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NOTE

Parasitology

Identification and phylogenetic analysis of *Babesia* parasites in domestic dogs in Nigeria

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ABSTRACT. The present study examined the presence of *Babesia* parasites in 104 domestic dogs in Nigeria. Sequentially, *Babesia* parasites infecting domestic dogs underwent genetic and phylogenetic analyses. The results of nested PCR based on the Piroplasmida 18S rRNA gene illustrated that 13.5% (14/104) of the samples were positive. The obtained positive samples determined the nucleotide sequences of the 18S rRNA genes. In the genetic and phylogenetic analyses, four of five nucleotide sequences were similar to *Babesia canis rossi*, and one sample exhibited a close similarity to a *Babesia* sp. isolated from a raccoon in Hokkaido, Japan. The present study revealed the widespread presence of *B. canis rossi* among domestic dogs in Nigeria.

KEY WORDS: 18S rRNA, *Babesia canis*, domestic dog, Nigeria, phylogenetic analysis

Canine babesiosis is caused by the intra-erythrocytic protozoa of the genera *Babesia* belonging to the order Piroplasmida. *Babesia* classification in dogs is performed microscopically according to the parasite morphology in erythrocytes. Large *Babesia* parasites in canines are usually referred to as *Babesia canis*, whereas small *Babesia* parasites are classified as *B. gibsoni* [3, 8, 14, 25]. Recently, the detection and identification of Piroplasmida in infected domestic animals including dogs and wild animals was enabled by molecular techniques [1–3]. Moreover, the canine *Babesia* parasites include *B. annae*, which was initially classified in the genus *Theileria*, in domestic dogs in Spain [6], as well as *B. conradae* [13] and *Rangelia vitalii* [4, 5, 23]. *B. canis* has been classified into three subspecies by molecular methods: *B. canis canis*, *B. canis rossi*, and *B. canis vogeli* [3, 14]. *B. canis rossi* is the most pathogenic of the three subspecies, and it was first isolated from the side-striped jackal (*Canis adustus*) in Kenya [17]. This protozoan has been detected in sub-Saharan Africa, and it is known to be transmitted by ticks such as *Haemaphysalis leachi* [15–17, 21]. Conversely, *B. canis vogeli* has been detected in southern Europe, Asian countries including Japan, and South Africa [1, 16–18], and its virulence is weaker than that of the other two subspecies. *B. canis vogeli* is transmitted by *Rhipicephalus sanguineus* [15–17]. In Nigeria, the detected *Babesia* spp. include *B. canis rossi* and *B. canis vogeli*, as well as other tick-borne pathogens such as *Hepatozoon canis*, *Ehrlichia canis*, and *Rickettsia* spp. [1, 11, 21]. In addition, *Babesia* parasites have been found in dogs in Nigeria [10, 22], Sudan [16], Egypt [20], and South Africa [17], but reports about canine babesiosis in the African continent are sparse. In this study, we collected blood samples from 104 domestic dogs in 2013 to assess the presence of *Babesia* parasites. Sequentially, genetic and phylogenetic analyses of the 18S rRNA gene were performed using *Babesia* parasites obtained from domestic dogs.

In 2013, ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples were aseptically collected on Whatman FTA® classic cards (Whatman International Ltd., Maidstone, UK) from 104 domestic dogs that underwent rabies vaccination at the Veterinary Hospital of Federal University of Agriculture, Abeokuta (FUNAAB), in Nigeria. The dogs did not display any clinical signs of parasitemia. Samples were collected in accordance with the Guidelines for the Use of Experimental Animals. All procedures were reviewed and approved by the FUNAAB (approval number: FUNAAB/COLVET/CREC/202/1/09/02). FTA cards spotted with blood specimens from domestic dogs were used for DNA extraction using the phenol–chloroform–isoamyl alcohol (PCI) method [9]. Briefly, 100 µl of red blood cell (RBC) pellets were mixed with a 10-fold volume of DNA extraction buffer (0.1 M Tris-HCl, pH 8.0; 0.1 M NaCl; 10 mM EDTA containing 1% Sodium dodecyl sulfate (SDS)). The contents of the tube were

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mixed thoroughly and incubated at 55°C for 2 hr with 50 µg of proteinase K (10 mg/ml), followed by the addition of an equal volume of PCI. Finally, genomic DNA (gDNA) from the aqueous phase was precipitated with ethanol, and the pellet was dried and dissolved in 25 µl of TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). The dissolved gDNA samples were stored at −20°C until use in PCR.

