NOTE *Internal Medicine*

Ehrlichia canis Infection in Two Dogs that Emigrated from Endemic Areas

Kenji BABA[1)]*, Kazuhito ITAMOTO[2)], Akiteru AMIMOTO[3)], Kozue KITAGAWA[1)], Hiroko HIRAOKA[4)], Takuya MIZUNO[5)], Hiroshi SATO[1)] and Masaru OKUDA[5)]

[1)]*Laboratory of Veterinary Parasitology, Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan*
[2)]*Laboratory of Veterinary Clinical Diagnostics, Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan*
[3)]*Amica Pet Clinic, 3–3–2 Onda-Cho, Ube, Yamaguchi 755–0023, Japan*
[4)]*Laboratory of Veterinary Clinical Pathology Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan*
[5)]*Laboratory of Veterinary Internal Medicine, Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan*

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ABSTRACT. Two dogs, emigrated from Zambia and China to Japan, were diagnosed with *Ehrlichia canis* infection. Both cases had thrombocytopenia, non-regenerative anemia, and hyperglobulinemia with polyclonal gammopathy. Case 1 had ataxia of the hind limbs. Severe meningitis was revealed by magnetic resonance imaging examination. Intracytoplasmic inclusions were observed in mononuclear cells of cerebrospinal fluid. Case 2 had a history of bilateral epistaxis, and severe pancytopenia was noticed in complete blood count. Diagnosis was finally achieved by nested polymerase chain reaction and sequence analysis. Thus, even in non-endemic areas, *E. canis* infection should be included in the differential diagnosis of clinically ill dogs that emigrated from endemic areas.
KEY WORDS: canine, diagnosis, *Ehrlichia canis*, polymerase chain reaction.

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Canine ehrlichiosis is a tick-borne disease that is found worldwide and is caused by a variety of ehrlichial bacteria species [6]. *Ehrlichia canis* is the most common etiological agent in dogs and is known to cause canine monocytic ehrlichiosis (CME), a systemic disorder manifested by fever, hemorrhagic tendencies associated with thrombocytopenia and platelet dysfunction, and non-regenerative anemia [2, 3, 6]. Diagnosis of CME is based on the detection of ehrlichial morulae in monocytic cells by microscopic examination, measurement of serum antibody titers by indirect fluorescent antibody test, and detection of *E. canis* DNA by polymerase chain reaction (PCR) [4]. Incidence of CME is likely related to the geographic distribution of *Rhipicephalus sanguineus*, the primary vector for *E. canis* [8]. *R. sanguineus* is not a common tick in Japan, except in Okinawa Prefecture [7]. Therefore, CME is an uncommon disorder in Japan and has been reported in one dog only that emigrated from an endemic area [9]. In this report, we describe the clinical and clinicopathological findings for 2 dogs diagnosed with CME that emigrated from Zambia and China to Japan.

Case 1 was a 4-year-old neutered mixed-breed dog that had emigrated from Zambia 3 years ago. The dog was referred to a veterinarian for vomiting and ataxia of the hind limbs. A history of tick infestation was unknown. Blood examination revealed mild anemia [packed cell volume (PCV): 29.5%], leukocytosis (25,600/μl) with a left shift, thrombocytopenia (78,000/μl), hyperproteinemia (8.8 g/dl), and increased levels of blood urea nitrogen (BUN: 74.5 mg/dl) and alkaline phosphatase (ALP: 2,073 IU/l). Proteinuria was also detected by urinary examination. The referring veterinarian treated the dog with food formulated for renal insufficiency. Three months later, the dog was referred to Yamaguchi University Animal Medical Center (YUAMEC) for anorexia, weight loss, and ataxia. Complete blood count (CBC) and serum biochemical findings included non-regenerative anemia (PCV: 21%), thrombocytopenia (27,000/μl), hypoalbuminemia (1.5 g/dl), and increased levels of BUN (50.2 mg/dl), ALP (1,090 IU/l), and C-reactive protein (CRP: >20 mg/dl). Serum protein electrophoresis indicated polyclonal gammopathy [albumin/globlin (A/G) ratio: 0.15]. Splenomegaly and hepatomegaly were also detected by abdominal radiography. Neurological examination revealed depressed postural reactions of the left hind limb.

