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Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in Dogs in a Village of Eastern Sudan by Using a Screening PCR and Sequencing Methodologies

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Babesia and _Hepatozoon_ infections of dogs in a village of eastern Sudan were analyzed by using a single PCR and sequencing. Among 78 dogs, 5 were infected with _Babesia canis rossi_ and 2 others were infected with _B. canis vogeli_. Thirty-three dogs were positive for _Hepatozoon_. _Hepatozoon canis_ was detected by sequence analysis.

Both *Babesia* and *Hepatozoon* infections are important tick-borne protozoal diseases of dogs (2, 8). The diagnosis of infections with these protozoa is usually based on the detection of pathogens in peripheral blood under a microscope. However, such morphology-based methods are labor- and time-consuming because of their low sensitivities. Recently, molecular techniques, including PCR and sequence analysis, have been used for the diagnosis and epidemiological studies of canine *Babesia* and *Hepatozoon* infections (1, 3, 10, 11, 21, 22). The advantages of the molecular methods over other techniques are their higher sensitivities and specificities for the detection of the target pathogens in peripheral blood. *Babesia canis* is divided into three subspecies, *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* (7, 14, 19). By using these molecular methods, the diagnosis of *Babesia* infection is easily performed at the subspecies level. *Hepatozoon* has also been analyzed by using molecular technologies to identify two species, *Hepatozoon canis* and *H. americanum* (1, 2, 20). Because most epidemiological studies of protozoal infections in African countries are performed based on morphology, little information is available on canine *Babesia* and *Hepatozoon* infections in Africa (5, 9, 17). Thus, the objective of this study was to clarify the infection rates and subspecies of *Babesia* and *Hepatozoon* in dogs in Barbar el Fugara, a village in eastern Sudan, by using a combination of screening PCR and the sequencing methodology. We used a screening PCR to detect both *Babesia* and *Hepatozoon* simultaneously, followed by sequencing to identify the organisms to the species or the subspecies level.

Peripheral blood was obtained from 78 randomly selected dogs in the village during May 1997, May 1998, April 1999, and January 2000 (6). As these dogs were all free roaming around the village, the ages and histories of dogs were unknown. The sex and health status of the dogs were not recorded. Ticks were recovered from 61 dogs for identification. *Rhipicephalus sanguineus* was the most dominant tick species: it was recovered from 44 of 61 dogs (72.1%), in agreement with the findings presented in a previous report (12), followed by *Rhipicephalus evertsi evertsi* (3 of 61 dogs [4.9%]) and *Amblyomma lepidum* (4 of 61 dogs [6.6%]). Total DNA was extracted from each sample of canine blood with a QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany), adjusted to 200 μl with TE (Tris-EDTA) buffer, and stored at −20°C until it was used. Detection of DNA fragments of *Babesia* and *Hepatozoon* was attempted by PCR with primers of Babesia-F (GTG-AAA-CTG-CGA-ATG-GCT-CA) and Babesia-R (CCA-TGC-TGA-AGT-ATT-CAA-GAC). This primer set was previously reported to be specific for the genus *Babesia* (11), but it could amplify both *Babesia* and *Hepatozoon* simultaneously in our preliminary experiments. To confirm the results of PCR and to identify the infectious agents at the species or subspecies level, selected products of the PCR were purified with a QIAPCR purification kit (QIAGEN) or QIAquick gel extraction kit (QIAGEN) for direct sequence analysis. A fluorescence-labeled dideoxynucleotide technology was used for the DNA sequencing reactions (Perkin- Elmer, Applied Biosystems Division, Foster City, CA). The samples were then sequenced by using a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University. The sequences of the agent determined were analyzed for phylogenetic relationships with other sequences registered in GenBank. Multiple-sequence alignment analysis, the determination of pairwise percent identities of the sequences, distance matrix calculations, and the construction of phylogenetic trees were all performed with the ClustalW program (18), version 1.8, in the DNA data bank of Japan (DDBJ; Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]), as described in a previous report (11). The distance matrices for the aligned sequences with

