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Molecular characterization of *Hepatozoon* sp. and *Babesia* sp. isolated from endangered Asiatic lion (*Panthera leo persica*)

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ABSTRACT

Hemoparasitic infections are frequently encountered in wild carnivores. Although, mostly, the infections are typically asymptomatic, they can be pathogenic under certain circumstances, viz. concurrent disease and stress due to captivity, habitat degradation, adverse climatic conditions or immunosuppression. The present study was undertaken to genotype *Babesia* sp. and *Hepatozoon* sp. isolated from lions of Lion Safari, Etawah, Uttar Pradesh, India and establishing phylogenetic relationship based on 18S rRNA sequence with other isolates around the globe. Blood samples of five Asiatic lions, received in the Clinical and Wildlife Parasitology Laboratory, Division of Parasitology, Indian Veterinary Research Institute, were screened microscopically for any haemoparasitic infection. Out of five, one sample was positive for *Hepatozoon* sp. and another sample was positive for *Babesia* sp. Polymerase chain reaction of 18S rRNA with genomic DNA amplified 1775 bp and 1665 bp segments for *Hepatozoon* sp. and *Babesia* sp., respectively. Sequencing of PCR amplicon and BLAST analysis indicated that *Hepatozoon* spp. in Asiatic lion was 99% similar to *Hepatozoon felis* isolate of Spain and *Babesia* spp. like organism was 95% similar to *Babesia canis* 18S ribosomal RNA gene of Israeli cat and *Babesia canis canis* of domestic dogs. Based on the literature available in public domain and the findings of present study, it can be concluded that these haemoprotozoa are not restricted to their respective hosts, and more than one genotype can be found in the same habitat. Cryptic babesiosis and hepatozoonosis can flare up in immuno-compromised animals and may result into fatal consequences in endangered Asiatic lion.

Key words: Asiatic lion, *Babesia*, *Hepatozoon*, Phylogeny, Polymerase chain reaction

The Asiatic lion (*Panthera leo persica*) is a magnificent feline which exists as single population in and around Gir forests of Gujarat. Most wildlife species commonly encounter hemoparasitic infections with *Babesia*, *Hepatozoon*, *Ehrlichia* and *Mycoplasma* (Rar and Golovijova 2011). Although these infections are typically asymptomatic, they can be pathogenic under certain circumstances, viz. unnatural hosts, stress due to captivity, habitat degradation, adverse climatic conditions and immunosuppression (Penzhorn 2006, East *et al.* 2008).

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Hepatozoon, which normally causes asymptomatic infections in carnivores, is suspected to have caused mortality in juvenile spotted hyenas in Tanzania (East *et al*. 2008). There is lot of ambiguity regarding identification of *Hepatozoon* species in field and its vectors. Some researchers have reported *Hepatozoon felis* (Tabar *et al*. 2008); whereas, in some cases, *H. canis* has been incriminated as the causative agent of feline hepatozoonosis (Jittapalapong *et al*. 2006). Babesiosis can range from an asymptomatic or mild infection to a severe illness depending on the virulence of the infecting *Babesia* species and the susceptibility of the individual host. Criado-Fornelio *et al.* (2003) provided initial molecular evidence for infection by *Babesia canis canis* in cats. The arrival of molecular diagnostic methods has led to the discovery of some new piroplasmids as well as *Hepatoozoon*-related organisms in the last few years (Vincent-Johnson *et al.* 1997, Penzhorn *et al.* 2001). The use of such methods has prompted a better knowledge of epizootiology in quantitative terms, but as sequencing of isolates is seldom undertaken, qualitative aspects remain less well understood. Hence it is imperative to exemplify these parasites particularly when it concerns

the endangered species like Asiatic lion. Therefore, the present study was undertaken for genotyping of *Babesia* sp. and *Hepatozoon* sp. infecting lions at Lion Safari, Etawah and establishing phylogenetic relationship with other isolates around the globe, based on 18S rRNA sequence.

