NOTE *Parasitology*

A PCR-Based Epidemiological Survey of *Hepatozoon canis* in Dogs in Nigeria

Mizuki SASAKI[1)], Olutayo OMOBOWALE[2)], Kaisaku OHTA[1)], Morito TOZUKA[1)], Aya MATSUU[3)], Haruyuki HIRATA[4)], Helen Oyebukola NOTTIDGE[2)], Hiromi IKADAI[1)]* and Takashi OYAMADA[1)]

[1)]*Department of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, Japan,* [2)]*Department of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria,* [3)]*Department of Veterinary Internal Medicine, Faculty of Agriculture, Tottori University, Koyama-Minami, Tottori 680–8553 and* [4)]*Section of Animal Research, The Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113–0033, Japan*

(Received 8 November 2007/Accepted 4 April 2008)

ABSTRACT. The prevalence of *Hepatozoon canis* infections in dogs in Nigeria was surveyed using molecular methods. DNA was extracted from blood samples obtained from 400 dogs. A primer set that amplified the *Babesia canis* 18S rRNA gene, which has high similarity to the *H. canis* 18S rRNA gene, was used for the PCR. As a result, samples from 81 dogs (20.3%) produced 757 bp bands, which differed from the 698 bp band that corresponded to *B. canis* infection. The sequence of the PCR products of 10 samples were determined, all of which corresponded with the *H. canis* sequence.

KEY WORDS: *Hepatozoon canis*, Nigeria, PCR.

J. Vet. Med. Sci. 70(7): 743–745, 2008

Hepatozoon canis is a protozoan that is transmitted by the ixodid *Rhipicephalus sanguineus*. *H. canis* is present in southern Europe, Asia, Africa and Latin America [2–4, 8, 9]. This parasite is transmitted to dogs by the ingestion of ticks containing mature oocysts. In infected dogs, gametocytes are present within peripheral blood leukocytes. *H. canis* infections range from an asymptomatic type to a lethal type in which symptoms such as fever, anemia, emaciation and debility are displayed. Concurrent infections with pathogens such as *Babesia canis* and *Ehrlichia* spp. may aggravate the clinical condition of dogs infected with *H. canis* [8].

Diagnosis of canine hepatozoonosis is usually accomplished by microscopic examination of stained peripheral blood smears for the presence of gametocytes within neutrophils. However, it is sometimes difficult to detect the parasite when the number of circulating gametocytes is low. Serological tests such as an indirect fluorescent antibody test and an enzyme-linked immunosorbent assay have been developed for the diagnosis of canine hepatozoonosis [1, 7, 8, 12]. Recently, molecular methods have been used to detect *Hepatozoon* spp. Molecular PCR methods are more sensitive and specific for pathogens in peripheral blood and arthropod vectors than other methods [2, 8]. Moreover, PCR analysis followed by sequence analysis can be used for phylogenic characterization of *Hepatozoon* isolates [2]. These molecular methods can play an important role in the diagnosis of canine hepatozoonosis and research into its distribution and prevalence.

Molecular methods have been used worldwide to conduct epidemiological studies of canine hepatozoonosis [1, 2, 8, 10]. A study demonstrated that *H. canis* and its arthropod vector, *R. sanguineus*, are present in the Sudan [10]. As *R. sanguineus* is thought to be widely distributed throughout Africa, it is possible that *H. canis* is widespread on this continent. *H. canis* was reported to be present in Nigeria, in the Midwest of Africa [5], but this has not been validated by an epidemiological survey in which molecular methods have been used.

In a previous study, we surveyed the prevalence of *B. canis* infection among 400 dogs in Nigeria using nested PCR and primer sets that amplified the *B. canis* 18S rRNA gene [11]. *B. canis* infection resulted in a 698 bp band after nested PCR. Some of these samples also produced a 757 bp band, which was larger than the *B. canis* band (698 bp). Therefore, the 757 bp PCR products were used for nucleotide sequence determination. The sequences of samples were shown to correspond to that of *H. canis* 18S rRNA genes (99.3%, GenBank accession no. AF176835). In the present study, we analyzed the sequences of these PCR products and investigated the incidence of *H. canis* infection in dogs in Nigeria.

Peripheral blood samples from 400 dogs were obtained randomly from many parts of Nigeria between October 2004 and August 2005. Each 200 μL of blood sample was fixed on an FTA Card (Whatman, UK) and stored at room temperature in the dark. DNA was extracted from blood on the FTA Cards using the Whatman FTA Card DNA Isolation kit (Whatman).