Magnetic resonance imaging (MRI) of the brain, cerebrospinal fluid (CSF) aspiration, and bone marrow aspiration were performed to determine the cause of neurological signs, non-regenerative anemia, and thrombocytopenia. MRI of the brain disclosed hypertrophy and high signal intensity in T2-weighted images of the meninges, indicating meningitis. CSF abnormalities included xanthochromia, increased protein levels (2.4 g/dl), pleocytosis (3,200/μl), and intracytoplasmic inclusions (suspected as morulae)

*CORRESPONDENCE TO: BABA, K., Laboratory of Veterinary Parasitology, Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753-8515, Japan.
e-mail: kbaba@yamaguchi-u.ac.jp

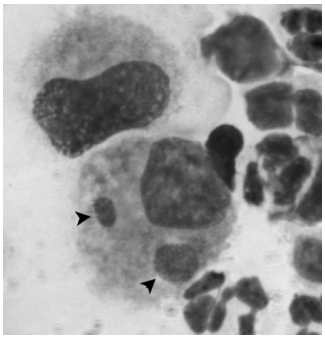

Fig. 1. Photomicrograph of cerebrospinal fluid cytology in Case 1. Round-shaped intracytoplasmic inclusions (arrowheads) were observed in mononuclear cells of cerebrospinal fluid (Hemacolor stain; Merck, Darmstadt, Germany).

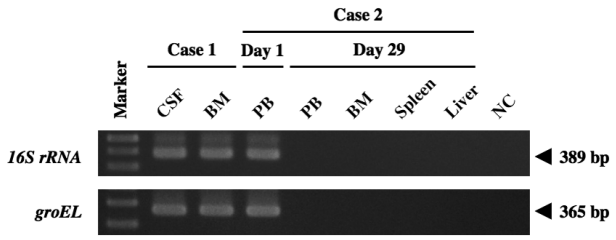

Fig. 2. Detection of *Ehrlichia canis* DNA by nested polymerase chain reaction (PCR). Genomic DNA was extracted from specimens of Cases 1 and 2 and subjected to nested PCR for detecting the partial 16S ribosomal RNA gene of *E. canis* and groEL gene of *anaplasmataceae* bacterium [10, 11]. Lane 1: 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA); Lane 2: cerebrospinal fluid (Case 1); Lane 3: bone marrow (Case 1); Lane 4: peripheral blood (Case 2, Day 1); Lane 5: peripheral blood (Case 2, Day 29); Lane 6: bone marrow (Case 2, Day 29); Lane 7: spleen (Case 2, Day 29); Lane 8: liver (Case 2, Day 29); Lane 9: peripheral blood from an uninfected healthy dog (negative control).

in infiltrated macrophages (Fig. 1). The serum immunoglobulin G (IgG) antibody titer for *E. canis* was considered as negative (1:25; reference range, <1:40) (Monolis, Inc., Tokyo, Japan). *E. canis* infection was suspected based on intracytoplasmic inclusions found in mononuclear cells in CSF.

To confirm *E. canis* infection, nested PCR and sequence analysis were performed using CSF and bone marrow aspirate samples. DNA was purified from each sample using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, U.S.A.), according to the manufacturer's instructions. Nested PCR for detecting the partial 16S ribosomal RNA (rRNA) gene of *E. canis* and groEL gene of *anaplasmataceae* bacterium were performed as described previously, except for modifying the annealing temperature from 60 to 63°C for the amplification of 16S rRNA gene [10, 11]. The PCR products were electrophoresed on 1.5% agarose gels. Results are shown in Fig. 2. The PCR products were purified using RECOCHIP (TaKaRa, Kyoto, Japan) and then subjected to direct DNA sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of genes amplified by each PCR showed 100% identity to the partial 16S rRNA (GenBank accession number: AF162860) and groEL (U96731) genes of *E. canis*, respectively. We excluded co-infection with *Babesia* spp. and/or *Hepatozoon* spp., which were most common tick-borne diseases worldwide, by the blood smear examination and the PCR targeting for the 18S rRNA genes [1] (data not shown). The dog was finally diagnosed with *E. canis* infection. The following day, the dog became unconscious and was euthanized at the owner's request.

