Molecular epidemiological study of

Enterocytozoon bieneusi infection in dogs, in Japan

Totsapon PHROMPRAPHAI

日本国内の犬における

Enterocytozoon bieneusi 感染の分子疫学的研究

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Introduction

Enterocytozoon bieneusi (*E. bieneusi*) is an obligate intracellular parasite belonging to a fungal group identified as Microsporidia [27]. *E. bieneusi* infection has been reported worldwide in many animals including domestic animals and over 90 genotypes have been identified [19, 27, 38]. Nine groups were recognized in *E. bieneusi* genotypes: group 1; containing the zoonotic genotypes, groups 2-9; containing host-adapted genotypes including dogs [13, 41]. The infective spores of *E. bieneusi* are excreted with feces from an infected host into the environment [38]. Since the fecal-oral route of infection is the most frequent, the primary site of infection is through the enterocytes of small intestine. However, *E. bieneusi* spores can be ingested or inhaled, so the transmission route in the case presented herein remains unclear [16]. *E. bieneusi* can cause abdominal cramping, nausea, weight loss, and severe diarrhea in humans, especially in elderly people, children, and immunocompromised patients with human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) [4, 26, 30, 44]. Studies on *E. bieneusi* infection in humans have been widely reported in the world [4, 26, 27, 30, 45, 48]. Approximate prevalences of 0.2-22.5% were demonstrated in China [22, 43, 46, 48], Australia [49], Iran [40], Nigeria [33], India [15], and Thailand [30]. Unfortunately, the molecular epidemiological studies on *E. bieneusi* infection in humans were not available in Japan. Presently, data released by the Internal Affairs and Communications Ministry showed that the total population of Japan is 126.71 million people as of October 1, 2017. The number of the peoples aged over 65 years old is 35.15 million and the percentage of those population reaches to 27.7% [28]. So, Japan has the highest percentage of the elderly in the world and the National Institute of Population and Social Security Research estimates that the ratio of elder population will occupy 35.3% in 2040 [31]. In addition, from 2000 to 2016, newly reported cases of HIV/AIDS in Japan are more than twice, with 70% of the cases being individuals in

their 20s and 30s (early adulthood) [32]. In 2016, around 1,011 HIV and 437 AIDS cases were newly reported in Japan. Therefore, a large proportion of the Japanese, especially in elder people and HIV/AIDS patients, have the high risk potential for *E. bieneusi* infection. On the other side, *E. bieneusi* has also been reported worldwide in dogs. Previous studies have detected *E. bieneusi* in dogs in Portugal [23], Poland [34], Colombia [37], Switzerland [25], and China [14]. According to relationship between dogs and humans, the dogs have the potential to be most important reservoir of *E. bieneusi* infection due to their close contact. Indeed, some *E. bieneusi* isolates from dogs have the potential for zoonotic transmission, because several same genotypes have been determined in both humans and dogs, such as genotypes D, Type IV, WL11, and Peru 6 [20, 27, 38] (Table 1). However, only a few reports are available regarding the molecular determination of *E. bieneusi* in dogs, in Japan. In 2009, a previous study demonstrated that the prevalence of *E. bieneusi* in dogs was 2.5% (2/79) in Japan [1]. The molecular epidemiological study of *E. bieneusi* infection in dogs, in Japan has not been updated.

The purpose of the present study was to investigate the molecular prevalence and determine the genotypes of *E. bieneusi* isolates in dogs and to evaluate the potential for zoonotic transmission from dogs to humans, in Japan. The present study is divided into three chapters as follows: Chapter 1 - Molecular determination and genotyping of *E. bieneusi* in family pet dogs, in Japan; Chapter 2 - Molecular determination and genotyping of *E. bieneusi* in pet shop puppies, in Japan; and Chapter 3 - Molecular determination and genotyping of *E. bieneusi* in breeding kennel dogs, in Japan.