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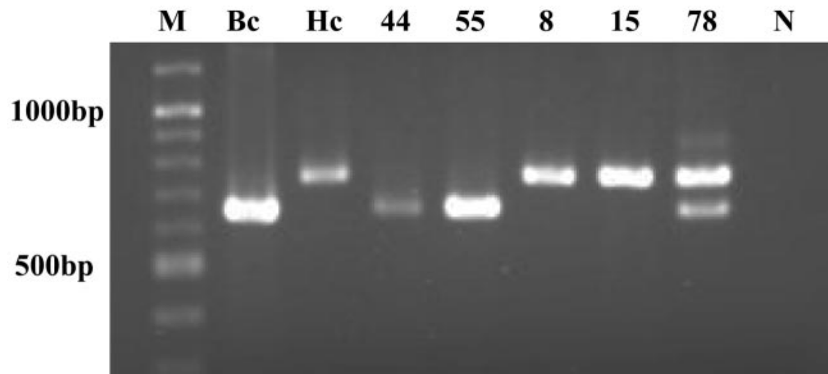

FIG. 1. Results of PCR for five positive samples. Screening by PCR produced a 645-bp fragment for *Babesia* (lanes Bc, 44, and 55) and a 780-bp fragment for *Hepatozoon* (lanes Hc, 8, and 15). Isolate 78 showed dual positivity for *Babesia* and *Hepatozoon*, with 645- and 780-bp fragments. Lane M, molecular size marker; lane N, negative control.

all gaps ignored were calculated by using the Kimura two-parameter method (13), and the neighbor-joining method was used to construct a phylogenetic tree (16). The stability of the tree obtained was estimated by bootstrap analysis for 100 replications by using the same program. Tree figures were generated by using the Tree View program, version 1.61 (15). The GenBank accession numbers of the 18S rRNA gene sequences of other species used to analyze the data are as follows: *Babesia divergense*, GenBank accession no. U16370; *Babesia odocoilei*, GenBank accession no. U16369; *Babesia gibsoni* Asia-1, GenBank accession no. AF175300; *B. gibsoni* Asia-2, GenBank accession no. AF175301; *B. canis vogeli*, GenBank accession no. AY072925; *B. canis canis*, GenBank accession no. AY072926; *Babesia caballi*, GenBank accession no. Z15104; *Babesia bigemina*, GenBank accession no. X59607; *Babesia bovis*, GenBank accession no. L19078; *Theileria sergenti*, GenBank accession no. AB000271; *Hepatozoon canis* Japan, GenBank accession no. AF418558; *Hepatozoon canis* Italia, GenBank accession no. AF176835; *Hepatozoon americanum*, GenBank accession no. AF176836; *Hepatozoon catesbianae*, GenBank accession no. AF176837; and *Neosporum caninum*, GenBank accession no. U03069.

Among the 78 dogs examined, 7 (9.0%) dogs (dogs 44, 55, 59, 69, 74, 76, and 78) showed a band positive for *Babesia* at about 645 bp. A total of 33 (42.3%) dogs were positive for *Hepatozoon* with a band of about 780 bp. Among these, three dogs (dogs 59, 74, and 78) showed dual positivity for bands at both 645 and 780 bp (Fig. 1). By analyzing the seven sequences of the *Babesia* 645-bp PCR products, excluding the primer region, five were identified as *B. canis rossi* (GenBank accession no. L19079) with percent identities of 99.7 to 99.8% (Fig. 2). The other two were very similar to *B. canis rossi* (GenBank accession no. AY072925), with percent identities of 99.8% (Fig. 2). Nine PCR products were randomly selected from among 33 *Hepatozoon*-positive PCR products for sequence analysis. All nine samples examined showed higher similarities with *H. canis* (GenBank accession no. AF176835), with percent identities of 99.1 to 100% (Fig. 2).

