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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Detection of *Babesia canis rossi, B. canis vogeli* and *Hepatozoon canis* in dogs in a village of eastern Sudan using a screening PCR and sequencing methodologies

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Abstracts: *Babesia* and *Hepatozoon* infection of dogs in a village of eastern Sudan were analyzed using a single PCR and sequencing. Among 78 dogs, 5 were infected with *B. canis rossi* and 2 others with *B. canis vogeli*. Thirty-three were positive for *Hepatozoon*. *Hepatozoon canis* were detected by the sequence analysis.

Both *Babesia* and *Hepatozoon* infections are important tick-borne protozoa diseases of dogs (2, 8). The diagnosis of these protozoa is usually based on the detection of pathogens in peripheral blood under the microscope. However, such morphological methods are labor- and time-consuming because of their low sensitivity. Recently, molecular techniques, including the polymerase chain reaction (PCR) and sequence analysis, have been used for the diagnosis and epidemiological studies of canine *Babesia* and *Hepatozoon* infection (1, 3, 10, 11, 21, 22). The advantages of the molecular methods over other techniques are their higher sensitivity and specificity for the detection of the target pathogens in peripheral blood. *Babesia canis* is divided into three sub-species, *B. canis canis*, *B. canis vogeli* and *B. canis rossi* (7, 13, 19). By using these molecular methods, the diagnosis of *Babesia* infection is easily performed at the sub-species level. *Hepatozoon* has also been analyzed using molecular technologies to identify two species, *H. canis* and *H. americanum* (1, 2, 20). Because most epidemiological studies of protozoa infections in African countries are performed based on morphology, little information is available on canine *Babesia* and *Hepatozoon* infection in Africa (5, 9, 17). Thus, the objective of this study was to clarify the infection rates and sub-species of *Babesia* and *Hepatozoon* in dogs in Barbar el Fugara, a village of eastern Sudan, using a combination of screening PCR and sequencing methodology. We used a screening PCR to detect both *Babesia* and

Hepatozoon simultaneously, followed by sequencing to identify the species or sub-species level.

Peripheral blood was obtained from 78 randomly selected dogs in the village during May 1997, May 1998, April 1999 or January 2000 (6). As these dogs were all free-roaming around the village, the age 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 among 61 dogs (72.1%), in agreement with previous report (12), followed by *Rhipicephalus evertsi evertsi*（3/61, 4.9%）and *Amblyomma lepidum*（4/61, 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 buffer and stored at –20 °C until used. Detection of DNA fragments of *Babesia* and *Hepatozoon* was attempted using PCR using 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 genus-specific for *Babesia* (11), but it can 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 sub-species level, selected products of the PCR were purified with a QIAPCR purification kit (QIAGEN) or QIA quick Gel Extraction Kit (QIAGEN) for direct sequence analysis. Fluorescence- labeled dideoxynucleotide

technology was used for DNA sequencing reactions (Perkin- Elmer, Applied Biosystems Division, Foster City, CA). Samples were then sequenced using a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University. The determined sequences of the agent were analyzed for phylogenetic relationships with other sequences registered in GenBank. Multiple alignment analysis, the determination of pair-wise 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 all gaps ignored were calculated using the Kimura two-parameter method (14), and the neighbor-joining method was used for constructing a phylogenetic tree (16). The stability of the tree obtained was estimated by bootstrap analysis for 100 replications using the same program. Tree figures were generated 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: *B. divergense*, U16370; *B. odocoilei*, U16369; *B. gibsoni* Asia-1, AF175300; *B. gibsoni* Asia-2, AF175301; *B. canis vogeli*, AY072925; *B. canis canis*, AY072926; *B. caballi*, Z15104; *B. bigemina*, X59607; *B. bovis*, L19078; *Theileria sergenti*, AB000271; *Hepatozoon canis* Japan, AF418558; *Hepatozoon canis* Italia, AF176835; *Hepatozoon*

americanum, AF176836; *Hepatozoon catesbianae*, AF176837 and *Neosporum caninum*, U03069.

Among 78 dogs examined, 7 (9.0%) dogs (Nos. 44, 55, 59, 69, 74, 76 and 78) showed a positive band of *Babesia* at around 645bp. A total of 33 (42.3%) dogs were positive for *Hepatozoon* with a band of about 780 bp. Among these, 3 dogs (Nos. 59, 74 and 78) showed dual positivity at both 645 and 780 bp (Fig.1). By analyzing the 7 sequences of the *Babesia* 645-bp PCR products excluding the primer region, 5 were identified as *B. canis rossi* (L19079) with percent identities of 99.7 to 99.8% (Fig.2). The other 2 were very similar to *B. canis rossi* (AY072925), with percent identities of 99.8% (Fig.2). Nine PCR products were randomly selected among 33 *Hepatozoon*- positive PCR products for sequence analysis. All of the 9 samples examined showed higher similarities with *H. canis* (AF176835), with percent identities of 99.1 to 100% (Fig.2).