Chapter 1

Molecular detection and genotyping of *E. bieneusi* in family pet dogs, in Japan

1. Introduction

In the present society, dogs are increasingly viewed as an important part of the family. Most of pet owners consider their dogs to be true family members, equal in status to children. However, many people don't realize that dogs can transmit the some infectious diseases to humans by direct and/or indirect contact. *E. bieneusi* is one of the most commonly identified Microsporidia in humans and has also been reported worldwide in many animals including pets and livestock [38]. *E. bieneusi* can cause severe diarrhea in humans, particularly in children and the elder ones [27, 38]. A parts of *E. bieneusi* isolates from dogs have the potential for zoonotic transmission [37]. Therefore, dogs have the potential to act as a reservoir of *E. bieneusi* transmission to human.

Considering the scale of research, to date, only a few reports are available regarding the molecular epidemiological discussion of *E. bieneusi* infection in family pet dogs that are in close contact with humans [14, 20, 34]. In 2009, a previous study demonstrated that the prevalence of *E. bieneusi* infection in pet dogs was 0% (0/1) in Japan [1], however, the samples size was too small. Therefore, 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

1) Fecal samples

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, and 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 the 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) DNA extraction

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

3) PCR amplification

A nested polymerase chain reaction (PCR) assay targeting the internal transcribed spacer (ITS) region of ribosomal DNA was employed for the detection of *E. bieneusi*. For primary reaction, forward primer EBITS3 (5'-GGTCATAGGGATGAAGAG-3') and reverse primer EBITS4 (5'-TTCGAGTTCTTTCGCGCTC-3') were used to amplify a DNA fragment of approximately 435 bp. In the secondary reaction, forward primer EBITS1 (5'-GCTCTGAATATCTATGGCT-3') and reverse primer EBITS2.4 (5'-ATCGCCGACGGATCCAAGTG-3') were used to amplify a DNA fragment of approximately 390 bp [36]. 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 preformed, each consisting of 30 sec 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 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.

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 complication 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.

5) Statistical analysis

The data were stratified according to age group (<1-year-old vs. ≥1-year-old), 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, and Okinawa). Data were analyzed statistically using Fisher's exact probability test, with values of $P < 0.05$ considered significant.

3. Results

As determined by conventional PCR, the present study could confirm the positive samples for *E. bieneusi* infection via the specific DNA fragments at the positive of approximately 390 bp (Figure 1). 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 in ≥1-year-old dogs (3.4%) (Table 2). In the obtained routes, the prevalence of dogs from breeding kennel (14.3%) was significantly higher 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 isolates positive for *E. bieneusi* were found to have 99 to 100% similarity to the sequence of genotype PtEb IX (accession number DQ885585) in GenBank.

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 [34], 11.7% (23/197) in China

[14], and 1.4% (2/141) in China [20]. The author 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. Author does 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 [6, 27, 29, 42]. 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 [2, 5]. In addition, the response to stimulation of lymphocytes in younger dogs is lower than that in adult dogs [2]. 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 kennel, 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 owner, and 21.2% (99/467) of pet shop origin. In addition, if author includes the animals aged just 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 indicate that the breeding kennel is a major source of *E. bieneusi* infection in dogs. The same factor caused the higher presence of *E. bieneusi* infection 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 [13, 20], as there is no correlation between the fecal condition and the detection of *E. bieneusi*. Further in humans, same 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 [6, 27, 29, 42].

Based on the PCR amplicon sequencing, all 26 samples were identified as *E. bieneusi* genotype PtEb IX. This genotype has been recognized as dog-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 [1, 14, 20, 27, 38, 47]. Therefore, the role of family pet dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan.

The present study is the first report investigating the molecular prevalence of *E. bieneusi* in family pet dogs, in Japan. The results suggest that *E. bieneusi* infection is at low level but is common in family pet dogs, in Japan. The risk for zoonotic transmission of *E. bieneusi* from

family pet dogs to humans is likely to be low, because all sequenced samples were identified as dog-specific genotype.