B. canis has three subspecies: *B. canis canis*, *B. canis rossi*, and *B. canis vogeli.* Each subspecies has a different vector and has a different pathogenesis in canine hosts. *B. canis rossi* is known to be the most pathogenic among the three subspecies and is transmitted by *Hemaphysalis leachi* (7). The pathogenesis of *B. canis vogeli* is comparatively weaker than those of the other two subspecies, and it is transmitted by *Rhipicephalus sanguineus* (7). In the present study, the predominant tick species recovered from dogs was *R. sanguineus*, and *H. leachi* was not detected. *Babesia canis rossi* may also be transmitted by ticks, such as *R. sanguineus*, *R. evertsi evertsi*, or *A. lepidum*, which were recovered from dogs in this study. Although the clinical symptoms of the infected dogs were not recorded in this study, infection with *B. canis rossi* might cause clinical disease in the canine host. The findings reported here are the first evidence of infection with *B. canis rossi* and *B. canis vogeli* in dogs in Sudan.

Our findings are also the first evidence of *Hepatozoon canis* infection in dogs in Sudan. *H. canis* is also known to be transmitted by *R. sanguineus* (4), which was the most common tick found in the present study. The rate of infection with *H. canis* was higher than that with *B. canis* in the present study. The weak pathogenesis of *H. canis* infection in canine hosts might contribute to the higher infection rate in this group, although the clinical symptoms of the infected dogs were not recorded.

Infections with *B. canis rossi*, *B. canis vogeli*, and *H. canis* in dogs may have a clinical impact on the quality of dogs' lives in this area. Dogs may also be reservoirs for continued propagation or may be the cause of increased infection rates. Furthermore, *R. sanguineus* may play an important role in the transmission of *Babesia* and *Hepatozoon* in Sudan.

In the present study, a single PCR was successfully used to detect *Babesia* and *Hepatozoon* simultaneously in canine blood samples. This provided an easy screening method for the detection of both *Babesia* and *Hepatozoon* in a single PCR. In combination with subsequent sequence analysis, this PCR assay may provide accurate information about the infectious agents. There were no difficulties in determining the subspecies of *Babesia* or the species of *Hepatozoon* in the sequence analysis in the present study. A dog might be infected with more than one subspecies of *Babesia* or more than one species of *Hepatozoon* at the same time. In such a case, the results of subsequent sequence analysis would be more difficult to interpret, because the results of the direct sequencing of the PCR products could not be read accurately. A subspecies-specific PCR for *Babesia canis* and a species-specific PCR for *Hepato-*