Case 2 was a 2-year-old female Chow Chow dog that had emigrated from China 3 days prior admission. The dog was referred to a veterinarian for anorexia, weight loss, a 2-month history of bilateral epistaxis, and pale visible mucous membranes (Day 1). A history of tick infestation at China was also obtained. CBC disclosed severe anemia (PCV: 14.9%), leukopenia (2,900/μl), and thrombocytopenia (71,000/μl). The reticulocyte count was 49,700/μl (2.2%), indicating reduced red blood cell regeneration. Serum biochemical findings included increased levels of ALP (10,983 IU/*l*), alanine aminotransferase (ALT: 1,090 IU/*l*), aspartate transaminase (AST: 417 IU/*l*), γ-glutamyltransferase (GGT: 150 IU/*l*), total bilirubin (T-Bil.: 0.8 mg/d*l*), total cholesterol (T-Chol.: 408 mg/d*l*), and hyperproteinemia (10.7 g/d*l*). Serum protein electrophoresis indicated polyclonal gammopathy (A/G ratio: 0.22). Abdominal radiography revealed mild splenomegaly.

To determine the cause of pancytopenia, bone marrow aspiration and serological test for *E. canis* infection were performed (Day 2). The bone marrow aspirate smears revealed normocellularity with a distinctly decreased myeloid to erythroid (M/E) ratio (0.17) without morphological abnormalities, indicating erythroid hyperplasia (Fig. 3A). Megakaryocytic cells were occasionally observed. Increased phagocytosis of nucleated erythrocytes was also observed (Fig. 3B). Microscopic examination showed no intracytoplasmic inclusions on the bone marrow aspirate or peripheral blood smears. Serological test for *E. canis* was negative (<1:25) (Monolis, Inc.). Because CME was suspected based on the history, clinical symptoms, and laboratory abnormalities, the referring veterinarian treated the dog with doxycycline (7 mg/kg, every 12 hr, PO) and ursodeoxycholic acid (6 mg/kg, every 24 hr, PO).

Upon presentation to YUAMEC (Day 24) for diagnosis confirmation, the dog had no clinical signs. Anemia and thrombocytopenia were improved, whereas neutropenia (1,836/μl) was persistent. Serum biochemical abnormalities were unchanged, including ALP (10,503 IU/*l*), ALT (1,491 IU/*l*), AST (852 IU/*l*), GGT (211 IU/*l*), T-Bil. (0.6 mg/d*l*), T-Chol. (487 mg/d*l*), and hyperproteinemia (10.1 g/d*l*). The serum IgG antibody titer for *E. canis* was considered as negative (1:25, Monolis, Inc.). To confirm the diagnosis, bone marrow aspiration, liver and spleen tru-cut