Chapter 2

Molecular detection and genotyping of *E. bieneusi* in pet shop puppies, in Japan

1. Introduction

It is no doubted that pet shops are the most common source of puppies for private owners. In addition, previous studies demonstrated that pet shop puppies had the high prevalences of intestinal parasites including zoonotic protozoan such as *Giardia duodenalis* and *Cryptosporidium* spp. [10, 12]. In Chapter 1, author showed that *E. bieneusi* infection was higher prevalent in young dogs <1-year-old. Therefore, it is easy to have the hypothesis that *E. bieneusi* infection is significantly higher prevalent in pet shop puppies. However, there are only a few available reports regarding *E. bieneusi* infection in pet shop puppies [1]. Additionally, to my knowledge, the genotypes of *E. bieneusi* in pet shop puppies are unknown in Japan.

The purpose of the present study was to determine the prevalence of *E. bieneusi* in pet shop puppies and to characterize the genotype of isolates using molecular technique. And author discussed the potential for zoonotic transmission of *E. bieneusi* from pet shop puppies to humans in Japan.

2. Materials and Methods

1) Fecal samples

A total of 621 fresh feces were randomly collected on a single occasion from pet shop puppies (≤3-months-old), 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. All fecal samples were naturally defecated,

and were donated by the managers, who granted permission to include their dogs in the examination.

2) DNA extraction, PCR amplification, and sequencing analysis

The isolation of *E bieneusi* spores, DNA extraction, PCR technique, and sequencing analysis were same as Chapter 1.

3) Statistical analysis

The data were stratified according to fecal condition (formed vs. soft vs. diarrhea), and pet shop location. Data were analyzed statistically using Fisher's exact probability test, with values of $P < 0.05$ considered significant.

3. Results

Overall, *E. bieneusi* infection was positive in 38 animals (6.1%) of the 621 pet shop puppies and was determined in all facilities (Table 3). The prevalence of each pet shop 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). No significant differences were observed in fecal condition in pet shop puppies.

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. Only one sample from PS-A corresponded to genotype CD7 (accession number KJ668734) with 100% similarity (Figure 2).

4. Discussion

The present study is the first large-scale investigation of the prevalence and genotype determination of *E. bieneusi* in pet shop puppies, in Japan. Previously, the prevalence of *E. bieneusi* in pet shops and pet market have been recorded as 7.7% (2/26), 14.8% (16/108), and 5.9% (19/322) in China [20, 47, 48] and 5% (1/20) in Japan [1]. As same as Chapter 1, the author 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. 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. The lower prevalence determined here in some pet shops were the same as the level recorded previously in private household dogs of 4.9% (4/82) in Poland [34], 1.4% (2/141) in China [20], and 4.4% (26/597) in Chapter 1. It is well demonstrated that the immune response is important to control the opportunistic Microsporidia infections [6, 29, 42]. In young animals, the immune immaturity and immune suppressive status is a risk factor for microsporidiosis [2, 5]. 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 are significantly different depending on facilities and appointed facilities maintained high levels of *E. bieneusi* infections. *E. bieneusi* spores appear to be relatively resistant and can survive for several months under environmental conditions [18, 21]. Therefore, there is a potential for infection from environmental contamination [3, 9]. The present study suggests that the major factor influencing the higher *E. bieneusi* infection in pet shop puppies was a high density of dogs in pet shops with insufficient sanitation control [50]. Unfortunately, the information of sanitary management in each facility was not evaluated in the present study, but it is likely to be a major factor. The results of the present study suggest that there is no correlation between *E. bieneusi* infection and fecal condition disorder (soft or diarrhea). In addition, it is suggested that

the dogs, even when they are apparently normal, can shed the spores in feces at any times and become an asymptomatic carrier of *E. bieneusi* [26, 39].

The sequencing data of PCR positive samples demonstrates the dominancy of *E. bieneusi* genotype PtEb IX in pet shop puppies, in Japan, because this genotype was isolated from all pet shops. The genotype PtEb IX is recognized as a dog-specific genotype and its isolation is restricted to dogs worldwide, for example in Switzerland [25], in Portugal [23], in Colombia [37], in China [14, 20, 47], and in Poland [34]. One isolates of the *E. bieneusi* genotype CD7, which derive from one pet shop (PS-A), has been determined here for first time in Japan. Genotype CD7 has recently been described in China as the non-dominant dog-specific genotype [14, 20].