MATERIALS AND METHODS

Parasite and isolation of genomic DNA: Blood samples were taken from 5 Asiatic lions after properly anaesthetizing during routine health check up. Genomic DNA was isolated from 300 µl of EDTA anticoagulated whole blood using Genomic DNA Mini Kit (IBI Scientific, USA) following manufacturer's protocol and stored at –20°C for future use. The blood samples were also examined microscopically for the presence of parasite.

Polymerase chain reaction amplification, cloning and sequencing: In order to amplify the partial 18S rRNA sequence, TGGTTGATCCTGCCAGTA and CTTCTC-CTTCCTTTAAGTGA primers sequences were used. The PCR reaction, cloning and sequencing was carried out as per Mandal *et al.* (2014).

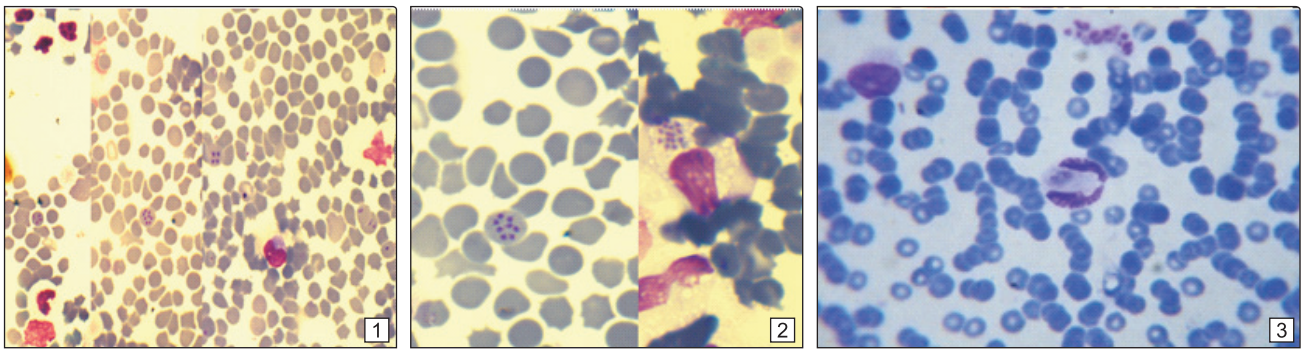
Sequence analysis: In order to analyze sequence variations, the newly generated sequences of 18S rRNA gene of *Hepatozoon sp.* and *Babesia canis* like organism were compared with published sequences in the nucleotide database in GenBank by BLAST program of the National Center for Biotechnology Information and Megalign in DNASTAR.

Phylogenetic analysis: Phylogenetic analyses of the 18S rRNA gene of generated sequences as well as other similar sequences from different hosts available in the GenBank, was independently done using the MEGA6 (Tamura *et al.* 2013). A total of fifteen 18S rRNA gene sequences were used in the analysis for *Hepatozoon* sp. (depicted in the figure as Query) while 15 nucleotide sequences were used for *Babesia canis* like organism (depicted in the figure as Query).

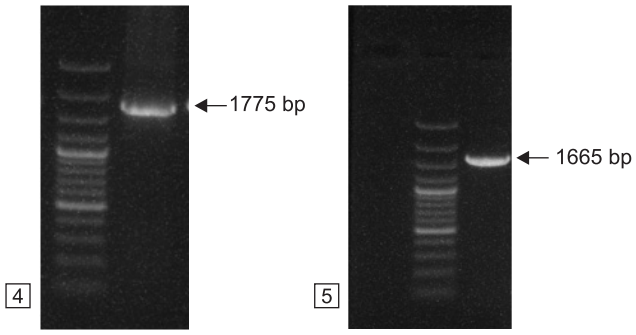
RESULTS AND DISCUSSION

Arthropod borne diseases have emerged as major pathogens over few decades due to ecological and climatic changes (Shaw *et al.* 2001). Extensive wildlife conservation attempts involving capturing of wild animals for establishment of new populations or translocation to safer places create conditions favourable for development of arthropod borne disease like babesiosis, hepatozoonosis etc. In the present study, thin blood smear examination revealed presence of haemoprotozoon infection in two samples out of 5 samples examined. One of the samples was positive for *Hepatozoon* sp. while the other was harbouring *Babesia canis* like organism (Figs 1, 2, 3).

