Molecular Identification of a Novel Species, Babesia panickeri *sp. nov., from a Naturally Infected Domestic Cat of India and Its Comparison with Canine* Babesia *Isolates*

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ORIGINAL PAPER

Molecular Identification of a Novel Species, *Babesia panickeri* sp. nov., from a Naturally Infected Domestic Cat of India and Its Comparison with Canine *Babesia* Isolates

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Abstract

Introduction Babesiosis is a tick-borne hemo-parasitic disease of domestic and wild animals. Parasites causing babesiosis are considered to infect only specific hosts but some sporadic reports in recent past are in strong disagreement with their host specificity. This is the first report of a domestic cat being naturally infected with a novel *Babesia* sp. in India.

Methods Blood samples collected from dogs ($n=6$) and a 3-month-old cat, with clinical symptoms of babesiosis, were submitted to two different laboratories for hematology analysis, light microscopical examination, and molecular confirmation of *Babesia* sp. using PCR, sequencing, and phylogenetic analysis.

Results Hematological alterations noticed in canine and feline samples were severe anemia and thrombocytopenia. Pear-shaped merozoites were visualized on light microscopic examination of both canine and feline blood smears. Size of the merozoites in feline blood sample was smaller when compared to canine samples. Molecular analysis using *Babesia* species-specific primers showed that all canine samples were positive for *B. vogeli* and feline sample was negative for *B. canis*, *B. rossi*, and *B. vogeli* infecting dogs. Amplification and sequencing of full-length ssrRNA using universal apicomplexan primers followed by molecular and phylogenetic analysis revealed that the Indian domestic cat was infected with a novel *Babesia* sp.

Conclusion This work presents the first molecular and phylogenetic evidence of a novel *Babesia* sp. causing feline babesiosis in a naturally infected domestic cat in India. We propose to name this novel species as *Babesia panickeri* sp. nov.

Keywords Babesiosis · ssrRNA gene · Indian feline isolate

Introduction

Babesiosis is a tick-borne hemo-parasitic disease caused by an apicomplexan parasite *Babesia* with its worldwide occurrence [2]. *Babesia* spp. have a broad range of hosts including mammals, birds and reptiles [4]. These intra-erythrocytic piroplasms primarily infect and multiply within the erythrocytes causing hemolytic anemia [12]. Babesiosis in domestic cat is caused by multiple babesial species including small babesial organisms like *B. felis* and *B. cati* (1.0–2.5 µm) and large babesial organism like *B. herpailuri* and *B. pantherae* (2.5–5.0 µm) [19, 29]. In the recent past, sporadic incidence of *Babesia* infection in domestic cats by unidentified species of *Babesia* was reported from different parts of the world [15, 23, 33]. Feline babesiosis caused by *B. felis* was first presented as clinical babesiosis from Africa in 1937 and was wide spread in coastal areas [8, 13, 22]. *Babesia cati* is the only species of *Babesia* reported from domestic cats in India [24]. Till date, most of the reports on feline babesiosis from India were based only on morphological identification of the piroplasms in blood smears [4, 20]. Vectors transmitting babesiosis in domestic cats are still unidentified [12]. Clinical signs in feline babesiosis are lethargy, weakness, and anorexia [14]. Domestic cats infected with canine *Babesia* spp. were reported from different countries like Spain and Portugal (*B. canis*), Israel (*B. canis presentii*), Portugal

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(*Babesia microti*-like sp.), Poland (*Babesia canis*-like species), and Thailand (*B. vogeli*) [3, 7, 27, 31, 36].

Babesia gibsoni (small form < 1.5 µm), *B. canis*, *B. rossi*, and *B. vogeli* (large form > 2.5 µm) are the major species causing babesiosis in canines [6, 32, 39]. *Babesia canis* distributed in Europe causes disease with variable degrees of hemolytic anemia, and is transmitted by the tick *Dermacentor reticulatus* [35]. *Babesia rossi* found in South Africa causes severe clinical illness in dogs and is transmitted by *Haemaphysalis leachi* [28]. *Babesia vogeli* distributed in tropical and subtropical countries causes mild to moderate clinical symptoms in dogs and is transmitted by *Rhipicephalus sanguineus* s.l., commonly called as brown dog tick [21]. Since all these large *Babesia* have morphologically similar merozoites, it is not possible to classify these organisms based on their morphology alone. Reports on genetic characterization of canine piroplasms using ssrRNA showed that in addition to *B. gibsoni*, some other small forms of *Babesia* can also cause clinical babesiosis [16, 17, 37, 38]. The abovementioned studies suggest that molecular characterization of ssrRNA is a method of choice for the identification of the piroplasm and its phylogenetic analysis.

