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Molecular determination of *Enterocytozoon bieneusi* in pet shop puppies and breeding kennel dogs

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Abstract

Enterocytozoon (*E.*) *bieneusi* is the most common opportunistic pathogen in humans with immunocompromised status. This microorganism has been reported in various mammals including humans and companion animals. There are at least 90 genotypes of *E. bieneusi*, and some of them are considered zoonotic. Although pet shops and breeding kennels are the major source of dogs for owners, only a few reports are available regarding the molecular determination of *E. bieneusi* in pet shop puppies, and there are no reports in breeding kennels dogs. The present study reports the molecular prevalence and genotypes of *E. bieneusi* in 621 pet shop puppies and 314 breeding kennel dogs in Japan. The overall prevalences of *E. bieneusi* infection were 6.1 and 11.8% in pet shop puppies and breeding kennel dogs, respectively. The prevalences are significantly different depending on facilities, and appointed facilities maintained high levels of *E. bieneusi* infection. Due to the concentrated environmental contamination and the stressful situation, which can induce an immunosuppressive status, the reinfection and/or the reactivation of microsporidiosis are likely to be the major reasons for the high levels of infection in some facilities. Regarding the sequencing data of 75 polymerase chain reaction (PCR)-positive samples, 70 isolates correspond to *E. bieneusi* genotype PtEb IX and the remaining five are identified as genotype CD7. The importance of pet shop puppies and breeding kennel dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan, since both isolated genotypes here are recognized as canine specific.

Enterocytozoon (*E.*) *bieneusi* is most commonly determined worldwide as an opportunistic pathogen in symptomatic (chronic diarrhea) human patients with immunocompromised status due to factors, such as acquired immunodeficiency syndrome (AIDS), organ transplant, and malignant diseases [1-3]. Although the accurate transmission routes and origins for human infections are poorly understood, the spore of *E. bieneusi* are shed into the feces of the infected host, and the new host can infect via the ingestion of environmental spores in contaminated water or food [2-4]. This microorganism has been reported in various mammals, including humans, wild, domestic, and companion animals [5,6]. In addition, recent molecular approaches have demonstrated that *E. bieneusi* has more than 90 genotypes, and some of them are considered zoonotic genotypes because of the determination in both humans and animals [2,5]. Therefore, animals have the potential to act as a reservoir of *E. bieneusi* transmission to humans. Dogs are likely the most important reservoir due to their close contact with humans. Epidemiological reports of *E. bieneusi* infection in dogs are increasing [7-11]. Considering the scale of research, however, only a few reports are available regarding the molecular determination of *E. bieneusi* in dogs from pet shops, which is the major source of puppies for private owners [10]. Furthermore, there is no report on the molecular detection of *E. bieneusi* in dogs of breeding kennels that are occupied the upper stream for pet shops as the place of puppy reproduction. The purpose of the present study was to investigate the molecular prevalence and was to characterize the genotypes of *E. bieneusi* in pet shop puppies and breeding kennel dogs in Japan.

A total of 621 and 314 fresh fecal samples were randomly collected on a single occasion from pet shop puppies (≤3 months old) and breeding kennel dogs (from 2 months old to 11 years old and divided into two groups: <1 year old vs. ≥1 year old), respectively, between August 2014 and July 2017. The pet shops included four pet shops located in three different prefectures (Aomori: 2 pet shops; PS-A and B, Saitama: 1 pet shop; PS-C, Ibaraki: 1 pet shop; PS-D) in east Japan. The breeding kennels consisted five breeding kennels located in five different prefectures (Miyagi: 1 breeding kennel; BK-1, Niigata: 1 breeding kennel; BK-2, Gunma: 1 breeding kennel; BK-3, Shizuoka: 1 breeding kennel; BK-4, Aichi: 1 breeding kennel; BK-5) in Japan. All fecal samples were naturally defecated, and were donated by the managers, who granted permission to include their dogs in the examination. After the evaluation of fecal condition (formed vs. soft vs. diarrhea), the spores of *E. bieneusi* were recovered using a sucrose gradient concentration method with a specific gravity of 1.26. The DNA extraction was performed using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The obtained DNA samples were stored at -20°C prior to analysis.

