

Molecular Evidence of *Enterocytozoon bieneusi* in JapanNiichiro ABE^{1)*}, Isao KIMATA²⁾ and Motohiro ISEKI³⁾

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ABSTRACT. *Enterocytozoon bieneusi* is an emerging and clinically significant enteric pathogen in humans associated mainly with chronic diarrhea. It has been found in a variety of wild, domestic and companion mammals and birds. To date, epidemiological surveys of *E. bieneusi* infection in humans, other mammals and birds have been performed in more than 21 countries in Africa, the Americas, Australasia and Europe. In Asia *E. bieneusi* has been found in India, Thailand, Vietnam and Korea, but it has been quite unclear whether this pathogen is present in Japan. In the present study, we examined 149 DNAs extracted from 45 human (9 of them HIV-positive) and 104 animal fecal samples by PCR. Two dogs and a cat were positive and their genotypes were found to be dog specific and zoonotic (genotype K) types, respectively. Present study is the first record of *E. bieneusi* in Japan.

KEY WORDS: *Enterocytozoon bieneusi*, microsporidia, PCR, zoonotic parasite.

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Microsporidia is an obligate intracellular pathogen in humans and animals. This pathogen is considered to be highly diverged and specialized parasites that are related to the fungi, but their exact relationship to the fungi remains to be unclear [13]. *Enterocytozoon bieneusi*, first detected in a HIV-positive patient in 1985, has been the most frequently diagnosed microsporidian species in humans, particularly in immunocompromised patients with AIDS and chronic diarrhea [18]. In addition, *E. bieneusi* has been found in a variety of wild, domestic and companion mammals and birds [18]. Based on subtle sequence differences at the internal transcribed spacer (ITS) and a portion of the small and large subunit ribosomal RNA gene region, *E. bieneusi* consists of multiple genotypes. Recent molecular epidemiological studies show that some genotypes are host-specific and others are zoonotic [18]. Therefore, identification of the isolates at the genotype level is important for the control *E. bieneusi* infections and for understanding the population structure of *E. bieneusi* genotypes. To date, epidemiological surveys of *E. bieneusi* infection in humans, other mammals and birds have been performed in more than 21 countries in Africa, the Americas, Australasia and Europe [10, 18, 22]. In Asia *E. bieneusi* has been found in India, Thailand, Vietnam and Korea [14, 15, 18]. The present study was undertaken to investigate whether this pathogen is present in Japan.

As shown in Table 1, a total of 149 DNA samples from humans and animals (45 diarrheic humans, 9 of them HIV-positive, 6 head of cattle, 5 pigs, 1 raccoon dog, 1 mongoose, 3 ferrets, 2 cockatiels, 7 cats and 79 dogs) used previously [1-9, 19, 20] for the examination of intestinal

protozoan parasites by PCR were provided for the present study. All DNA samples from humans were originated from diarrheic patients infected with *Cryptosporidium hominis*, *C. parvum*, *C. meleagridis*, *Cyclospora cayentanensis*, *Giardia duodenalis*, *Isospora belli* or *Entamoeba histolytica*. The 45 animal samples with the exception of 6 and 53 samples from cats and dogs, respectively, were from individuals infected with *C. parvum*, *C. andersoni*, *C. suis*, *C. canis*, *Cryptosporidium ferret* or mongoose genotypes, *C. baileyi*, *C. meleagridis*, *G. duodenalis*, *Isospora rivolta* or *Eimeria furonis*. Sixty-six samples from 7 cats and 59 dogs were from stray animals in Osaka City, and 20 dog samples were from animals at a pet shop in Kanazawa City and from a house pet in Niigata City. It was unclear whether the animals examined showed diarrhea or not. These DNAs had been extracted and purified as follows. The oocysts or cysts in fecal samples were concentrated by centrifugal flotation using sucrose or zinc sulfate solution and the genomic DNAs were extracted and purified using Qiagen kits (QIAamp DNA Stool or DNA Mini Kit) (Qiagen, Hilden, Germany) after boiling the concentrates for 10 min. In the present study, the internal transcribed spacer (ITS) and a portion of the large and small subunit ribosomal RNA gene region were amplified by nested PCR using primers (EBITS3 and EBITS4 for the first PCR, EBITS1 and EBITS2.4 for the second PCR) specific for *E. bieneusi* [11]. DNA of *E. bieneusi* originating from cattle [21] was used as a positive control for PCR. Amplification products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced in both directions with the EBITS1 and EBITS2.4 primer pair on an ABI 310 automated sequencer (Applied Biosystems, CA, U.S.A.). Sequence chromatograms from each strand were aligned and inspected using the SEQUENCHER Version 4.1 (Gene Codes Corp., MI, U.S.A.). The sequences of approximately

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Table 1. Information of 149 DNA samples examined in the present study