In the present study, we report the clinical manifestations, morphology, and complete ssrRNA gene analysis of a novel feline *Babesia* sp. isolate and its comparison with that of canine isolates.

Materials and Methods

Collection of Clinical Samples and Hematology

EDTA-blood samples collected from dogs ($n = 6$) and one 3-month-old domesticated cat suspected positive for babesiosis were submitted to Molecular biology lab, Teaching Veterinary clinical complex, Mannuthy, Kerala, India, and Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences (CoVAS), Mannuthy, Thrissur. Dogs and cat were presented with clinical signs of anorexia and lethargy. Blood samples collected were stored at 4 °C and processed within 12–24 h for analyzing hematological parameters using a hematology analyzer (Orphee Mythic-18, Switzerland). Field's stained blood smears were prepared and examined under Labomed microscope with 100X oil immersion objective. Molecular confirmation was carried out using canine samples and the results were compared with that of feline isolate.

DNA Isolation and Molecular Identification

Genomic DNA was isolated from 100 µL blood of each dog and cat, confirmed positive for *B. canis* infection on microscopic examination, according to the manufacturer's instruction using DNeasy® blood and tissue kit (Qiagen, Germany). DNA was eluted in 200 µL of Tris–EDTA buffer and stored at − 20 °C until further study. Concentration of the eluted DNA was measured using a NanoDrop™ 2000 C spectrophotometer (Thermo Fisher Scientific, USA). All the DNA samples were screened for *B.canis*, *B. rossi*, and *B. vogeli* using following forward primer: BAB1- 5′-GTG AAC CTT ATC ACT TAA AGG-3′ and reverse primers: BAB3- 5′-CTA CAC AGA GCA CAC AGC C-3′ (*B. canis*), BAB5- 5′-AGG AGT TGC TTA CGC ACT CA-3′ (*B. rossi*) and BAB4- 5′-CAA CTC CTC CAC GCA ATC G-3′ (*B. vogeli*) targeting regions between 18S rRNA and 28S rRNA genes. Amplification was carried out in an automated thermal cycler (BIO-RAD T100™, USA) following PCR cycle parameters as reported earlier [10]. Amplification of full-length ssrRNA gene was carried out in cat sample using universal apicomplexan primers-5′-AAC CTG GTT GAT CCT GCC AGT AGT CAT-3′ (forward primer) and 5-GAA TGA TCC TTC CGC AGG TTC ACC TAC-3 (reverse primer) by following the same reaction conditions as reported by Caccio et al. [5].

Sequencing and Phylogenetic Analysis

Amplicons obtained after PCR amplification were gel-extracted and purified using QIAquick Gel Extraction Kit (Qiagen, Germany) as per the manufacturer's protocol. Sequencing was done using sequencing services available at Agri Genome Labs Private Ltd., Cochin, Kerala, and the sequences were aligned with published sequences in the public database using the programme Clustal W [34]. Identity search of the sequences was carried out using BLAST program of NCBI (https://www.ncbi.nlm.nih.gov/BLAST) and the unique sequences thus obtained were submitted to GenBank database. Sequences of different species of *Babesia* and *Cytauxzoon felis*, used for sequence alignment and phylogenetic analysis, were obtained from GenBank database (www.ncbi.nlm.nih.gov). Phylogenetic analysis was done with MEGA seven software [18] and tree was constructed using all the different options available in the software. Stability of tree was assessed using bootstrap value of 1000 replicates.