A nested polymerase chain reaction (PCR) assay targeting the internal transcribed spacer (ITS) region of ribosomal DNA was applied to the detection of *E. bieneusi*. In the primary reaction, forward primer EBITS3 (5' -GGTCATAGGGATGAAGAG-3') and reverse primer EBITS4 (5'-TTCGAGTTCTTTCGCGCTC-3') were used for amplification of

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an approximately 435 bp DNA fragment. In the secondary reaction, forward primer EBITS1 (5'-GCTCTGAATATCTATGGCT-3') and reverse primer EBITS2.4 (5'-ATCGCCGACGGATCCAAGTG-3') were used to amplify an approximately 390 bp fragment [12]. For the primary reaction, the PCR mixture was composed 1× buffer containing 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), and 3.0 μl of template DNA in a total reaction volume of 25 μl. For the secondary reaction, the PCR mixture was the same as that for the primary reaction, with the exception of primary PCR amplicons, which were used as a template. The following cycling parameters were used for the primary reaction: after an initial denaturation of 3 min at 94°C, 35 cycles were performed, each consisting of 30 sec at 94°C for denaturation, 30 sec at 57°C for annealing, and 40 sec at 72°C for extension, with a final extension of 10 min at 72°C. For the secondary reaction, the parameters were the same as those for the primary reaction, except that the annealing temperature was 55°C.

All secondary PCR products were examined by electrophoresis on 1.5% agarose gels. The specific DNA fragments (approximately 390 bp) were confirmed after alternative ethidium bromide staining under ultraviolet (UV) light using a transilluminator. Secondary PCR amplicons were purified using a QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the secondary primer set. Sequences were analyzed by a commercial laboratory (FASMAC Co., Ltd., Atsugi, Kanagawa, Japan). Sequence alignment and compilation were performed using the MEGA 6.06 (www.megasoftware.net) program. To determine the genotypes of *E. bieneusi*, the DNA sequences were compared to GenBank references by BLAST (Basic Local Alignment Search Tool) searches (http://www.ncbi.nlm.nih.gov/), and their similarity was determined based on the degree of sequence identity. Data were analyzed statistically using Fisher's exact probability test, with values of $P<0.05$ considered significant.

As determined by conventional PCR, we could confirm the positive samples for *E. bieneusi* infection via the specific DNA fragments at the position of approximately 390 bp (Figure 1). Overall, *E. bieneusi* infection was positive in 38 animals (6.1%) of the 621-pet shop puppies and was determined in all facilities (Table 1). The prevalence of each pet shops ranged from 1.3 to 12.2%, and the highest prevalence of PS-D (12.2%) was statistically significant in comparison to the other three pet shops (PS-A, PS-B, and PS-C). In 314 breeding kennel dogs, overall prevalence of *E. bieneusi* infection was 11.8%, which was significantly ($P<0.01$) higher than that of pet shop puppies. As in the pet shop puppies, *E. bieneusi* infection was confirmed in all breeding kennels ranging from 2.1 to 20.3%. The prevalence of BK-5 (20.3%) was significantly higher than those of three breeding kennels (BK-1, BK-2, and BK-3). In addition, although there was no statistical significance, the prevalence in <1-year-old breeding kennel dogs (22.5%) revealed a higher tendency than that of ≥1-year-old dogs (10.2%). No significant differences were observed in fecal condition in both pet shop puppies and breeding kennel dogs.