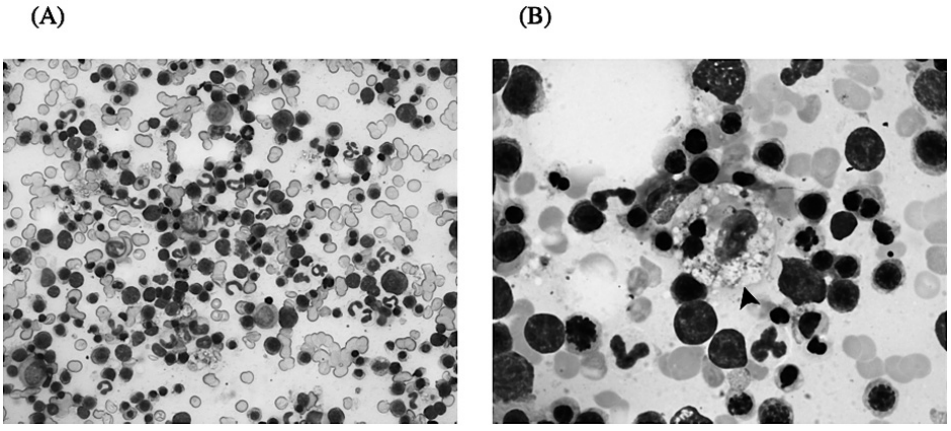

Fig. 3. Photomicrographs of bone marrow aspiration cytology obtained on Day 2 for Case 2 (Hemacolor stain). (A) Erythoid hyperplasia and (B) phagocytosis of nucleated erythrocytes (arrowhead) were observed. Intracytoplasmic inclusions were not found in the bone marrow aspirate.

biopsies, and nested PCR examination were performed on Day 29. The findings from bone marrow aspirate smears were almost the same as that on Day 2, including marked erythroid hyperplasia (M/E ratio: 0.075) and phagocytosis of erythroid cells. Histopathological examination revealed no intracytoplasmic pathogens in the bone marrow aspirate smear and liver and spleen specimens. Nested PCR examination for *E. canis* infection was performed as described for Case 1. *E. canis* DNA was present in the peripheral blood sample on Day 1, but absent in the peripheral blood, bone marrow, spleen, and liver samples on Day 29 after treatment with doxycyclin for 4 weeks (Fig. 2). The sequences of genes amplified by each PCR showed 100% identity to the partial 16S rRNA (AF162860) and groEL (U96731) genes of *E. canis*, respectively. *Babesia* spp. and *Hepatozoon* spp. could not be detected by the blood smear examination and the PCR targeting for the 18S rRNA genes [1] (data not shown). The dog was finally diagnosed with *E. canis* infection. Currently, 2 years after diagnosis, the dog is clinically healthy.

Both cases had common clinical symptoms and laboratory abnormalities, including anorexia, weight loss, thrombocytopenia, anemia, and hyperproteinemia with polyclonal gammopathy, which have been reported as the most typical findings for CME [2, 3]. In addition to these findings, ataxia and pancytopenia were observed in Cases 1 and 2, respectively. Neurological signs of CME are attributed to plasma cell infiltration of the meninges or hemorrhage into the cerebral or spinal cord parenchyma [12]. As revealed by MRI examination, severe meningitis associated with *E. canis* infection is a possible cause of the ataxia observed in Case 1. In chronic CME cases, pancytopenia can typically result from hypoplasia of all bone marrow cells [6, 12]. Pancytopenia can, however, occur in both normocellular and hypercellular bone marrow [5]. Indeed, pancytopenia was observed in Case 2 with normocellular bone marrow. Interestingly, the marrow showed marked erythroid hyperplasia and phagocytosis of erythroid cells, although we cannot elucidate the reason.

Screening of *E. canis* infection is usually based on serological testing [4]. Dogs with chronic infection frequently have high antibody titers that persist after treatment [2]. In Cases 1 and 2, however, the anti-*E. canis* IgG titers were considered as negative. In a previous report, 5 of 61 dogs with ehrlichiosis had antibody titer lower than 1:40 [2]. As another possibility, the examination in the submitted diagnostic laboratory may be somewhat less sensitive. Whatever the reason, because the results of serological examinations were ambiguous, definitive CME diagnosis was based on the detection of *E. canis* DNA by nested PCR and sequence analysis of amplified fragments using CSF and bone marrow samples for Case 1 and peripheral blood samples for Case 2. PCR examination and sequence analysis may be useful for confirming suspected *E. canis* infection in dogs that do not exhibit remarkably elevated antibody titers. In Case 2, however, *E. canis* DNA could not be detected after treatment with doxycyclin, indicating that PCR examination should be carried out using samples collected before treatment begins in order to confirm diagnosis.