The present results suggest that the importance of pet shop puppies as reservoirs for *E. bieneusi* transmission to humans is low in Japan, since PtEb IX and CD7 genotypes isolated here are recognized as dog-specific genotypes.

Chapter 3

Molecular detection and genotyping of *E. bieneusi* in breeding kennel dogs, in Japan

1. Introduction

Breeding kennels occupy the upper stream for pet shops as the most major provider of puppies. It is well known that breeding kennel dogs including adult animals harbor intestinal parasites at high level percentage [11]. In addition, the high prevalent intestinal parasites in breeding kennel dogs are including zoonotic protozoan such as *Giardia duodenalis* and *Cryptosporidium* spp. [8, 11]. Therefore, breeding kennel dogs have the potency as reservoirs of zoonotic intestinal parasites. In Chapter 1, author showed that *E. bieneusi* infection was significantly higher prevalent in dogs from breeding kennel route. Moreover, in Chapter 2, the appointed pet shop puppies revealed high prevalence of *E. bieneusi* infection. Consideration these situation, author suspects that breeding kennel dogs have high level *E. bieneusi* infection. However, there is no available epidemiological report worldwide on the molecular detection of *E. bieneusi* infection in dogs of breeding kennels.

The purpose of the present study was to investigate the molecular prevalence and was to characterize the genotypes of *E. bieneusi* in breeding kennel dogs, in Japan.

2. Materials and Methods

1) Fecal samples

A total of 314 fresh fecal samples were randomly collected on a single occasion from breeding kennel dogs (from 2-months-old to 11-years-old and divided into two groups: <1-year-

old vs. ≥1-year-old), between August 2014 and July 2017. The breeding kennels consisted five breeding facilities 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, and 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.

2) DNA extraction, PCR amplification, and sequencing analysis

The isolation of *E bieneusi* spores, DNA extraction PCR technique, and sequencing analysis were performed as same as in Chapter 1.

3) Statistical analysis

The data were stratified according to age group (<1-year-old vs. ≥1-year-old), fecal condition (formed vs. soft vs. diarrhea), and breeding kennel location. Data were analyzed statistically using Fisher's exact probability test, with values of P <0.05 considered significant.

3. Results

Overall, *E. bieneusi* infection was positive in 37 animals (11.8%) of the 314 breeding kennel dogs, and was confirmed in all breeding kennels ranging from 2.1 to 20.3%. (Table 4). 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 breeding kennel dogs.

A sequencing analysis of the ITS region of ribosomal DNA fragments demonstrated that 33 isolates of 37 PCR positive samples shared 99 to 100% similarity with the sequences of *E. bieneusi* genotype PtEb IX (accession number KJ668719) retrieved from the GenBank database, 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 3).

4. Discussion

The hypothesis of the breeding kennel dogs indicated the markedly high prevalence of *E. bieneusi* infection, likely due to the intestinal protozoan *Giardia* spp. and *Cryptosporidium* spp. [7, 10-12]. Contrary to author's expectation, the present study demonstrated the low level of *E. bieneusi* infection such as 11.8%, but which was significantly (P<0.01) higher than that of pet shop puppies in Chapter 2 (6.1%). The current prevalence recorded in breeding kennel dogs is impossible to compare due to the lack of previous data from breeding kennels. However, 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 breeding kennels were the same as the level recorded previously in private household dogs of 4.9% (4/82) in Poland [34], and 1.4% (2/141) in China [20]. 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 [3, 4, 9, 27, 29]. Therefore, younger dogs are at high risk due to their immature immune systems [2, 5]. However, as same as Chapter 2, 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 breeding kennel dogs are significantly different depending on facilities and appointed breeding kennels 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 in Chapter 2 (6.1%). Microsporidia spores are resistant in environments for extended time periods; therefore, there is a potential for infection from environmental contamination [3, 9]. Contaminated water is one of the primary causes of human microsporidiosis [4, 9, 27]. The present study 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, 24, 29, 35]. 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 [4, 13, 20, 27]. The immunosuppressed trigger, for example human immunodeficiency virus (HIV) infection, is also indicated to contribute to the symptomatic infections [6, 27, 29, 42].