Results

Hematology

Hematological abnormalities noticed in the cat were severe macrocytic hypochromic anemia and thrombocytopenia [red blood cells 2.27×10^6/µL (normal range 5.92–9.93×10^6/µL), hemoglobin concentration 4.1 g/dL (normal range 9.3–15.9 g/dL), hematocrit 13.8% (normal range 29–48%),

platelet count 25×10^3/µL (normal range 200–500×10^3/µL), MCV 64 fL (normal range 37–61 fL), MCHC 28% (normal range 30–38%)]. Total and differential leukocytes showed no characteristic difference in their normal values. Major hematological alterations noticed in canine samples were severe anemia and thrombocytopenia [red blood cells-$3 \pm 0.90 \times 10^6$/µl (normal range 4.8–9.3×10^6/µL), hemoglobin concentration 5.53 ± 0.96 g/dL [normal range 12.12–20.3 g/dL), hematocrit 17.21 ± 2.65% (normal range 36–60%), platelet count $96.33 \pm 75.92 \times 10^3$/µL (normal range 170–$400 \times 10^3$/µL), MCV 59.71 ± 11.10 fL (normal range 58–79 fL), MCHC 32.15 ± 3.65% (normal range 30–38%)].

Taxonomic Review

Babesia panickeri sp. nov.

Type-host Cat, *Felis catus* (Carnivora, Felidae, Felinae).

Type-locality Mannuthy, Thrissur, Kerala, India.

Vector Currently unkown.

Etymology The species is named after M.N Purushothama Panicker father of the first author.

ZooBank reference numbers Pub: D60FFE7F-CDBD-497A-BD99-1D0313829DAD and act: 6D30B774-5400-48FE-845A-F3279459FADF.

Light Microscopic Examination

Pear-shaped merozoites were visualized on microscopic examination of both cat and dog blood smears (Fig. 1). Of the 50 parasitized erythrocytes examined in the cat, 15 merozoites (30%) were single form, 31 (62%) were in pairs and the rest of the erythrocytes contained up to four merozoites in a single cell. Densely stained disc-shaped nuclei of the merozoites were seen towards the distal pole.

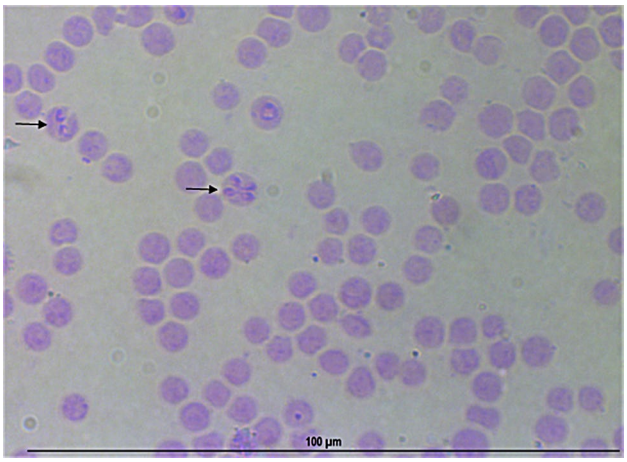

Fig. 1 Merozoites of *Babesia panickeri* sp. nov. in Indian feline blood sample

Mean size of the merozoites observed in cat blood smear was 2.7 ± 0.30 µm by 1.3 ± 0.21 µm (range 3.6—1.9 µm by 1.7–1.02 µm). Size of the merozoites was smaller when compared with that of dog. The samples which were positive on microscopic examination were later sent to two different labs for molecular identification.

Molecular Identification of the *Babesia* spp.

Dogs with clinical babesiosis were confirmed positive for *B. vogeli* infection by species-specific PCR. All the six sequences showed 100% sequence similarity between the sequences and BLAST search revealed 99.62% identity with *B. vogeli* sequences isolated from Delhi (Accession no. MN165668). Cat sample did not produce any product on PCR with primers specific for all three *Babesia* spp. Hence, full-length ssrRNAgene of the *Babesia* seen in cat was amplified with apicomplexan primers. Single amplified product of approximate sequence length 1845 bp was obtained and the sequence information was deposited in GenBank database (Accession no. MN267560). On comparison of the submitted sequences, highest similarity score (99.1%) was observed with *Babesia* sp. isolated from Indian lion (*Panthera leo persica*) (Accession no. KY026486), followed by *B. rossi* (96%) isolated from dogs of South Africa (Accession no. DQ111760), *B. canis presentii* (94.62%) isolated from domestic cats of Israel (Accession no. AY272047), *B. canis* (94.61%) isolated from European dogs (Accession no. AY072926) and *B. vogeli* (94%) isolated from Italian dogs (Accession no. AY072925). Since 100% sequence similarity could not be found with any of the *Babesia* spp. sequences in the public databases, it is presented as a novel *Babesia* sp. infecting domestic cat in India.

