGACTCCAGTCTG			
A. marginale	*msp4*	AmargMSP4Fw	CTGAAGGGGGAGTAATGGG	60	344	Torina et al. 2012
		AmargMSP4Rev	GGTAATAGCTGCCAGAGATTCC			
A. ovis	*msp4*	MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	55	869	De La Fuente et al. 2005
		MSP43	CCGGATCCTTAGCTGAACAGAATCTTGC			

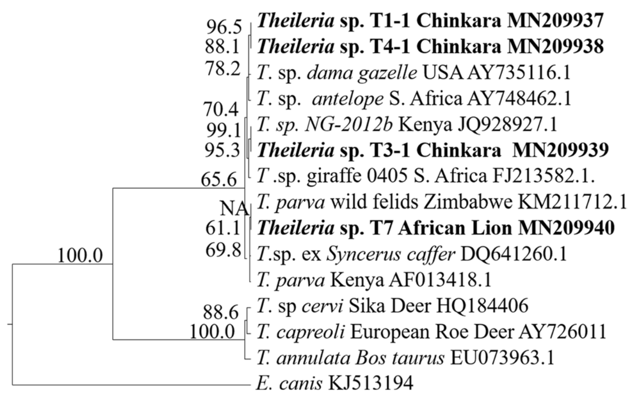

Fig. 1 Phylogenetic analysis of the *Theileria* species identified in this study based on the 18S rRNA gene. *Ehrlichia canis* was used as outgroup. Boldface indicates the sequences obtained in this study

Nucleotide sequence accession numbers

The representative sequences of the identified pathogens in this study were deposited in the GenBank database and assigned accession numbers as follows: MN209937 and MN209938 for 18S rRNA gene sequences of *Theileria sp. dama gazelle*; MN209939 and MN209941 for 18S rRNA gene sequences of *Theileria* sp. *NG-2012b*; MN209940 for 18S rRNA gene sequences of *Theileria parva*; and MN213735 for 16S rRNA gene sequences of *A. bovis*.

Results and discussion

Among 22 blood samples collected from 22 tick-infested captive wild animals, five were detected positive for the presence of tick-borne pathogens, with one mixed infection of Piroplasm and *Anaplasma* sp. Piroplasm DNA was detected in five captive wild animals included in this study by nested PCRs that amplified partial 18S rRNA gene sequences (approximately 430 bp) of *Babesia/Theileria* spp. (Table 1). All obtained amplicons were sequenced, and BLAST analysis revealed that the obtained sequences belonged to *Theileria* species. None of the animals tested positive for *Babesia* (Table 1). The sequence analysis of all positive samples was based on 18S rRNA sequences and revealed the presence of three species of *Theileria* species in selected captive wild animals. *Theileria sp. dama gazelle* was found in two chinkara and *Theileria sp. NG-2012b* in chinkara and a giraffe. *Theileria parva* was detected in African Lion (Table 1). Sequence analysis revealed that the 18S rRNA sequences of *Theileria* sp. *dama gazelle* detected in this study in two chinkaras were 100% identical. These were 98.12% identical to the Texas dama gazelle (AY735116) isolate of *Theileria sp. dama gazelle* identified in dama gazelle from the USA (Fig. 1). The 18S rRNA sequences of *Theileria sp. NG-2012b* from a giraffe showed 99.4% similarity with *Theileria sp. NG-2012b* isolate 115 (JQ928929) in Giraffe from Kenya (Githaka et al. 2013). *Theileria parva* found in the African Lion in this study was 99.3% identical to *T. parva* from Cheetah (M211712) and 99.1% similar to *Theileria.* Sp. Ex *Syncerus caffer* detected in African Buffalo (DQ641260.1), respectively (Fig. 1). To verify the particular species of *Theileria* identified in this study, a 1410 bp fragment for the 18S RNA of *Theileria sp. NG-2012b* was amplified, but none amplified for *Theileria sp. dama gazelle* and *T. parva*.

Most *Theileria* spp. infect domestic animals, but increasing evidence suggests that some *Theileria* spp. are identified in unexpected hosts, particularly wild animals (Mans et al. 2015). In this study, *Theileia parva* was identified in African Lion; *Theileria sp. dama gazelle* and *Theileria sp. NG-2012b* were detected in chinkara and giraffe (Githaka et al. 2013). According to a study in Kenya, a blood smear from a giraffe contained a hemoparasite identified as *Theileria sp. NG-2012b* (Githaka et al. 2013). *Theileria sp. dama gazelle* was first identified from clinical cases of farmed dama gazelle from Texas, USA (Bendele 2004). Based on another molecular marker, the study grouped *Theileria* sp. identified from dama gazelle with different *Theileria* spp. in the phylogenetic tree. During the phylogenetic analysis, it appeared to be a previously unreported isolate and another species. There is no further information about the pathogenicity of *Theileria sp. NG-2012b* and *Theileria*