The gDNA extracts from dog blood samples were first screened for the presence of *Babesia* and *Theileria* using previously reported primers [7], which target a 1,616-base pair (bp) portion of the 18S rRNA gene. The 18S rRNA gene was amplified using Piro0F (5′-GCCAGTAGTCATATGCTTGTGTTA-3′) and Piro6R (5′-CTCCTTCCTYTAAGTGATAAGGTTCAC-3′) for the first-round PCR and Piro1F (5′-CCATGCATGTCTWAGTAYAARCTTTTA-3′) and Piro5.5R (5′-CCTYTAAGTGATAAGGTTCACAAAACTT-3′) for nested PCR as previously described [7, 12]. All PCR products were isolated by 1.0% (w/v) agarose gel electrophoresis in TAE buffer and purified using a GENECLEAN kit (BIO 101, Vista, CA, USA). The 18S rRNA gene was successfully amplified from the gDNA of spotted blood samples using nested PCR (data not shown). The prevalence of Piroplasmida infection according to nested PCR using the aforementioned primer set for the 18S rRNA gene was 13.46% (14/104). Genetic and phylogenetic analyses were performed in all positive samples (n=14) by nested PCR. The 18S rRNA genes were directly determined using a CEQ8000 automated sequencer (Beckman Coulter, Inc., Brea, CA, USA) with the DTCS DNA Sequence kit (Beckman Coulter). DNA sequences were analyzed using Mac Vector software package, version 12.5.1, and their identity was confirmed by comparison to homologous sequences available in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). In a BLAST search analysis, 10 positive were closely related to the genus *Babesia*, and the remaining four were closely related to the genus *Hepatozoon*. We determined that the complete nucleotide sequence of the 18S rRNA genes from 10 samples (1,560 bp) with high homology to the genus *Babesia*, followed by genetic and phylogenetic analyses. Six of ten positive samples had identical nucleotide sequences, whereas the other four samples had different nucleoside sequences. We obtained a total of five positive samples. None of these sequences was identical to any sequence in public databases. In a BLAST search of GenBank, four of five 18S rRNA genes were found to be closely related to that of *B. canis rossi* (AB303075, identity: 99.7, 99.5, 99.7, and 99.5%, respectively) detected in dogs in Nigeria, and one positive sample (AB935162) was revealed to be closely related to *Babesia* sp. (AB251610, 99.5%), which was isolated from a wild raccoon in Hokkaido, Japan [12]. All positive samples of 18S rRNA genes were related to the *B. gibsoni* 18S rRNA sequence (AF271081, identity: 99.4%). We registered the nucleotide sequences of the 18S rRNA gene as *Babesia canis rossi* strain N1 (AB935163), *Babesia canis rossi* strain