Phylogenetic Analysis

Sequences selected for phylogenetic analysis were trimmed to remove all the gaps and ambiguous characters. Phylogenetic trees having same topologies were obtained using all the tests (Maximum Likelihood Tree, Neighbour-Joining Tree, Minimum-Evolution Tree, UPGMA Tree and Maximum Parsimony Trees) available in the software. *Cardiosporidium cionae* was selected and kept as outgroup for the tree construction. *Babesia panickeri* sp. nov. isolated from domestic cat formed a single clade with all other large babesial forms in carnivores, which is a sister clade to small form of *Babesia* in dogs, *B. gibsoni*. A representative tree for ssrRNA sequence obtained using Tamura 3-parameter (I+G) model of Maximum Likelihood method of analysis is presented in Fig. 2.

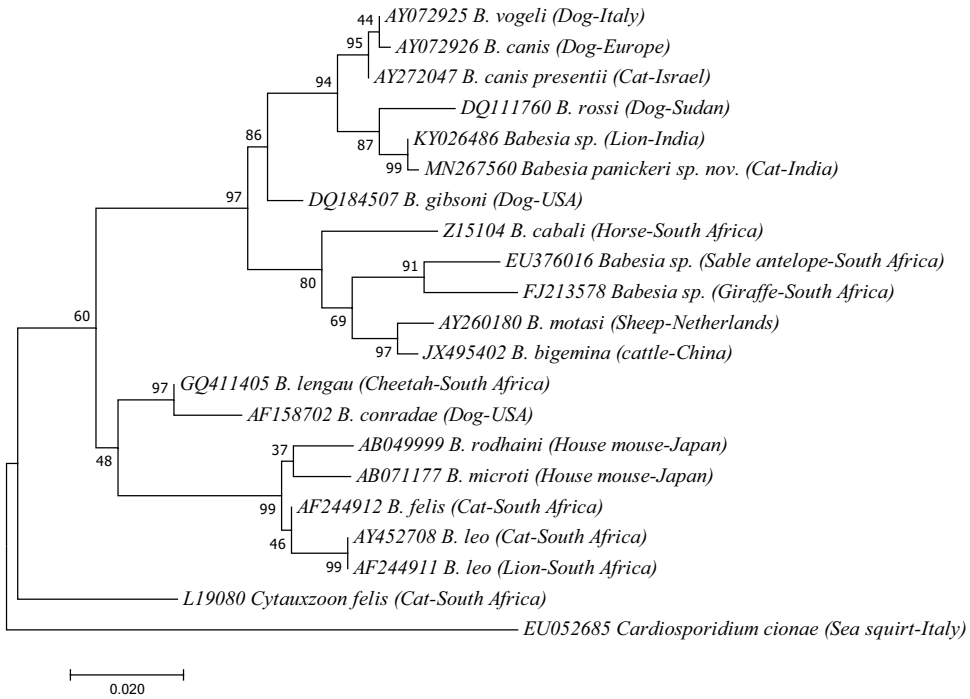

Fig.2 Phylogenetic tree was generated on the basis of full-length ssrRNA sequences of Indian feline isolate and sequences of related species retrieved from public database. Tree was constructed using maximum likelihood method by keeping *Cardiosporidium cionae* as outgroup and evolutionary history was inferred based on Tamura 3-parameter (I + G) model. Scale bar represents the evolutionary distance of 0.02 and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 21 nucleotide sequences and all the positions containing gaps and missing data were eliminated