Although, *Hepatozoon* infections in wildlife are normally subclinical, high prevalence has been reported from numerous free-ranging African carnivores (East *et al.* 2008). In India, barring few reports (Pawar *et al.* 2012), literature on feline and canine hepatozoonosis in Indian wildlife is totally lacking. Canine hepatozoonosis has been reported in domestic dogs (Pawar and Gatne 2005), in most cases however, subclinical infections occurred with a prevalence ranging from 3% to 9% (Sharma *et al.* 1997, Singh *et al*. 2017). *Babesia* and Canine distemper coinfections have caused severe mortality in African lions (Munson *et al.* 2008). There are several reports of babesiosis infecting wild animals from all over the globe (Solano-Gallego and Baneth 2011). Few reports of sporadic occurrence of babesiosis in wild carnivores from India are also available (Haque 2007, Mishra *et al.* 2008). Most of the times, parasites were identified by blood smear examination. However, numerous variants and cryptic species make the diagnosis erroneous. Therefore, PCR amplification of 18S rRNA was carried out and the amplified products resolved into 1775 bp and 1665 bp for *Hepatozoon* sp. and *Babesia canis* like organism respectively (Figs 4,5), after agarose gel (1.5%) electrophoresis. Subsequently, 18S rRNA genes were custom sequenced. The sequence of the 18S rRNA gene (1665 bp) generated in this study was submitted to GenBank (KY026486). Sequence similarity searches in BLAST revealed that newly generated sequence of 18S rRNA gene was similar (95% identity) with the published sequences of *Babesia canis* 18S ribosomal RNA available in GenBank. A closer comparison of the sequence after alignment revealed that it shared 95% identity with the sequences of *Babesia canis* 18S ribosomal RNA (L19079.1), *Babesia canis* 18S ribosomal RNA gene, partial sequence from cat



Figs 1–3. **1**. *Babesia sp.* in the blood smear of a lioness (×1000). **2**. a. *Babesia* sp. in the blood smear of a lioness (×2000); b. Infected erythrocyte engulfed by a macrophage. **3**. *Hepatozoon felis* gamont inside neutrophil of a lioness (×1000).



Figs 4–5. **4**. PCR amplification of *Hepatozoon* sp. (1775 bp). **5**. PCR amplification of *Babesia* sp. (1665 bp) .

(Israel) (AY272047.1), Italy (KX839231.1) and *Babesia canis canis* 18S ribosomal RNA gene, partial sequence from Croatia (AY072926.1). The generated sequence was also similar (95%) to *Babesia canis vogeli* gene for small subunit rRNA, partial sequence from Japan (AB083374.1), China (HM590440.1), Venezuela (DQ297390.1) and Italy (AY072925.1). Similarly, the sequence of 18S rRNA gene of *Hepatozoon* sp. (1770 bp) generated in this study was submitted to GenBank (KX017290). Sequence similarity searches in BLAST revealed that newly generated sequence of 18S rRNA gene was similar (99% identity) with the published sequences of *Hepatozoon felis* isolate Spain (AY628681.1, AY620232.1). The sequence was also found to be similar (97% identity) to *Hepatozoon* sp. European pine marten 1 18S ribosomal RNA gene, partial sequence (EF222257.1) and *Hepatozoon canis* isolate fox 1-2 18S ribosomal RNA gene, partial sequence (KU893118.1). Murugesan *et al.* (2017) reported 100% identity of *H. canis* from Namakkal, Tamil Nadu with Hungary isolate.

