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Short Communication

Molecular detection and genotyping of *Enterocytozoon bieneusi* in family pet dogs obtained from different routes in Japan

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ABSTRACT

The present study determined the prevalence of *Enterocytozoon (E.) bieneusi* infection and genotyped the isolates in family pet dogs obtained from three different routes in Japan. Fresh fecal samples were collected from 597 family pet dogs. A nested polymerase chain reaction (PCR) assay targeting the internal transcribed spacer (ITS) region of ribosomal DNA was employed for detection of *E. bieneusi*, and the obtained positive PCR amplicons were sequenced for genotyping. The overall prevalence of *E. bieneusi* in family pet dogs was 4.4%. Significant differences in prevalence were found among the categories of age (< 1 year: 8.3% vs. ≥1 year: 3.4%), obtained route (from private owner: 3.2% vs. from pet shop: 3.9% vs. from breeding kennel: 14.3%), and living regions (ranged 0–10.3%). However, no significant differences were observed in the prevalence of living condition (indoor: 4.6% vs. outdoor: 2.0%), and fecal condition (formed: 4.5% vs. soft: 4.7% vs. diarrhea: 0%). Of the 26 sequenced samples, all were identified as genotype PtEb IX, which belongs to the dog-specific genotype. In conclusion, although *E. bieneusi* infections are relatively common in family pet dogs in Japan, the risk of zoonotic transmission from dogs to humans is likely to be low.

1. Introduction

Microsporidia are single-celled obligate intracellular protozoans, and widely infect animals. There are approximately 1200 species in this group [1,2]. *Enterocytozoon (E.) bieneusi* is the most commonly identified Microsporidia in humans and has also been reported worldwide in many animals including pets and livestock [2]. *E. bieneusi* is considered to be an opportunistic pathogen, including > 90 genotypes, and can cause diarrhea in humans [2,3]. Thus far, there has been no evidence that *E. bieneusi* can induce digestive obstruction in dogs until now, although the relationship to diarrhea has been suggested [4]. In contrast, some *E. bieneusi* isolates from dogs have the potential for zoonotic transmission, because some specific genotypes have been detected in both dogs and humans [5]. Therefore, dogs have the potential to act as reservoirs of human *E. bieneusi* infection. Considering the scale of research, to date, only a few reports are available regarding the molecular epidemiological discussion of *E. bieneusi* in family pet dogs that are in close contact with humans [4,6,7]. The purpose of the present study was to determine the molecular prevalence and genotypes of *E. bieneusi* in family pet dogs obtained from different routes in Japan.

2. Materials and methods

2.1. Study design, study animals and sampling

Between October 2013 and December 2016, a total of 597 fresh fecal samples were randomly collected on a single occasion from family pet dogs, from nine veterinary clinics located in six different regions (Hokkaido: 1 clinic, Tohoku: 3 clinics, Kanto: 2 clinics, Kinki: 1 clinic, Kyushu: 1 clinic, Okinawa: 1 clinic) in Japan. All animals were kept in families as pet dogs and were presented to veterinary clinics with or without the history of illness. The owners obtained their dogs from three different routes (from private owner, pet shop, and breeding kennel) at time of puppies (2–3 months old). The fecal samples were donated by the owners, who granted permission to include their dogs in the examination.

2.2. DNA extraction from fecal samples

The spores of *E. bieneusi* were isolated using a sucrose gradient concentration method with a specific gravity of 1.26, and DNA extraction was performed using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The

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obtained DNA samples were stored at −20 °C prior to analysis.

2.3. PCR amplification

A nested polymerase chain reaction (PCR) assay targeting the ITS region of ribosomal DNA was employed for the detection of *E. bieneusi*. For primary reaction, forward primer EBITS3 (5′ -GGTCATAGGGATG AAGAG-3′) and reverse primer EBITS4 (5′-TTCGAGTTCTTTCGCG CTC-3′) were used to amplify a DNA fragment of approximately 435 bp. In the secondary reaction, forward primer EBITS1 (5′-GCTCTGAATAT CTATGGCT-3′) and reverse primer EBITS2.4 (5′ -ATCGCCGACGGATC CAAGTG-3′) were used to amplify a fragment of approximately 390 bp [8]. For the primary reaction, the PCR mixture comprised 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 s at 94 °C for denaturation, 30 s at 57 °C for annealing, and 40 s 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 identified by electrophoresis on 1.5% agarose gels. The specific DNA fragments (approximately 390 bp) were confirmed after alternative ethidium bromide staining under UV light using a transilluminator.

2.4. Sequencing analysis

Secondary PCR amplicons of the predicted size 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 Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/), and their similarity was determined based on the degree of sequence identity.

2.5. Statistical analysis

The data were stratified according to age group (< 1 year vs. ≥ 1 year), fecal condition (formed vs. soft vs. diarrhea), living condition (indoor vs. outdoor), obtained route (from private owner vs. from pet shop vs. from breeding kennel), and living region (Hokkaido, Tohoku, Kanto, Kinki, Kyushu, Okinawa). Data were analyzed statistically using Fisher's exact probability test, with values of $P < 0.05$ considered significant.

