Short communication

Analysis of the 18S rRNA gene sequence of a Hepatozoon detected in two Japanese dogs

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Abstract

Partial sequences of the 18S rRNA gene (625 bp) from a Hepatozoon detected in two canine hepatopzoonosis cases, one clinical and one subclinical, in Japan were analyzed. Both sequences were identical to each other and they were closely related to the Hepatozoon canis strain found in Israel with 99% (617/625) nucleotide identity. Both Hepatozoon americanum and Hepatozoon catasbianae were distantly related to the Japanese Hepatozoon with 94% (586/625) and 91% (566/625) identities, respectively. In a phylogenetic tree, the Japanese Hepatozoon was most closely related to H. canis from Israel but was significantly different than H. americanum and H. catasbianae. These results suggest that the Hepatozoon detected in the Japanese dogs might be a strain variant of H. canis, but is apparently a different species than H. americanum. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Canine hepatopzoonosis is a tick-borne protozoan disease caused by two known pathogens: Hepatozoon americanum and Hepatozoon canis. The geographic distribution, vectors and pathogenesis of these two pathogens are reported to be different. H. americanum is a causative agent of canine hepatopzoonosis in the US, which is transmitted by Amblyomma
maculatum ticks (Vincent-Johnson et al., 1997b). The agent causes a distinct clinical syndrome in dogs characterized by fever, lethargy, weight loss, stiffness, signs of pain, paralysis, and ocular discharge (Vincent-Johnson et al., 1997a,b; Macintire et al., 2001). Diagnosis of American hepatozoonosis is made based on a muscle biopsy to detect the pathogens (Craig et al., 1984). H. canis is the cause of Old World canine hepatozoonosis reported from southern Europe, the Middle East, Africa and the Far East. The main vector of the disease is the brown dog tick, Rhipicephalus sanguineus (Baneth et al., 2001). Pathogenesis of H. canis is thought to be weak, because subclinical infections are common, usually causing a milder disease that affects the spleen, lymph nodes, and bone marrow, resulting in anemia and lethargy (Baneth and Weigler, 1997). Gametocytes in circulating white blood cells are often an incidental finding in dogs without clinical signs and the disease is easily diagnosed by observation of the blood film (Baneth et al., 1996).

Both clinical and subclinical cases of canine hepatozoonosis have been reported in Japan since 1991. Most cases in Japan are subclinical with gametocytes appearing in the peripheral blood (Murata et al., 1993a,b,c). These findings are similar to those of cases reported from Israel (Baneth et al., 1996, Baneth and Weigler, 1997). On the other hand, clinical cases with lethargy, weight loss, obvious signs of pain, leukocytosis or periosteal new-born proliferation were also reported similar to the American type of hepatozoonosis (Makimura et al., 1991, Murata et al., 1991, Nakayama et al., unpublished data). Furthermore, Haemaphysalis spp. is suspected to be the vector tick of canine hepatozoonosis in Japan (Murata et al., 1995). Given this evidence, one may suspect that the pathogen of the Japanese hepatozoonosis is different from that of USA and Israel. Although genetic analysis revealed that Hepatozoon strains found in the US and Israel are quite different (Baneth et al., 2000), no genetic analysis has been done for the pathogen of canine hepatozoonosis in Japan. Thus, we analyzed the partial 18S rRNA gene sequences of Hepatozoon detected in two canine cases in Japan, one clinical and the other subclinical, to clarify the phylogeny of the Japanese Hepatozoon in dogs.

2. Materials and methods

Two canine cases of Hepatozoon infection were used in this study. The first case was a clinical hepatozoonosis. A male Beagle, 1 year and 9 months old, born in Fukuoka Prefecture was admitted to animal hospital in Yamaguchi University for stiffness, paralysis, depression and anorexia on 21 March 1997 (day 1). The mother was also infected with Hepatozoon without clinical signs. The second case was a mongrel dog, 1 year and 6 months old, in Yamaguchi Prefecture. The dog was presented at the animal hospital in Yamaguchi University for ferialia control in May 1998. No clinical signs were found in the second case.

PCR and sequencing were performed with EDTA-anticoagulated peripheral blood taken on the first day for both cases. The buffy coat of each blood sample was separated and kept frozen at −80 °C until used. DNA from each sample was extracted following the QIAamp Tissue Kit procedure (QIAGEN GmbH, Hilden, Germany). Finally each DNA sample was eluted in 200 µl of TE buffer. A primer set, HepF (5 ATA-CAT-GAG-CAA-AAT-CTC-AAC 3) and HepR (5 CTT-ATT-ATT-CCA-TGC-TGC-AG 3), was designed to amplify a partial 18S rRNA gene sequence of Hepatozoon spp. based upon alignment data from the H. canis
from a dog in Israel (GenBank accession number AF176835), *H. americanum* from a dog in the US (AF176836) and *H. catesbianae* from a frog (AF176837). PCR conditions were as previously described (Inokuma et al., 2001) with an annealing temperature of 57 °C. Five millilitre of each DNA was used as the template. The positive amplification product with 666 base pairs was then extracted using the QIA PCR purification kit (QIAGEN GmbH) for sequence analysis. The fluorescence-labeled dideoxynucleotide method 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 nucleotide sequence was confirmed by two independent PCR amplification and sequencing experiments. The sequences of *Hepatozoon* Japan and the registered sequences of other related protozoal species deposited in GenBank were analyzed for phylogenetic relationships. The GenBank accession numbers of the 18S rRNA gene sequences used to construct phylogenetic trees are as follows: *Eimeria peromysci*, AF339492; *Plasmodium vivax*, U93233; *Sarcocystis cruzi*, AF017120; *Neospora caninum*, U16159; *Toxoplasma gondii*, L37415; *Cryptosporidium parvum*, AF115377; *Theileria parva*, L02366; *Babesia gibsoni*, AF158702 and *Trypanosoma cruzi*, AF359496. Multiple alignment analysis, distance matrices calculation, and construction of a phylogenetic tree were performed with the ClustalW program (Thompson et al., 1994) version 1.8 in the DNA Data Bank of Japan (DDBJ; Mishima, Japan). The distance matrices for the aligned sequences with all gaps ignored were calculated using the Kimura two-parameter method (Kimura, 1980), and the neighbor-joining method was used for constructing a phylogenetic tree (Saitou and Nei, 1987). 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 (Page, 1996). The sequence of the partial 18S rRNA gene of Japanese *Hepatozoon* determined in this study has been deposited in GenBank data library under accession number AF418558.