Recent genetic characterization has revealed that numerous variants, subspecies, or cryptic species of *H. canis* exist worldwide (Starkey *et al.* 2013). Similarly, subspeciation of *B. canis* was subsequently supported by DNA analyses indicating genetic dissimilarity between the subspecies (Carret *et al.* 1999). A recent PCR study on wild felids and canids in India detected *H. felis* only in felids (Lions, Tigers and Leopards) and *H. canis* only in canids (domestic dogs and Indian wild dogs) (Pawar *et al.* 2012); however, these parasites are not considered to be specific to the suborders of Carnivora and *H. canis* and *H. felis* have been reported in both canids and felids (Baneth *et al.* 2013). The phylogenetic tree of 1775 bp region of 18S rRNA gene is presented in Fig. 6. In this tree, all isolates of *Hepatozoon canis* formed a separate major clade with high boot strap value (97%) indicating monophyletic nature of all the *Hepatozoon canis* isolates except *Hepatozoon canis* Spain 2 isolate. Another major clade comprised *Hepatozoon felis* parasitizing Asiatic lion and Indian leopard. However, *Hepatozoon felis* from domestic cat and Bengal tiger moved away from the Indian clade. *Hepatozoon felis* from Asiatic lion in the present study also didn't occupy the Indian clade and formed parallel clade with *Hepatozoon felis* from Bengal Tiger (Boot strap value 73%). *Hepatozoon felis* from Spain occupied a separate clade in the phylogenetic tree. The phylogenetic tree based on 1665 bp region of 18S rRNA gene is presented in Fig. 7. In the tree representing phylogeny of *Babesia canis* like organism, *Babesia canis canis* and *Babesia canis vogeli* formed separate clades altogether. *Babesia canis* like organism formed a separate major clade with *Babesia canis vogeli* and most of the