3. Results

Of the 597 family pet dogs, 26 dogs (4.4%) were positive for *E. bieneusi*. The prevalence of *E. bieneusi* in < 1 year-old dogs (8.3%) was significantly higher than that in ≥ 1 year-old dogs (3.4%) (cf. Table 1). In the obtained routes, the prevalence of dogs from breeding kennel was significantly higher (14.3%) than those of pet shop (3.9%) and private owner (3.2%). *E. bieneusi* was determined in all regions, except for Okinawa. The prevalence in Kinki (10.3%) was significantly higher than those in Kanto (3.1%) and in Okinawa (0%), respectively. In contrast, no significant differences were observed in fecal condition and living condition. All 26 samples positive for *E. bieneusi* were found to have 99% to 100% similarity to the sequence of genotype PtEb IX (accession number DQ885585) in GenBank.

Table 1
Prevalence of *Enterocytozoon bieneusi* in family pet dogs obtained from different routes in Japan.

Category	Examined number	Positive	Prevalence (%)	*P* value
Age				
< 1 year old	121	10	8.3	–
≥ 1 year old	476	16	3.4	< 0.05
Fecal condition				
Formed	529	24	4.5	NS
Soft	43	2	4.7	–
Diarrhea	25	0	0	NS
Living condition				
Indoor	547	25	4.6	–
Outdoor	50	1	2.0	NS
Obtained route				
From private owner	95	3	3.2	< 0.05
From pet shop	467	18	3.9	< 0.05
From breeding kennel	35	5	14.3	–
Living region				
Hokkaido	48	4	8.3	NS
Tohoku	291	14	4.8	NS
Kanto	142	3	2.1	< 0.05
Kinki	39	4	10.3	–
Kyusyu	25	1	4.0	NS
Okinawa	52	0	0	< 0.05

NS: Not significant.

4. Discussion

The present study is the first report investigating the prevalence of *E. bieneusi* in family pet dogs from veterinary clinics in Japan. The results suggest that *E. bieneusi* infection is at low level but is common in family pet dogs in Japan. Although the prevalence was 4.4%, *E. bieneusi* was widely detected in all analytic categories, except for diarrhea in fecal condition and Okinawa in the living region. Previous studies have reported that the molecular prevalence of *E. bieneusi* infection in pet dogs was 4.9% (4/82) in Poland [4], 11.7% (23/197) in China [6], and 1.4% (2/141) in China [7]. We cannot compare the prevalence simply due to the different composition of age, region, and living condition in examined dogs between the previous studies and the present study. However, those results suggest that *E. bieneusi* infection was not frequently diagnosed in family pet dogs.

According to the categories, the present study revealed a significantly higher presence of *E bieneusi* in dogs of < 1 year-old. Further, significantly higher prevalence was recorded for the dogs of breeding kennel origin. Moreover, the Kinki region showed significantly high levels of infection compared to that in the regions of Kanto and Okinawa. We do not have sufficient answers for the factors affecting the significantly higher prevalence, since the details of immune response to Microsporidia infections including *E. bieneusi* and the etiology in hosts remains unknown. Generally, it has been demonstrated that the cell-mediated immune response, especially CD8+ T lymphocytes rather than CD4+ T lymphocytes, is more critical to control Microsporidia infections than the humoral immune response because Microsporidia is capable of invading the host cells, and this microorganism subsequently undergoes intracellular multiplication [3,9–11]. Although the percentage in the blood CD4+ T cells are stable from birth to adulthood in dogs, the percentage of CD8+ T cells are lower in younger dogs of under 6 months-old than in adult dogs of over 1 year-old [12,13]. In addition, the response to stimulation of lymphocytes in younger dogs is lower than that in adult dogs [13]. Therefore, these insights, which suggest an immature immune system, are presumed to be one of the factors for the higher *E. bieneusi* prevalence in dogs of < 1 year-old. For the higher prevalence in dogs originating from breeding kennels, one of

the suspected causes is the moderately higher percentage of dogs < 1 year-old occupying total examined numbers, such as 31.4% (11/35), compared to those of 11.6% (11/95) originating from private owners, and 21.2% (99/467) of pet shop origin. In addition, if we include the animals aged 1 year-old with dogs < 1 year-old, the percentages of younger dogs in the total examined numbers drastically changed to 51.4% (18/35), 18.9% (18/95) and 27.8% (130/467) in dogs obtained from breeding kennel route, private owner route, and pet shop route, respectively. Thus, the principle factor that was responsible for the higher prevalence in dogs obtained from breeding kennel route is likely to be larger number of younger dogs, whose immune system is not sufficiently development. Although the information on the sanitary management of each facility was not evaluated here, the findings of the present study strongly indicates that the breeding kennel is a major source of *E. bieneusi* infection in dogs. The same factor caused the higher presence of *E. bieneusi* in dogs from the Kinki region because the percentages of dogs < 1 year-old were 64.1% (25/39) in Kinki, 14.8% (21/142) in Kanto, and 3.8% (2/52) in Okinawa, respectively. Similar to the previous reports, the results of the present study suggest that *E. bieneusi* infection is not always associated with digestive tract obstruction and many asymptomatic subclinical cases are recognized in dogs [7,14], as there is no correlation between the fecal condition and the detection of *E. bieneusi*. Further in humans, some reports suggest that Microsporidia can induce asymptomatic shedding of spores not only in immunocompromised populations but also in immunocompetent individuals, and the trigger, such as human immunodeficiency virus (HIV) infection contributes to the development of clinical signs [3,9–11].

Based on the PCR amplicon sequencing, all 26 samples were identified as *E. bieneusi* genotype PtEb IX. This genotype has been recognized as a canine-specific genotype because it has been reported only in dogs worldwide, including Japan, and there is no information about its detection in humans and other animals [2,3,6,7,15,16]. Therefore, the role of family pet dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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