Primers for the PCR were based on a fragment of the gene that encodes *B. canis* 18S rRNA (GenBank accession no. AY072925, AY072926, L19079) [11]. Forward primer B18S-1 (5'-GGGAGGTAGTGACAAGAAA- 3') and reverse primer B18S-2 (5'-TTCCCCGTGTTGAGTCAAA-3') were used for the PCR [6, 11]. The sequences of the *B. canis* and *H. canis* 18S rRNA genes were similar to those of the respective primer sets (Table 1).

The DNA extracted from the blood samples was added to

* CORRESPONDENCE TO: IKADAI, H., Department of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628,Japan.
e-mail: ikadai@vmas.kitasato-u.ac.jp

Table 1. Sequences of the B18S-1 and B18S-2 primers and *H. canis* 18S rRNA gene

Primer	Primer sequence (5'–3')	*H. canis* nucleotide sequence
B18S-1	GGGAGGTAGTGACAAGAAA	GAGAGGTAGTAACAAGAAA
B18S-2	TTCCCCGTGTTGAGTCAAA	TTTCCCGTGTTGAGTCAAA

Table 2. Sex and age of dogs positive for *H. canis*

		No. of examined	*H. canis* positive
	Total	400	81 (20.3%)
Sex	Male	169	34 (20.1%)
	Female	227	47 (20.7%)
	ND*	4	0
Age (yr)	0–2	292	58 (19.9%)
	3–5	77	19 (24.7%)
	6–8	17	2 (11.8%)
	9–	10	2 (20.0%)
	ND*	4	0

* ND: no data.

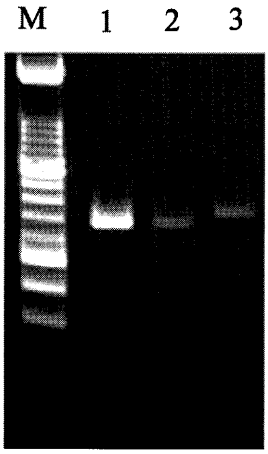

Fig. 1. Results of nested PCR analysis using primer sets that amplified *B. canis* 18S rRNA. Lane M, molecular size marker; lane 1, *B. canis rossi*; lane 2, *B. canis vogeli*; lane 3, *H. canis*.

a reaction mixture containing 10 pmol each of primers B18S-1 and B18S-2, 5 U/μL of AmpliTaq Gold DNA polymerase (Applied Biosystems, US), 2.0 mM dNTP (Applied Biosystems), 10 × PCR Gold Buffer (150 mM Tris-HCl [pH 8.0], 500 mM KCl) (Applied Biosystems) and 25 mM $MgCl_2$ Solution (Applied Biosystems), and adjusted to a volume of 25 μL with Ultra Pure Distilled Water. Amplification conditions were as follows: 95°C for 10 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 2 min and amplification at 72°C for 2 min, followed by final extension at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% TBE agarose gel and stained with ethidium bromide.

The amplified DNA was cloned into a pCR® 2.1 - TOPO® vector using a TOPO TA Cloning® Kit (Invitrogen, US) and the sequences were determined using the B18S-1 or B18S-2 primers and an ABI PRISM® 310 genetic Analyzer (Applied Biosystems). The sequences were analyzed using Genetyx® Version. 8 (Genetyx, Japan).

The PCR analysis showed that 81 of 400 samples (20.3%) contained fragments of the 757 bp PCR product which differed from the 698 bp band that corresponded to *B. canis* infection (Fig. 1). The PCR products of 10 samples selected at random from the 81 samples that produced fragments of the 757 bp band were used for nucleotide sequence determination (GenBank accession no. AB365071). The sequences of all 10 samples were shown to correspond to that of *H. canis*, indicating that all 81 samples were positive for *H. canis*. The sexes and ages of dogs infected with *H. canis* are shown in Table 1. Of the 81 dogs infected with *H. canis*, 34 were male and 47 were female. The ages of the infected dogs range from 5 weeks to 11 years. There was no correlation between the incidence of *H. canis* and the age and sex of the dogs. In none of the samples, gametocytes were detected in neutrophils by microscopic examination of Giemsa-stained peripheral blood smears.