A sequencing analysis of the ITS region of ribosomal DNA fragments demonstrated that 37 of 38 PCR-positive samples from the pet shop puppies shared 99 to 100% similarity with the sequences of *E. bieneusi* genotype PtEb IX (accession number KJ668719) retrieved from the GenBank database (Table 2). Only one sample from PS-A corresponded to genotype CD7 (accession number KJ668734) with 100% similarity. In the breeding kennel dogs, 33 isolates of 37 PCR-positive samples were genotype PtEb IX (accession number KJ668719), with 99 to 100% similarity, and the remaining 4 isolates from two breeding kennels (BK-2 and BK-5) corresponded to genotype CD7 (accession number KJ668734) with 99-100% similarity (Figure 2).

Table 1. Molecular determination of *Enterocytozoon bieneusi* in pet shop puppies and breeding kennel dogs

	Examined	Positives	Prevalence (%)	*P*-value
Pet shop puppies				
Overall	621	38	6.1	-
Fecal conditions				
Formed	550	32	5.8	N.S.
Soft	53	6	11.3	-
Diarrhea	18	0	0	N.S.
Pet shops				
PS-A	235	13	5.5	<0.05
PS-B	153	5	3.3	<0.01
PS-C	77	1	1.3	<0.01
PS-D	156	19	12.2	-
Breeding kennel dogs				
Overall	314	37	11.8	-
Age				
<1-year-old	40	9	22.5	-
≥1-year-old	274	28	10.2	N.S.
Fecal conditions				
Formed	271	36	13.3	-
Soft	41	1	2.4	N.S.
Diarrhea	2	0	0	N.S.
Breeding kennels				
BK-1	48	1	2.1*	<0.01
BK-2	87	4	4.6*	<0.01
BK-3	25	1	4.0	N.S.
BK-4	31	6	19.4	N.S.
BK-5	123	25	20.3	-

*The parameters were also significant differences ($P<0.05$) when compared with BK-4

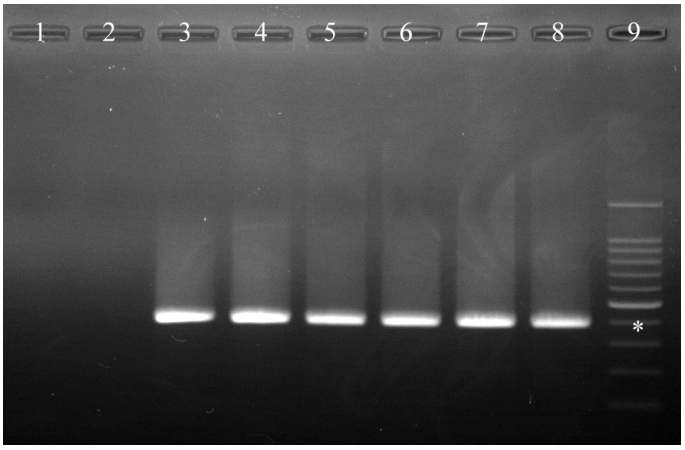

Figure 1. PCR products on 1.5% agarose gel. Lane 1-2: *Enterocytozoon bieneusi* negative control, Lane 3: *E. bieneusi* positive control, Lane 4: *E. bieneusi* genotype PtEb IX from pet shop (PS-B), Lane 5: *E. bieneusi* genotype PtEb IX from breeding kennel (BK-4), Lane 6: *E. bieneusi* genotype CD7 from pet shop (PS-A), Lane 7: *E. bieneusi* genotype CD7 from breeding kennel (BK-2), Lane 8: *E. bieneusi* genotype CD7 from breeding kennel (BK-5), Lane 9: 100 bp DNA ladder. *Indicates approximately 390 bp

We began the present study with the hypothesis that the dogs kept in pet shops and breeding kennels indicated the markedly high prevalence of *E. bieneusi* infection, likely due to the intestinal protozoan *Giardia* and *Cryptosporidium* [13-17]. Contrary to our expectation, the present study demonstrated the low levels of *E. bieneusi* infection, such as the overall prevalences of 6.1 and 11.8% in pet shop puppies and breeding