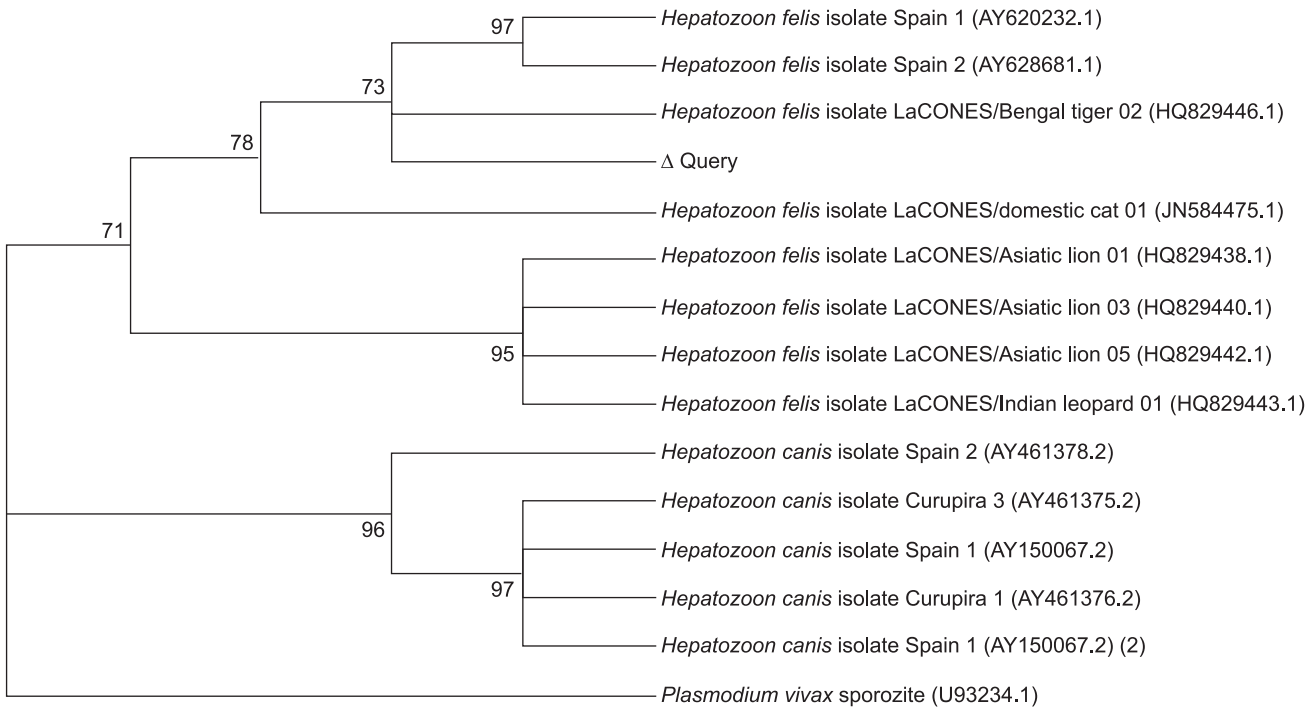


Fig. 6. Phylogenetic tree of *Hepatozoon* sp. isolated from a lioness.

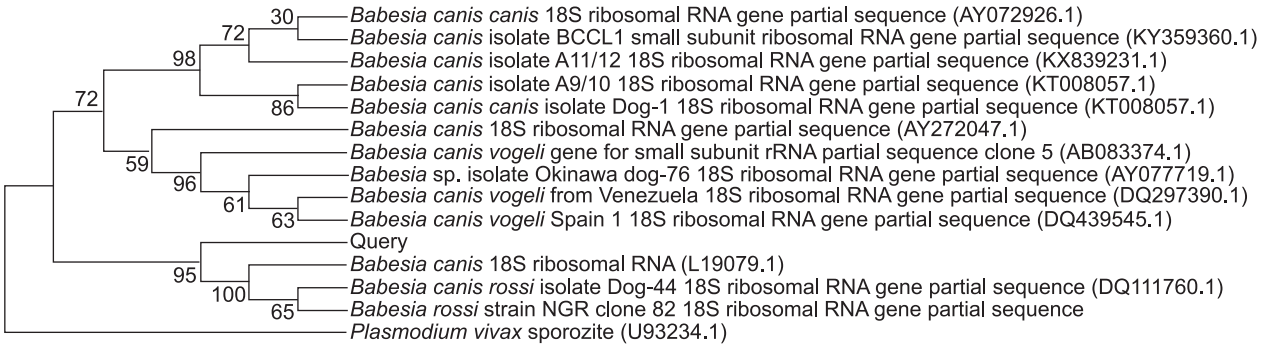


Fig. 7. Phylogenetic tree of *Babesia* sp. isolated from a lioness.

Babesia canis under study. However, it formed a minor separate clade with *Babesia canis rossi* with a bootstrap value of 95% and had less base substitutions with Babesia *canis rossi* in comparison to other sequences under the study.

The findings of the present study revealed that there is considerable variation in the genotype of *Hepatozoon* and *Babesia* parasites infecting wild carnivores in India. On the basis of sequence homology and phylogenetic analysis of 18S rRNA, the isolates of *Hepatozoon* and *Babesia* differed considerably with the isolates reported by Pawar *et al.* (2012). However, sequence similarity between different isolates is not a rule of law. In fact, at a particular place, two different genotypes were present in different host species indicating that wild and domestic animals in India are parasitized by more than one species, or genotype (Pawar *et al.* 2012). Although *H. felis* and *H. canis* are closely related genetically (Criado-Fornelio *et al.* 2006), they appear to target different host tissues. Not much is known about the arthropod vectors of *H. felis*, its transmission in general, and whether it exclusively infects domestic cats or can also infect other mammalian hosts. Due to dearth of genotyping reports regarding wild feline and canine haemoprotozoons in India, it's difficult to draw conclusions about phylogeny and diversity of genotypes present in India. Any pathogen that leads to immunosuppression may increase susceptibility to vector-borne pathogens such as *Hepatozoon, Babesia* etc. potentially leading to increased mortality. Identification of potential pathogens and the development of mitigation strategies to decrease their potential impact should be an important part of ongoing conservation efforts for endangered species.

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