In Nigeria, Ibrahim *et al.* [5] studied 18 dogs that exhibited clinical signs of *H. canis* infection and detected *H. canis* gametocytes in myeloperoxidase-deficient neutrophils obtained from these dogs. However, an extensive investigation of the prevalence of *H. canis* infection in Nigeria has not been performed using molecular methods. In the whole of the African continent, the only epidemiological study to have been performed using PCR was undertaken in the Sudan [10]. In the Sudan study, 33 of 78 dogs (42.3%) were infected with *H. canis*, and among these, three dogs were also infected with *B. canis* [10]. Our study showed that in Nigeria, 81 of 400 dogs (20.3%) were infected with *H. canis*, a much lower incidence of infection than in the Sudan. The incidence of *B. canis* infection in dogs is also lower in Nigeria than in the Sudan [10, 11]. *R. sanguineus* is the dominant tick species in the Sudan [10], and transmits both *H. canis* and *B. canis vogeli*. The disparity between the results of our study and those of the Sudan study may have been caused by differences in sizes of tick populations or their distribution.

The 400 blood samples used for this nested PCR study were identical to those used in our previous study of *B. canis* infection [11]. Of the 400 dogs, 81 samples produced a 757 bp band, and nine samples produced a 698 bp band. The sequences of these PCR products corresponded to those of *H. canis* and *B. canis*, respectively. Of the nine samples that

produced a 698 bp band, eight were identified as *B. canis* rossi, and one as *B. canis* vogeli [11]. Two samples were positive for both *H. canis* and *B. canis*. The primers used in this study made simultaneous diagnosis of both *H. canis* and *B. canis* infection possible. *H. canis* causes relatively mild symptoms in dogs, but the clinical condition of dogs infected with *H. canis* may deteriorate when they are concurrently infected with another pathogen [8]. In Nigeria, the presence of *R. sanguineus* may result in concurrent infections with *H. canis* and *B. canis*, because it is a vector for both these pathogens.

In conclusion, our molecular study revealed the infection of *H. canis* with dogs in Nigeria and demonstrated the existence of concurrent infection with *B. canis*. As infected dogs that do not display symptoms may be present throughout the African continent, it is important to detect carrier dogs and to control vector tick infestations.

REFERENCES

1. Baneth, G., Barta, J. R., Shkap, V., Martin, D. S., Macintire, D. K. and Vincent-Johnson, N. 2000. *J. Clin. Microbiol.* **38**: 1298–1301.
2. Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A. and Barba-Carretero, J. C. 2003. *Vet. Parasitol.* **114**: 173–194.
3. Ewing, S. A. and Pansiera, R. J. 2003. *Clin. Microbiol. Rev.* **16**: 688–697.
4. Gondium, L. F. P., Kohayagawa, A., Alencar, X., Biondo, A. W., Takahira, R. K. and Franco, S. R. V. 1998. *Vet. Parasitol.* 74: 319–323.
5. Ibrahim, N. D. G., Rahamathulla, P. M. and Njoku, C. O. 1989. *Int. J. Parasitol.* **19**: 915–918.
6. Ikadai, H., Tanaka, H., Shibahara, N., Matuu, A., Uechi, M., Itoh, N., Oshiro, S., Kudo, N., Igarashi, I. and Oyamada, T. 2004. *J. Clin. Microbiol.* **42**: 2456–2469.
7. Inokuma, H., Ohno, K. and Yamamoto, S. 1999. *J. Vet. Med. Sci.* **61**: 1153–1155.
8. Karagenc, T. I., Pasa, S., Kirli, G., Hosgor, M., Bilgic, H. B., Ozon, Y. H., Atasoy, A. and Eren, H. 2006. *Vet. Parasitol.* **135**: 113–119.
9. O'Dwyer, L. H., Massard, C. L. and Souza, J. C. P. 2001. *Vet. Parasitol.* **94**: 143–150.
10. Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto, K., Okuda, M. and Inokuma, H. 2005. *Clin. Diagn. Lab. Immunol.* **12**: 1343–1346.
11. Sasaki, M., Omobowale, O., Tozuka, M., Ohta, K., Matuu, A., Nottidge, H. O., Hirata, H., Ikadai, H. and Oyamada, T.2007. *J. Vet Med Sci.* **69**: 1191–1193.
12. Shkap, V., Baneth, G. and Pipano, E. 1994. *J. Vet. Diagn. Invest.* **6**: 121–123.