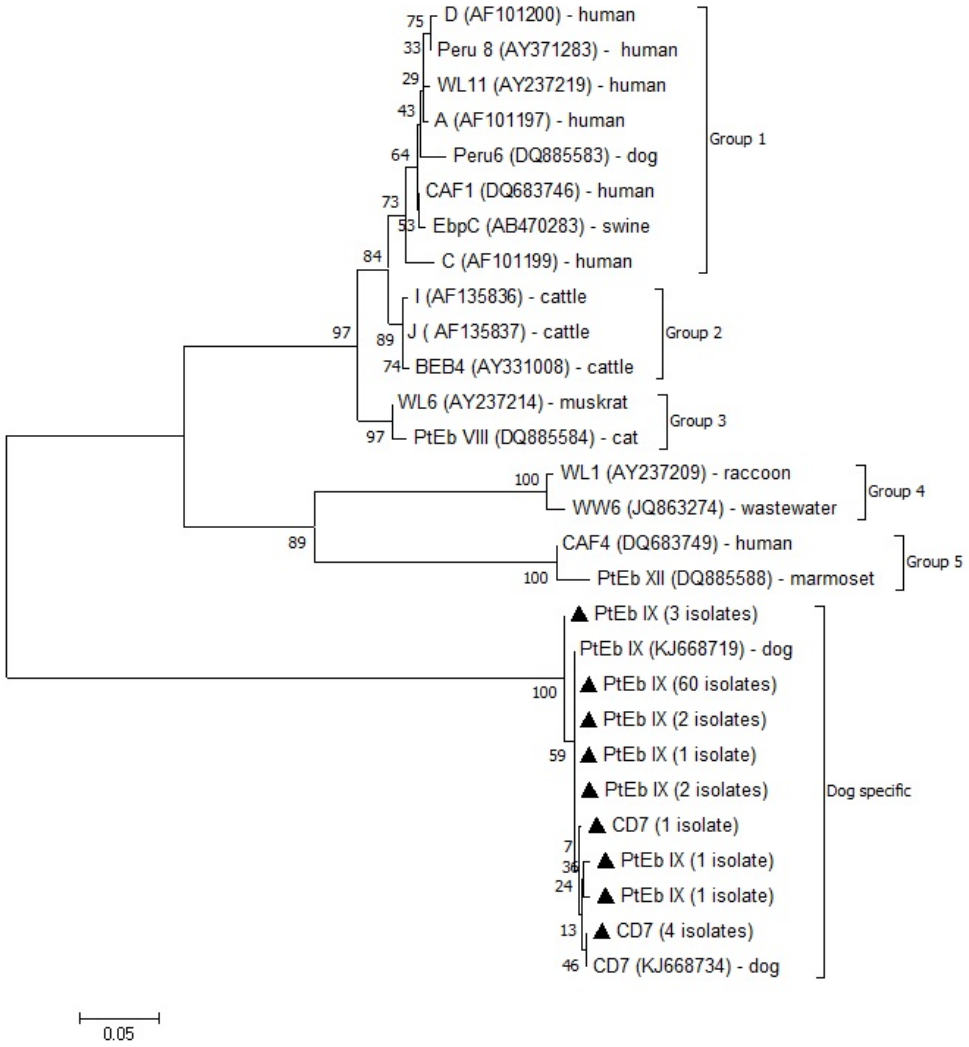

Figure 2. Phylogenetic analysis of ITS nucleotide sequences of *Enterocytozoon bieneusi* genotypes isolated from pet shop puppies and breeding kennel dogs in the present study
▲=Isolates from dogs in the present study