Because CME can display a variety of clinical symptoms, it may not be considered in differential diagnosis especially in non-endemic areas. Both cases in this report were known to have emigrated from areas endemic for *E. canis*. A review of travel history should indicate exposure to areas endemic for *E. canis* in order to better identify suspect CME in dogs with common clinical symptoms, such as thrombocytopenia, anemia, and hyperproteinemia with polyclonal gammopathy. To the best of our knowledge, in Japan CME has been reported in one dog only that emigrated from an endemic area [9]. Given the recent increase in travel across Japanese borders, *E.* canis infection should be included in the differential diagnosis of clinically ill dogs emigrated from endemic areas.

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REFERENCES

1. Cassini, R., Zanutto, S., Frangipane di Regalbono, A., Gabrielli, S., Calderini, P., Moretti, A., Tampieri, M. P. and Pietrobelli, M. 2009. Canine piroplasmosis in Italy: epidemiological aspects in vertebrate and invertebrate hosts. *Vet. Parasitol.* **165** 30–35. [Medline] [CrossRef]
2. Frank, J. R. and Breitschwerdt, E. B. 1999. A retrospective study of ehrlichiosis in 62 dogs from North Carolina and Virginia. *J. Vet. Intern. Med.* **13**: 194–201. [Medline] [CrossRef]
3. Harrus, S., Kass, P. H., Klement, E. and Waner, T. 1997. Canine monocytic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological investigation of prognostic indicators for the disease. *Vet. Rec.* **141**: 360–363. [Medline] [CrossRef]
4. Harrus, S. and Waner, T. 2011. Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview. *Vet. J.* **187**: 292–296. [Medline] [CrossRef]
5. Mylonakis, M. E., Koutinas, A. F. and Leontides, L. S. 2006. Bone marrow mastocytosis in dogs with myelosuppressive monocytic ehrlichiosis (Ehrlichia canis): a retrospective study. *Vet. Clin. Pathol.* **35**: 311–314. [Medline] [CrossRef]
6. Neer, T. M. 1998. Canine monocytic and granulocytic ehrlichiosis. pp. 139–147. *In*: Infectious Diseases of the Dog and Cat (Greene, C. E. ed.), WB Saunders Co., Philadelphia.
7. Shimada, Y., Beppu, T., Inokuma, H., Okuda, M. and Onishi, T. 2003. Ixodid tick species recovered from domestic dogs in Japan. *Med. Vet. Entomol.* **17**: 38–45. [Medline] [CrossRef]
8. Skotarczak, B. 2003. Canine ehrlichiosis. *Ann. Agric. Environ. Med.* **10**: 137–141. [Medline]
9. Suto, Y., Suto, A., Inokuma, H., Obayashi, H. and Hayashi, T. 2001. First confirmed canine case of *Ehrlichia canis* infection in Japan. *Vet. Rec.* **148**: 809–811. [Medline] [CrossRef]
10. Tabara, K., Arai, S., Kawabuchi, T., Itagaki, A., Ishihara, C., Satoh, H., Okabe, N. and Tsuji, M. 2007. Molecular survey of *Babesia microti, Ehrlichia* species and *Candidatus* neoehrlichia mikurensis in wild rodents from Shimane Prefecture, Japan. *Microbiol. Immunol.* **51**: 359–367. [Medline]
11. Wen, B., Rikihisa, Y., Mott, J. M., Greene, R., Kim, H. Y., Zhi, N., Couto, G. C., Unver, A. and Bartsch, R. 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of Ehrlichia canis infection in dogs treated with doxycycline. *J. Clin. Microbiol.* **35**: 1852–1855. [Medline]
12. Woody, B. J. and Hoskins, J. D. 1991. Ehrlichial diseases of dogs. *Vet. Clin. North Am. Small Anim. Pract.* **21**: 75–98. [Medline]