B. canis has three sub-species: *B. canis canis*, *B. canis rossi* and *B. canis vogeli.* Each sub-species has a different vector and shows different pathogenesis in canine hosts. *B. canis rossi* is known to be the most pathogenic among the three sub-species and is transmitted by *Haemaphysalis leachi* (7). The pathogenesis of *B. canis vogeli* is comparatively weaker than that of the other 2 sub-species, 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 of *B. canis rossi* might cause clinical diseases in the canine hosts. The findings reported here are the first evidence of infection of *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 infection rate of *H. canis* was higher than that of *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, though the clinical symptoms of the infected dogs were not recorded.

The infection of *B. canis rossi*, *B. canis vogeli* and *H. canis* in dogs may cause clinical impact on the quality of dogs' lives in this area. Dogs may also be reservoir for the propagation of continued or 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 to detect 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 sub-species of *Babesia* or the species of *Hepatozoon* in the sequence analysis in the present study. A dog might be infected with more than one sub-species of *Babesia* or more than one species of *Hepatozoon* at the same time. In such a case, subsequent sequence analysis would be more difficult to interpret, because the direct sequence method of the PCR products could not be read accurately. Sub-species specific-PCR for *Babesia canis* and species specific-PCR for *Hepatozoon* 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 gene of the *Babesia* and *Hepatozoon* detected from dogs in this study have been deposited in the GenBank database under the accession numbers, *Babesia canis rossi* Sudan-44, DQ111760; Sudan-55, DQ111761; Sudan-69, DQ111762; Sudan-74, DQ111763 and Sudan-76, DQ111764; *Babesia canis vogeli* Sudan-59, DQ111765 and Sudan-78, DQ111766; *Hepatozoon canis* Sudan-8, DQ111751; Sudan-12, DQ111752; Sudan-13, DQ111753; Sudan-26, DQ111754; Sudan-33, DQ111755; Sudan-47, DQ111756; Sudan-60, DQ111758; Sudan-68, DQ111759 and Sudan-78, DQ111757.

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Figure Captions

Fig.1. Results of PCR for 5 positive samples. Screening by PCR produced a 645-bp fragment for *Babesia* (Bc, 44, 55) and a 780-bp fragment for *Hepatozoon* (Hc, 8, 15). No.78 showed dual positivity for *Babesia* and *Hepatozoon*, with 645- and 780-bp fragments, respectively.