3. Results and discussion

Physical examination of the first case revealed a swelling of the hind legs, signs of pain and mild dehydration, but no fever was observed (38.7 °C). Results of hematological and biochemical analysis on the same day indicated nonregenerative anemia (red blood cell counts 312 × 10^6/ml, packed cell volume 19%, hemoglobin concentration 6.9 g/dl, reticulocytes 0.7%), mild leukocytosis (21,500/μl), thrombocytopenia (21,000/μl), high activities of serum alkaline phosphatase (1083 IU/l) and creatine kinase (834 IU/l), hypoalbuminemia (2.1 g/dl) and a high concentration of serum C-reactive protein (CRP, 8.4 mg/dl). Gametocytes were found in 11% of neutrophil-like cells in peripheral blood stained with Giemsa (Fig. 1). A muscle biopsy was performed, but nothing significant was found in the hind legs or hip. Radiographic findings showed periosteal new-borne proliferation at the bilateral humerus and femur. Treatment with toltrazuril, imidocarb, diminazene acetate and clindamycin did not improve the clinical signs. Prednisolone was also used occasionally to control the pain and inflammation suspected to be due to the high CRP levels. All treatment had been stopped at the end of October 1997, when the gametocytes in peripheral blood
disappeared, but the stiffness, signs of pain, and lethargy continued. The patient died of unknown causes in the summer of 1999, and post-mortem examination was not performed. In the second case, 3% of the white blood cells were infected with *Hepatozoon* gametocytes, results of blood examination were within the normal ranges, and radiographic findings in all the legs were also normal.

Canine hepatozoonosis is an endemic disease in the western part of Japan (Murata et al., 1993a, Inokuma et al., 1999). There have been other clinical cases like case no. 1 in the present study, which showed lethargy, stiffness, signs of pain and paralysis (Makimura et al., 1991, Murata et al., 1991). These findings were similar to those of *H. americanum* infection (Vincent-Johnson et al., 1997a, b, Macintire et al., 1997, 2001). Nonregenerative anemia, substantial leukocytosis, hypoalbuminemia and increased serum activity of alkaline phosphatase were also recorded as hematologic abnormalities in the American hepatozoonosis (Macintire et al., 1997, 2001). New-borne proliferation was also found in the radiographic findings (Macintire et al., 1997). On the other hand, most of the cases in Japan are subclinical with numbers of gametocytes appearing in peripheral blood as in case no. 2. These findings are similar to findings in *H. canis* infection reported as Old World canine hepatozoonosis, which usually causes a milder disease (Baneth et al., 1996, Baneth and Weigler, 1997). The *Hepatozoon* detected in Japanese dogs cannot be identified by its pathogenesis, vectors or morphological studies. Thus molecular analysis was performed to clarify the phylogeny of the Japanese *Hepatozoon*.

A positive PCR signal was detected with the DNA extracted from each dog. Analyzing the nucleotide sequences of the 625 bp PCR product excluding the primer regions revealed that both sequences were 100% identical to each other. Pairwise percent identities of the
Fig. 2. Phylogenetic relationship of *Hepatozoon* detected in two Japanese dogs, *H. canis* from a dog in Israel, *H. americanum* from a dog in the United States, *H. catesbiana* from a frog and other related protozoa species based on the partial sequences of the 18S rRNA gene. The neighbor-joining method was used to construct the phylogenetic tree using the ClustalW program. The scale bar represents 10% divergence. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown.
sequences with all gaps omitted were calculated. The sequence of the Japanese Hepatozoon was similar to that of *H. canis* from Israel with 99% (617/625) nucleotide identity. Both *H. americanum* and *H. catasbianae* were distantly related to Japanese *Hepatozoon* with 94% (586/625) and 91% (566/625) identities, respectively. The phylogenetic tree is shown in Fig. 2. All the *Hepatozoon* species analyzed in the present study were in the same group. *Hepatozoon* detected from dogs in Japan was the most closely related to *H. canis* in Israel, but showed significant distance from both *H. americanum* and *H. catasbianae*. These results suggest that *Hepatozoon* detected in Japanese dogs might be a strain variant of *H. canis*, but is apparently a different species from *H. americanum*.

The suspected vectors of Japanese *Hepatozoon* are *Haemaphysalis* ticks (Murata et al., 1995), which are different from the vector ticks of *H. americanum* and *H. canis*. Analyses of other gene sequences and antigens were needed to explain the differences in the pathogenesis and vectors of *Hepatozoon* in other countries. As partial sequences (625 bp) of the 18S rRNA gene of the agent from only two dogs were analyzed in the present study, pathogens from more dogs should be analyzed over longer sequences of the gene to confirm the results in this study. Furthermore, many western breeds of dogs are imported to Japan from other countries, including the USA where *H. americanum* infection is dominant. It is suspected that *H. americanum* also exists in Japan, though the vector tick *A. maculatum* has never been found there. More epidemiological studies using molecular tools are also required to clarify the canine hepatozoonosis in Japan.

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**References**