N2 (AB935164), *Babesia canis rossi* strain N3 (AB935165), *Babesia canis rossi* strain N4 (AB935166), and *Babesia* sp. Nigeria2014 (AB935162) in GenBank. These sequences were aligned using the CLUSTAL W Alignment program [24], and a phylogenetic tree was constructed by the neighbor-joining (NJ) method from the aligned sequences with the phylogenetic analysis in the Mac Vector software package, version 12.5.1. Support for tree nodes was calculated with 1,000 bootstrap replicates using the bootstrap tree algorithm [19]. The NJ phylogenetic tree constructed using the 18S rRNA gene sequences separated the *B. canis vogeli*, *B. canis canis*, *B. canis rossi*, *Babesia* sp. (raccoon), and *B. gibsoni* groups, respectively (Fig. 1). Four of the obtained 18S rRNA gene sequences (AB935163-AB935166) belonged to the clade of *B. canis rossi* (AB303075), which has been reported in Nigeria. Meanwhile, the remaining sample belonged to the clade of *Babesia* sp. (AB251610) isolated from Hokkaido, Japan (bootstrap value: 99%, Fig. 1). Two species of *Babesia* parasites, namely *B. canis rossi* and *B. canis vogeli*, have been detected in domestic dogs in Nigeria [1, 11, 21]. In a survey of canine babesiosis in Nigeria, the prevalence of *B. canis rossi* was 2.0% (8/400) in 2007 [21], 6.6% (12/181) in 2013 [11], and 38% (38/100) in 2014 [1]. Our study indicates that the prevalence of canine babesiosis has increased. We also detected new genotypes, which suggests that the parasitic lifecycle of domestic dogs, ticks, and *Babesia* parasites is widespread in Nigeria. However, few reports of canine babesiosis in Nigeria have been conducted using molecular techniques, and further investigation of the regional, seasonal, and epidemic variations of canine babesiosis in Nigeria and the African continent is needed. In addition, the vector ticks and the pathogen are spreading to new areas because of internationalization, increased trade, and climate change [10, 11]. The possibility of the spread of the disease to new areas in addition to the previously reported areas must be investigated.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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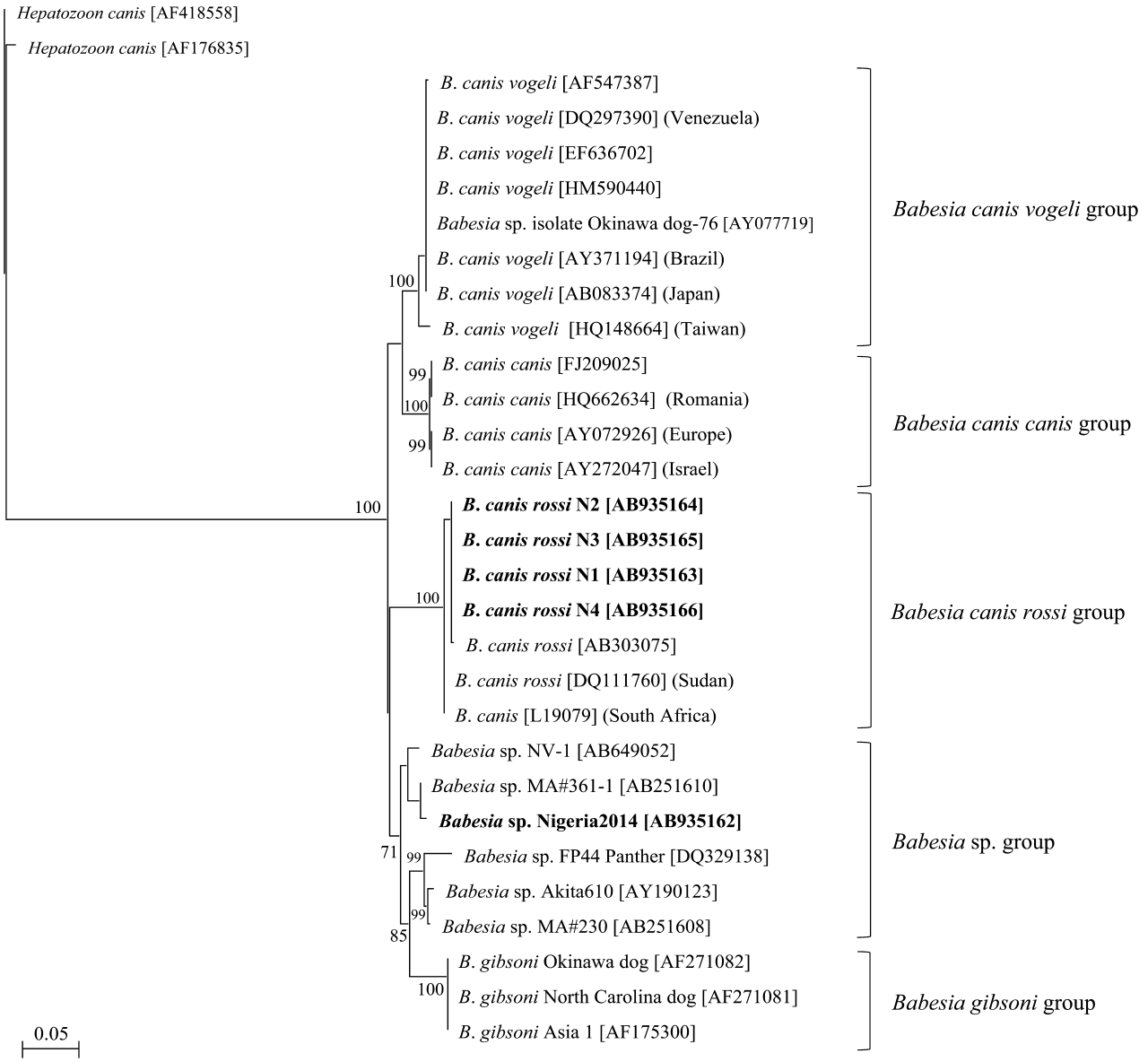

Fig. 1. Neighbor-joining phylogenetic tree presenting the relationships of 18S rRNA gene sequences from *Babesia* isolates. GenBank accession numbers are presented in the tree. The corresponding *Hepatozoon canis* (AF418558) sequence served as an outgroup. Numbers at the nodes indicate bootstrap support from 1,000 iterations.

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