Origin	Number of samples examined	Number of samples positive for <i>E. bieneusi</i> by PCR	<i>E. bieneusi</i> genotypes	Collection period of fecal samples	Host location	Other intestinal protozoan parasites identified by PCR (number of positive samples)
Humans ^{a)}	45 ^{b)}	0		2002–2007	Osaka, Tokyo, Tochigi	<i>Cryptosporidium hominis</i> (11), <i>C. parvum</i> (1), <i>C. meleagridis</i> (3), <i>Cyclospora cayentanensis</i> (4), <i>Giardia duodenalis</i> (2), <i>Isospora belli</i> (1), <i>Entamoeba histolytica</i> (23)
Cattle (farm animal)	6	0		2003–2005	Hokkaido	<i>C. parvum</i> (3), <i>C. andersoni</i> (1), <i>G. duodenalis</i> (2)
Pigs (farm animal)	5	0		2002	Gifu	<i>C. suis</i> (5)
Raccoon dog (wild animal)	1	0		2003	Osaka	<i>C. parvum</i> (1)
Mongoose (zoo animal)	1	0		2000	Osaka	<i>Cryptosporidium</i> mongoose genotype (1)
Ferrets (pet shop animal)	3	0		2002–2006	Ishikawa	<i>Cryptosporidium ferret</i> genotype (1), <i>Eimeria furonis</i> (1), <i>G. duodenalis</i> (1)
Cockatiels (pet shop animal)	2	0		2002	Ishikawa	<i>C. baileyi</i> (1), <i>C. meleagridis</i> (1)
Cats (stray animal)	7	1 ^{c)}	K	2001	Osaka	<i>I. rivolta</i> (1)
Dogs (stray animal)	59	1	Dog specific	2000–2002	Osaka	<i>Cryptosporidium canis</i> (17)
(pet shop animal)	19	1	Dog specific	2003	Ishikawa	<i>C. canis</i> (8)
(house pet animal)	1	0		2000	Niigata	<i>C. canis</i> (1)
Total	149	3				

a) All were diarrheic patients.

b) Including 9 HIV-positive patients.

c) Mixed infection with *I. rivolta*.

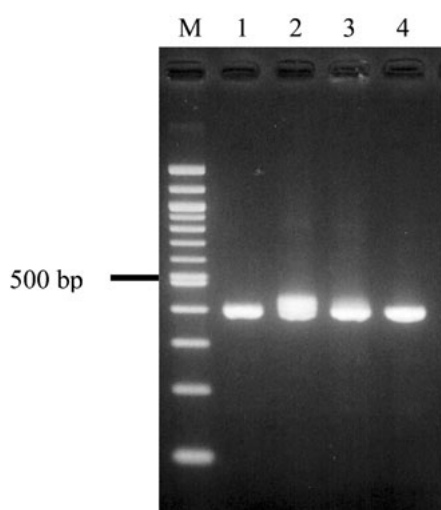


Fig. 1. PCR amplification using a set of primers specific for *E. bieneusi*. Lanes: M, molecular marker (100 bp ladder); 1, PCR with *E. bieneusi* DNA from cattle as a positive control; 2, PCR with DNA from a cat; 3 and 4, PCR with DNAs from two dogs.

360 bp fragments were compared with sequences in the DNA database (GenBank/DBJ/EMBL) by FASTA analysis (DBJ, <http://fasta.ddbj.nig.ac.jp/top-j.html>).

The specific fragment (approximately 360 bp) was successfully amplified in 3 DNA samples from a cat and two dogs (Fig. 1). The sequences of both isolates from dogs (DBJ accession numbers, AB359946 and AB359947) were identical with an *E. bieneusi* sequences from isolates that had been found in dogs only (GenBank accession number, AF059610) [17]. However, the sequence from the cat isolate (DBJ accession number, AB359945) had 100%

identity with a zoonotic genotype, genotype K, previously identified in cats but also in cattle and humans (GenBank accession numbers, AF267141 and DQ836343) [12, 17, 22, 23]. The other 146 DNA samples from humans and other animals were negative for *E. bieneusi* by PCR.

In the present study, we confirmed for the first time the presence of *E. bieneusi* in Japan. A zoonotic genotype, genotype K, was found in a cat and a dog specific genotype was found in two dogs. The genetically different novel type in dogs (accession number AF059610) was first found in farm dogs in Switzerland in 1999 [17]. The same type was found in a dog in Portugal in 2006 [16]. To our knowledge, many isolates from humans, other mammals and birds have been genotyped, but this genotype had been found in dogs only [16, 17] and therefore was considered to be a dog-specific genotype. In the present study, we found the same genotype from two dogs from Osaka and Kanazawa. This finding further supports that this genotype is dog specific. Two zoonotic genotypes, PtEbIX (identical to Peru 9, D, PigEBITS9, WL8) and PtEbVII (identical to Peru 6), were also found in the 8 isolates from dogs genotyped previously [16]. Therefore, cats and dogs that are in close contact with humans will be possible carriers of both human pathogenic and host-adapted genotypes.

In the present study, all samples from humans were negative and only a few individuals were positive in animals. The inadequate DNA extraction method was probably responsible for the low detection of the parasite. Microsporidian spores are very hardy, and most researchers mechanically or chemically break the spore wall first prior to conduct DNA extraction [10, 11, 15, 16]. The procedure using boiling and Qiagen kits were useful to prepare DNAs from oocysts and cysts, but would have left most spores intact.

To date, clinical cases of *E. bieneusi* infection remain to be reported in Japan. *Enterocytozoon bieneusi* would be

overlooked at Japanese medical centers because this pathogen is not known to Japanese physicians and medical technologists. Further fecal examinations in patients with chronic diarrhea and animals are necessary to clarify the epidemiology of *E. bienewsi* infection in Japan.

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