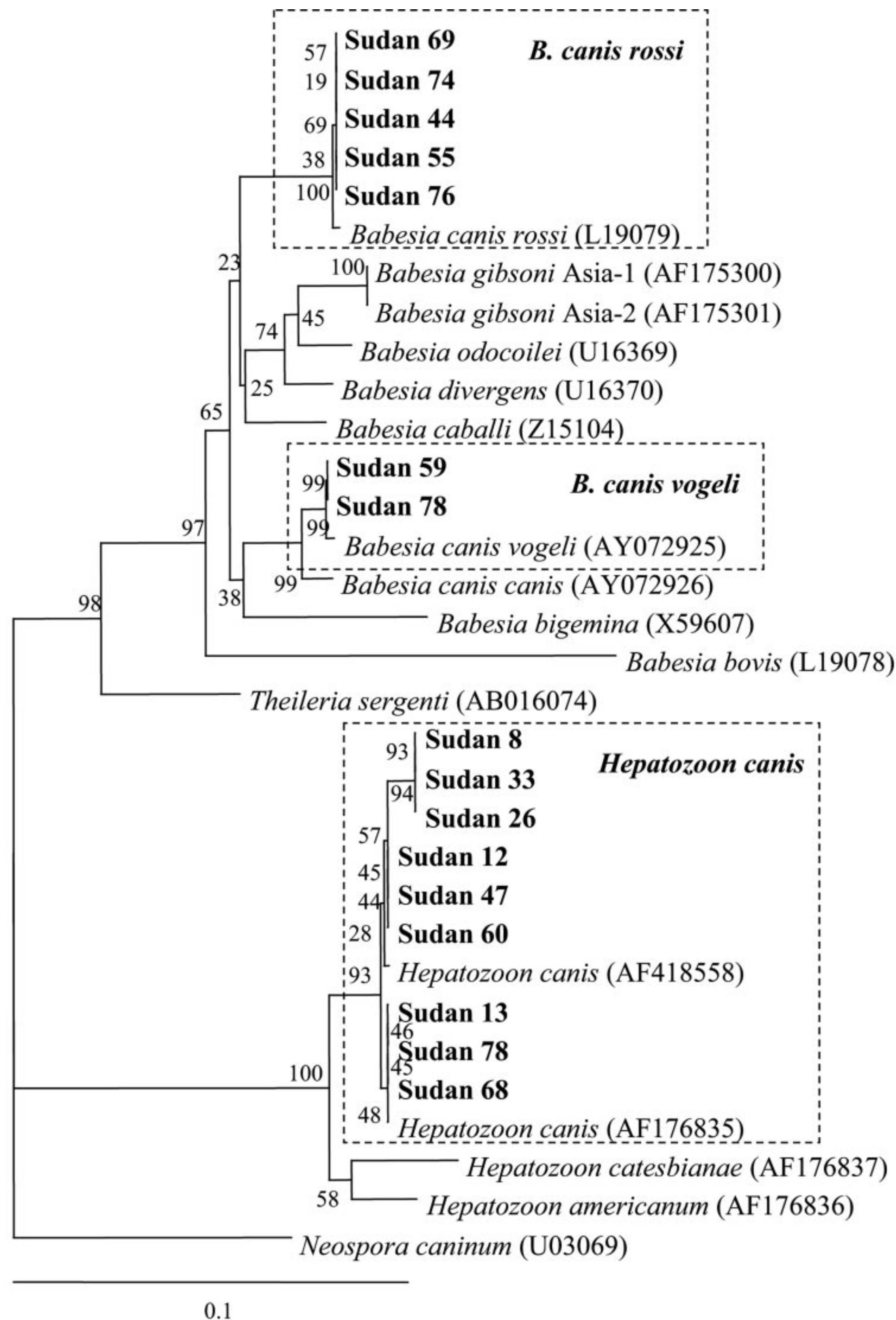

FIG. 2. Phylogenetic relationships between *Babesia* and *Hepatozoon* spp. in Sudan detected in this study and sequences registered in GenBank based on partial nucleotide sequences of the 18S rRNA gene. The numbers at the nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The scale bar represents 10% divergence.

zoon would be required to evaluate the infection rate with more accuracy in those cases.

Nucleotide sequence accession number. The nucleotide sequences of the 18S rRNA genes of the following *Babesia* and *Hepatozoon* isolates detected from dogs in this study have been deposited in the GenBank database under the indicated accession numbers: *Babesia canis rossi* Sudan-44, GenBank accession no. DQ111760; *Babesia canis rossi* Sudan-55, GenBank accession no. DQ111761; *Babesia canis rossi* Sudan-69, GenBank accession no. DQ111762; *Babesia canis rossi* Sudan-74, GenBank accession no. DQ111763; and *Babesia canis rossi* Sudan-76, GenBank accession no. DQ111764; *Babesia canis*

vogeli Sudan-59, GenBank accession no. DQ111765; *Babesia canis vogeli* Sudan-78, GenBank accession no. DQ111766; *Hepatozoon canis* Sudan-8, GenBank accession no. DQ111751; *Hepatozoon canis* Sudan-12, GenBank accession no. DQ111752; *Hepatozoon canis* Sudan-13, GenBank accession no. DQ111753; *Hepatozoon canis* Sudan-26, GenBank accession no. DQ111754; *Hepatozoon canis* Sudan-33, GenBank accession no. DQ111755; *Hepatozoon canis* Sudan-47, GenBank accession no. DQ111756; *Hepatozoon canis* Sudan-60, GenBank accession no. DQ111758; *Hepatozoon canis* Sudan-68, GenBank accession no. DQ111759; and *Hepatozoon canis* Sudan-78, GenBank accession no. DQ111757.

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