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

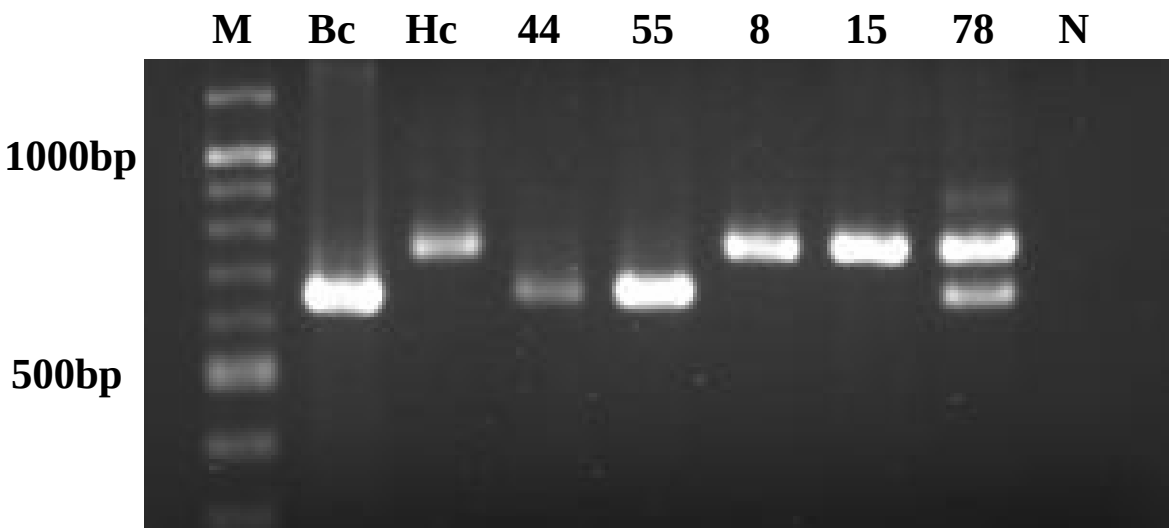

Fig.1

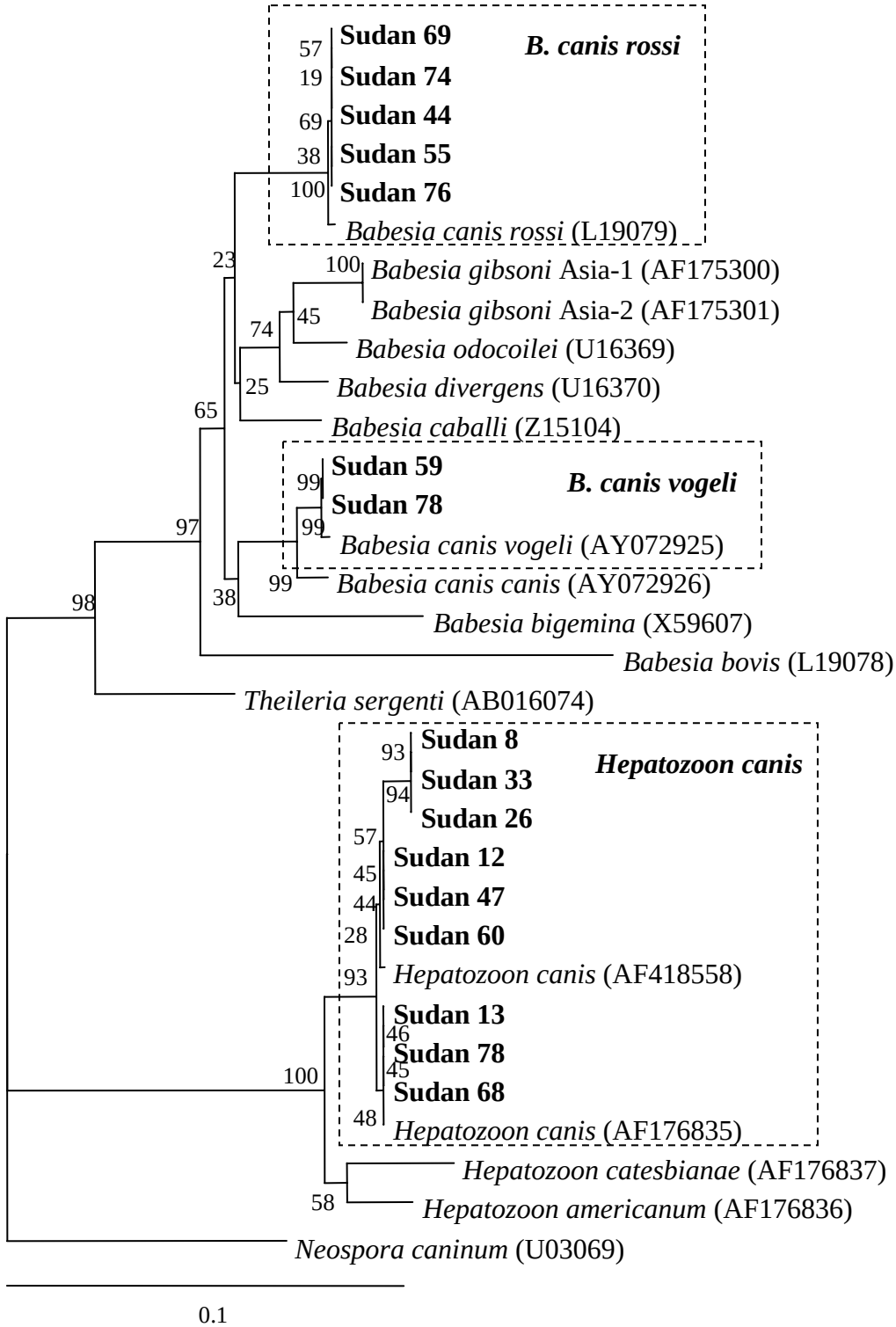
B. canis rossi
Sudan 69
Sudan 74
Sudan 44
Sudan 55
Sudan 76
Babesia canis rossi (L19079)
Babesia gibsoni Asia-1 (AF175300)
Babesia gibsoni Asia-2 (AF175301)
Babesia odocoilei (U16369)
Babesia divergens (U16370)
Babesia caballi (Z15104)
B. canis vogeli
Sudan 59
Sudan 78
Babesia canis vogeli (AY072925)
Babesia canis canis (AY072926)
Babesia bigemina (X59607)
Babesia bovis (L19078)
Theileria sergenti (AB016074)
Hepatozoon canis
Sudan 8
Sudan 33
Sudan 26
Sudan 12
Sudan 47
Sudan 60
Hepatozoon canis (AF418558)
Sudan 13
Sudan 78
Sudan 68
Hepatozoon canis (AF176835)
Hepatozoon catesbianae (AF176837)
Hepatozoon americanum (AF176836)
Neospora caninum (U03069)
0.1

Fig.2