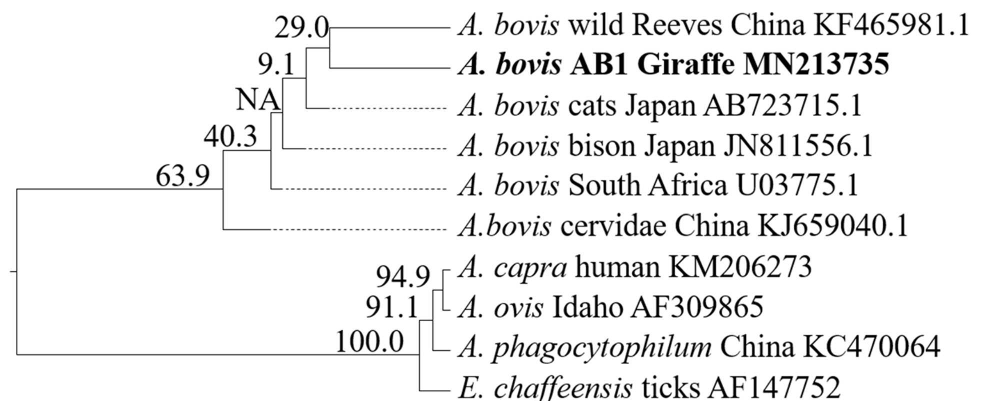

Fig. 2 Phylogenetic analysis of the *Anaplasma* species identified in this study based on the 16S rRNA gene. *Ehrlichia chaffeensis* was used as outgroup. Boldface indicates the sequence obtained in this study

sp. dama gazelle found in our study of giraffes and chinkara. *Theileria parva* is an apicomplexan parasite and causes East Coast fever in cattle, the most widespread tick-borne disease in Africa (de Castro 1997; Norval et al. 1991). Later, the disease spectrum expanded from domestic ruminants, and infection was detected in two lions in Zimbabwe (Kelly et al. 2014). It was also closely related to *T. sinensis* detected in yaks in China (Liu et al. 2013). The *Theileria parva* we identified in this study was less similar to *T. sinensis* and more identical to *Theileria* sp. ex Syncerus caffer MCO-2011 (HQ895982.1) detected from African buffalo in South Africa (Chaisi et al. 2011).

In this study, *Anaplasma bovis* was detected in a giraffe (Table 1). The 16S rRNA sequences of *A. bovis* obtained from the positive sample were 99.82 to 100% identical to the *A. bovis* isolates 2ax (KJ659040), Obihiro-bison (JN811556), and 2-44Ab (KF465981) derived from sika deer, American Bison, and reeves muntjac (Fig. 2). All animals included in this study were tested negative for *A. ovis*, *A. marginale*, and *A. phagocytophilum*. The co-infection of *Theileria* spp. and *Anaplasma* spp. was found in the giraffe (Table 1). *Anaplasma bovis* is an etiological agent of bovine anaplasmosis and an obligate parasite of monocytes (Donatien and Lestoquard 1936). Frequent reports document it as a domestic ruminant pathogen from bovine in Asia, Latin America, and Africa (Liu et al. 2012; Ogata et al. 2021; Peter et al. 2020). But recently, *A. bovis* was confirmed from wild canids in Korea (Kang et al. 2018) and Red deer and Sika deer in China (Li et al. 2016). In the present study, the detection of *A. bovis* in a giraffe indicates the concern that giraffes have the potential to act as a reservoir host for *A. bovis*, which requires further investigation.

Conclusion

This study is the first report from Lohi Bher Zoo, Pakistan, presenting tick-borne pathogen detection in captive wild animals from this particular setting. Three *Theileria* species (*Theileria sp. dama gazelle*, *Theileria sp. NG-2012b*, and *T. parva*) and an *Anaplasma* sp. (*A. bovis*) were identified molecularly in the investigated animals. This molecular investigation is the first to document *Theileria sp. dama gazelle* and *Theileria sp. NG-2012b* in Pakistan. A zoo generally has a diverse collection of exotic animals, holding a high concentration and large variety of animal species that require special attention. The animals are kept in a relatively small area, which increases the risk of spreading diseases. Probably, because of poor zoo practices and lack of active surveillance, a giraffe and an African lion tested positive for the non-native species. It indicates the possibility that it can be an emerging transboundary animal pathogen.

We stress that wild animals can play a critical role in the life cycle of these tick-borne pathogens and can act as reservoir hosts for the subsequent spread of these disease-causing agents. The findings also highlight the importance considering the human and domestic animal dimensions of captive wildlife management for effective wildlife management to reduce the risk of tick-borne diseases. These findings can enhance awareness of TAD trends while facilitating the prevention of TADs transmission in zoos through animal handling and management decisions, which are still underrepresented in zoo settings in particular and in the veterinary healthcare system in general. Due to limited resources, studies exploring the epidemiological role of these captive wild animals in the transmission of vector-borne pathogens, in general, are lacking. Therefore, further studies to understand their epidemiological role would be beneficial. The study emphasizes a need for a multi-faceted approach combining capacity building and training, wildlife disease surveillance, disease ecology studies, data sharing between zoos and outbreak investigation in zoos could prove beneficial.

Author contributions MUM and NI performed the experiments and data analysis and drafted the manuscript. ZN participated in the sample collection. JY and TLP designed this study and critically revised the manuscript. The authors read and approved the final manuscript.

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Data availability Sequences are submitted in the GenBank database under accession numbers: MN209937–MN209941 for 18S rRNA and MN213735 for 16S rRNA.

Declarations

Ethics approval and consent to participate Animal treatments and sample preparation complied with the Animal Ethics Procedures and Guidelines and were approved by the Animal Ethics Committee of Lohi Bher zoo.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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