Table 2. *Enterocytozoon bieneusi* genotypes in pet shop puppies and breeding kennel dogs

	genotypes
Pet shop puppies	
Pet shops	
PS-A	PtEb IX (12)*, CD7 (1)
PS-B	PtEb IX (5)
PS-C	PtEb IX (1)
PS-D	PtEb IX (19)
Breeding kennel dogs	
Breeding kennels	
BK-1	PtEb IX (1)
BK-2	PtEb IX (3), CD7 (1)
BK-3	PtEb IX (1)
BK-4	PtEb IX (6)
BK-5	PtEb IX (22), CD7 (3)

kennel dogs, respectively. Regarding dogs kept in pet shops and pet markets, approximately the same level molecular prevalence levels have been reported in China: 7.7% (2/26) [7], 14.8% (16/108) [9], and 5.9% (19/322) [10]. The current prevalence recorded in breeding kennel dogs is impossible to compare due to the lack of previous data from breeding kennels. However, in pet shop puppies, the overall prevalence of each pet shop ranged from 1.3 to 12.2%, and only one shop had a prevalence (PS-D; 12.2%) significantly higher than others. Moreover, in breeding kennels, including young dogs as well as adult animals, two facilities of BK-4 (19.4%) and BK-5(20.3%) recorded higher prevalences than others (2.1-4.6%). The lower prevalence determined here in some pet shops and breeding kennels were the same as the level recorded previously in private household dogs of 4.9% (4/82) in Poland [11], and 1.4% (2/141) in China [9]. It is well demonstrated that the immune response is important to control the opportunistic Microsporidia infections. The immature and suppressive status of immune systems is a risk factor for microsporidiosis [1-4,18]. Therefore, younger dogs are at high risk due to their immature immune systems [19,20]. However, the present results suggest that the prevalence of *E. bieneusi* infection is affected by the condition of the facility rather than the age of dogs because the prevalences of pet shop puppies and breeding kennel dogs are significantly different depending on facilities and appointed facilities maintained high levels of *E. bieneusi* infections. In addition, the prevalence of adult dogs kept in breeding kennels (10.2%) is significantly (P<0.05) higher than that of pet shop puppies (6.1%). Microsporidia spores are resistant in environments for extended time

periods; therefore, there is a potential for infection from environmental contamination [1,3]. Contaminated water is one of the primary causes of human microsporidiosis [2-4]. We did not evaluate the sanitary management and environmental condition in each facility. However, the reinfection and/or the reactivation of microsporidiosis according to the concentrated environmental contamination by *E. bieneusi* spores and the frequent close contact with other dogs in the limited space, which is a stressful situation for animals and can induce the immunosuppressive status, are likely to be major causes of high levels of infection in some facilities [4,17,18,21,22]. The results of the present study suggest that there is no correlation between *E. bieneusi* infection and fecal condition disorder (soft or diarrhea). Despite the fact that the etiology and pathogenicity of Microsporidia have not been clarified, the cases of asymptomatic spore shedding are recognized in dogs and humans, including both immunocompromised and immunocompetent individuals [2,4,9,23]. The immunosuppressed trigger, for example human immunodeficiency virus (HIV) infection, is also indicated to contributes to the symptomatic infections [2,18,24,25].

The present sequencing data of PCR-positive samples demonstrates the dominancy of *E. bieneusi* genotype PtEb IX in pet shop puppies and breeding kennel dogs in Japan, because this genotype was isolated from all facilities of pet shops and breeding kennels. It is easy to understand this result, because the genotype PtEb IX is recognized as a canine-specific genotype and its isolation is restricted to dogs worldwide, except for a few rare cases [8-11,26,27]. Five isolates of the *E. bieneusi* genotype CD7, which derive from one pet shop (PS-A) and two breeding kennels (BK-2 and BK-5), have been determined here for first time in Japan. Genotype CD7 has recently been described in China as the non-dominant canine-specific genotype, although it has not been reported in other countries [8,9]. The results suggest that at least two canine-specific *E. bieneusi* genotypes invade dogs in Japan, and the importance of pet shop puppies and breeding kennel dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict interest.

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