Discussion

Domestic cat with natural infection of babesiosis in the present study was only 3 months of age and this age predisposition was similar to other studies in felines, where natural infection of babesiosis was reported below 2 years of age [11]. The cat presented to the veterinary clinic was afebrile and anemic as observed in previous reports [11, 14]. Intra-vascular and extra-vascular hemolysis associated with feline babesiosis could have led to anemia and cats have the ability to adapt with severe anemia [29]. Even though severe thrombocytopenia was noticed in the present case, it is not considered as a consistent finding in earlier reports on feline babesiosis [26]. Thrombocytopenia recorded using automated methods may be misleading since the platelets of cat have a tendency to form clumps when it is measured electronically. Therefore, platelet count was taken manually and thrombocytopenia was confirmed. High level of parasitemia was observed in domestic cats when compared to dogs with babesiosis. This is due to a combination of various factors such as resistance of cats towards endotoxin, comparatively less virulence of the parasite, and visibility of the parasitized RBCs since all parasitized RBCs are presumably circulating [29]. Compared to endotoxin-sensitive species like dog, endotoxin-resistant species will show clinical symptoms only when level of parasitemia is high [2]. Total and differential leucocyte counts were normal which could rule out the chances of concurrent infections.

Morphologically, the pear-shaped merozoites of this novel piroplasm share common characteristics with *B.canis, B. rossi,* and *B. vogeli* in dogs. Maximum only up to four merozoites could be observed in a single erythrocyte which is contrary to the earlier reports from India, where up to eight merozoites could be observed in a single erythrocyte of cats infected with *B. cati* [24]. Unlike canine *B. vogeli* (5 by 3 µm), the merozoites of the feline isolate (2.7 by 1.3 µm) were remarkably smaller. In agreement with earlier reports, the smaller size of the merozoites might be due to the difference in the size of the erythrocytes of cat and dog [3].

Babesia panickeri sp. nov. in Indian feline are larger in size like *B. herpailuri* (2.7 by 2.2 µm) and *B. panthera* (2.0 by 1.8 µm) [8] than most of the *Babesia* seen in felines reported from elsewhere, like *B. cati* (0.5–2.0 µm) [24], *B. felis* (0.9 by 0.7 µm) [8] and *B. leo* (1.05 by 1.0 µm) [25]. Hence, according to the Denning and Brockles classification of feline *Babesia* species based on size of merozoites [9], the present isolate from domestic cat comes under large *Babesia*. All the six dog samples were found to be infected with *B. vogeli*, which is in accordance with earlier report [1]. Traditionally, classification of the piroplasms is based on blood smear morphology and host specificity, which are proved less specific with the recent developments in antigenic and genetic analyses. Also, methods based on partial ssrRNA polymorphisms and internal transcribed spacers showed potential drawbacks for the identification of species. Hence, the present isolate was identified by the most appropriate method of molecular characterization of full-length ssrRNA genes and phylogenetic analysis [5].

Homology search, pairwise identity search and phylogenetic analysis showed that in the present case, Indian

domestic cat is infected with a novel *Babesia* sp. Phylogenetic tree was constructed using multiple *Babesia* spp. having different evolutionary history to increase its reliability. Even though *B. felis* and *B. leo* (small form) showed close sequence similarity and were segregated as a single clade, *B. panickeri* sp. nov. reported in this study had only 87.90% sequence similarity with *B. felis* (Accession no. AF244912) and 87.73% sequence identity with *B. leo* (Accession no. AY452708). The maximum relatedness of *B. panickeri* sp. nov. with *Babesia* sp. isolated from lion in India may be due to its common evolutionary history.

On comparison with various fully characterized *Babesia* sp., the present *Babesia* isolate showed close similarity with *B. rossi* in dogs, the highly virulent strain seen in South Africa. Further research is required to know the pathogenicity, virulence, treatment, vectors responsible for the transmission and presence of the identified *B. panickeri* sp. nov. in dogs or any other hosts. Vector responsible for the transmission of this Indian feline isolate is unknown even though *R. sanguineus* is the potential vector seen in the Indian subcontinent [30]. Till date, to the best of our knowledge, there are no reports on the molecular identification of a *Babesia* species infecting domestic cats in India. This is the first report from India giving molecular evidence on the occurrence of a novel *Babesia* sp. infecting domestic cats in India.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by institutional ethical committee, and all applicable institutional, national, and international guidelines for the care and use of animals were followed.

Informed consent Written informed consent was obtained from the owners for the collection of samples from the animals.

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