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

Maremichi Oyamada,1 Bernard Davoust,2 Mickaël Boni,3 Jacques Dereure,4 Bruno Bucheton,5 Awad Hammad,6 Kazuhiro Itamoto,1 Masaru Okuda,1 and Hisashi Inokuma7*

Faculty of Agriculture, Yamaguchi University, 753-8515 Yamaguchi, Japan; Direction du Service de Santé en Region Sud-Est, BP16, 69998 Lyon Armes, France6; Groupe de Secteurs Vétérinaires Interarmées de Saint Germain en Laye, BP220, 00492 Saint Germain en Laye Armes, France7; Laboratoire de Parasitologie, Faculté de Médecine, 34090 Montpellier, France7; Laboratoire d’immunologie Parasitaire, Faculté de Médecine, Université de la Méditerranée, Marseille Cedex 5, France7; Département de Microbiologie et Parasitologie, Faculty of Medicine, University of Khartoum, P.O. Box 102, Khartoum, Republic of the Sudan7; and Obihiro University of Agriculture and Veterinary Medicine, 080-8555 Obihiro, Japan7*

Received 13 July 2005/Returned for modification 18 August 2005/Accepted 19 August 2005

*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 canis*. Detection of the target pathogens in peripheral blood was attempted by PCR with primers of *Babesia-F* (GTG-AAA-CTG-CGA-ATG-GCT-CA) and *Babesia-R* (CCA-TGC-TGA-ATG-ACTG-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 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 performed by using a pair of primers specific for the 18S ribosomal RNA gene of each species. The DNA fragments 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
all gaps were ignored 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 divergens, GenBank accession no. U16370; Babesia odocoi, 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 Neo- sporum 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-
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* Sudan-78, GenBank accession no. DQ111765; and *Babesia canis* Sudan-68, GenBank accession no. DQ111766; and *Hepatozoon canis* Sudan-13, GenBank accession no. DQ111767; *Hepatozoon catesbianae* (AF156835); and *Hepatozoon americanum* (AF156836).

**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.
vogeli Sudan-59, GenBank accession no. DQ111753; 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.

We acknowledge the technical expertise of the DNA Core Facility of the Center for Gene Research, Yamaguchi University.

Our study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Institut National de la Sante et de la Recherche Medicale, and the Japan Society for the Promotion of Science.

REFERENCES

1. Baneth, G., J. R. Barta, V. Shkap, D. S. Martin, D. K. Macintyre, and N. Vincent-Johnson. 2000. Genetic and antigenic evidence supports the separation of Hepatozoon canis and Hepatozoon americanum at the species level. J. Clin. Microbiol. 38:1298–1301.

2. Baneth, G., J. S. Mathew, V. Shkap, D. K. Macintyre, J. R. Barta, and S. A. Ewing. 2003. Canine hepatozoonosis: two disease syndromes caused by separate Hepatozoon spp. Trends. Parasitol. 19:27–31.

3. Caccio, S. M., B. Antunovic, A. Moretti, V. Mangili, A. Mariniculic, R. R. Baric, S. B. Slemenda, and N. J. Pleniazek. 2002. Molecular characterization of Babesia canis canis and Babesia canis vogeli from naturally infected European dogs. Vet. Parasitol. 106:285–292.

4. Christophers, S. R. 1907. The sexual life cycle of Leucocytozoon canis in the tick. Sci. Mem. Off. Med. Sanit. Dep. Gov. India 28:1–11.

5. Collett, M. G. 2000. Survey of canine babesiosis in South Africa. J. S. Afr. Vet. Assoc. 71:180–186.

6. Deregue, J., S. H. El-Safi, B. Bucheton, M. Boni, M. M. Kheir, B. Davoust, F. Pratlong, E. Feugier, M. Lambert, A. Dessein, and J. P. Dedet. 2003. Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships. Microbes Infect. 5:1103–1108.

7. Hauschild, S., and E. Schein. 1996. The subspecies specificity of Babesia canis. Berl. Munch. Tierarztl. Wochenschr. 109:216–219.

8. Homer, M. J., I. Aguilar-Delfin, S. R. Telford III, P. J. Krause, and D. H. Persing. 2000. Babesiosis. Clin. Microbiol. Rev. 13:451–469.

9. Ibrahim, N. D., P. M. Rahamatullah, and C. O. Njoku. 1989. Neutrophil myeloperoxidase deficiency associated with canine hepatozoonosis. Int. J. Parasitol. 19:915–918.

10. Inokuma, H., Y. Yoshizaki, K. Matsumoto, M. Okuda, T. Onishi, K. Nakagome, R. Kosugi, and M. Hirakawa. 2004. Molecular survey of Babesia infection in dogs in Okinawa, Japan. Vet. Parasitol. 121:341–346.

11. Inokuma, H., Y. Yoshizaki, Y. Shimada, Y. Sakata, M. Okuda, and T. Onishi. 2003. Epidemiological survey of Babesia species in Japan performed with specimens from ticks collected from dogs and detection of new Babesia DNA closely related to Babesia odocoilei. J. Clin. Microbiol. 41:3494–3498.

12. Jongejan, F., D. Zivkovic, R. G. Pegram, R. J. Tatchell, T. Fison, A. A. Latif, and G. Paine. 1987. Ticks (Acari:Ixodidae) of the Blue and White Nile ecosystems in the Sudan with particular reference to the Rhipicephalus sanguineus group. Exp. Appl. Acarol. 3:331–346.

13. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J. Mol. Evol. 16:111–120.

14. Kjemtrup, A. M., A. A. Kocan, L. Whitworth, J. Meinkoth, A. J. Birkenheuer, J. Cummings, M. K. Boudreaux, S. L. Stockham, A. Irizarry-Rovira, and P. A. Conrad. 2000. There are at least three genetically distinct small piroplasms from dogs. Int. J. Parasitol. 30:1501–1505.

15. Page, R. D. M. 1996. TREEVIEW: an application to phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

16. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Med. Biol. Evol. 4:406–425.

17. Shakespeare, A. S. 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. J. S. Afr. Vet. Assoc. 66:247–250.

18. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucl. Acids Res. 22:4673–4680.

19. Uilenberg, G., F. F. Frausen, N. M. Perie, and A. A. Spanjer. 1989. Three groups of Babesia canis distinguished and a proposal for nomenclature. Vet. Q. 11:33–40.

20. Vincent-Johnson, N. A., D. K. Macintyre, D. S. Lindsay, S. D. Lenz, G. Baneth, V. Shkap, and S. L. Blagburn. 1997. A new Hepatozoon species from dogs: description of the causative agent of canine hepatozoonosis in North America. J. Parasitol. 83:1165–1172.

21. Zahler, M., H. Rinder, E. Schein, and R. Gothe. 2000. Detection of a new pathogenic Babesia microti-like species in dogs. Vet. Parasitol. 89:241–248.

22. Zahler, M., H. Rinder, E. Zwyggarth, T. Fukata, Y. Maede, E. Schein, and R. Gothe. 2000. ‘Babesia gibsoni’ of dogs from North America and Asia belong to different species. Parasitology 120:365–369.