The present sequencing data of PCR positive samples demonstrates the dominancy of *E. bieneusi* genotype PtEb IX in breeding kennel dogs, in Japan, because this genotype was isolated from all facilities of breeding kennels. It is easy to understand this result, because the genotype PtEb IX is recognized as a dog-specific genotype and its isolation is restricted to dogs worldwide, except for a few rare cases [1, 14, 20, 34, 37, 47]. Four isolates of the *E. bieneusi* genotype CD7, which derive from 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 dog-specific genotype, although it has not been reported in other countries [14, 20].

The results suggest that at least two dog-specific *E. bieneusi* genotypes invade dogs in Japan, and the importance of breeding kennel dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan.

Summary

Introduction: *Enterocytozoon bieneusi* is the most common opportunistic pathogen in symptomatic (chronic diarrhea) humans with immunocompromised status such as acquired immunodeficiency syndrome (AIDS), organ transplant, and malignant diseases. 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. This microorganism has been reported in various mammals, including humans, wild, domestic, and companion animals. 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. 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. Considering the scale of research, however, only a few reports are available regarding the molecular determination of *E. bieneusi* in family pet dogs and pet shop puppies. 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 puppies reproduction. The purpose of the present study was to investigate the molecular prevalence and was to characterize the genotypes of *E. bieneusi* in dogs, in Japan. The present study is divided into three chapters. The title of each chapter is follows: Chapter 1 - Molecular determination and genotyping of *E. bieneusi* in family pet dogs, in Japan; Chapter 2 - Molecular determination and genotyping of *E. bieneusi* in pet shop puppies, in Japan; and Chapter 3 - Molecular determination and genotyping of *E. bieneusi* in breeding kennel dogs, in Japan.

Chapter 1: Molecular determination and genotyping of *E. bieneusi* in family pet dogs, in Japan. 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, and 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 to 3 months old). 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 obtained DNA samples were stored at -20°C prior to analysis. A nested polymerase chain reaction (PCR) assay targeting the ITS region of ribosomal DNA was employed for the detection of *E. bieneusi*. To determine the genotypes of *E. bieneusi*, the DNA sequences were compared to GenBank database. The present study is the first report investigating the prevalence of *E. bieneusi* in family pet dogs from veterinary clinics in Japan. 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%). In the obtained routes, the prevalence of dogs from breeding kennel (14.3%) was significantly higher than those of pet shop (3.9%) and private owner (3.2%). *E. bieneusi* was determined in all regions, except for Okinawa. 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). This genotype has been recognized as a dog-specific genotype. From the above results, it is suggested that *E. bieneusi* is at low level but is common in family pet dogs, in Japan. Immature immune system is presumed to be one of the risk factors for higher infection rate in dogs of <1-year-old. For the higher prevalence in dogs originating

from breeding kennel, one of the suspected causes is the moderately higher percentage of dogs <1-year-old occupying total examined numbers. The risk of zoonotic transmission from family pet dogs to humans is likely to be low, because all sequenced samples were identified as dog-specific genotype.

Chapter 2: Molecular determination and genotyping of *E. bieneusi* in pet shop puppies, in Japan. A total of 621 fresh fecal samples were randomly collected on a single occasion from pet shop puppies (≤ 3 months old), from four pet shops (PS-A to PS-D) located in three different prefectures in Japan. The isolation of *E. bieneusi* spores, DNA extraction, and PCR operations were performed as same as described in Chapter 1. As determined by conventional PCR, *E. bieneusi* infection was positive in 38 animals (6.1%) of the 621 pet shop puppies and was found in all facilities. The prevalence of each pet shop 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). No significant differences were observed in fecal condition. A sequencing analysis of the DNA fragments demonstrated that 37 of 38 PCR positive samples shared 99 to 100% similarity with the sequences of *E. bieneusi* genotype PtEb IX (accession number KJ668719) retrieved from the GenBank database. Only one sample from PS-A corresponded to genotype CD7 (accession number KJ668734) with 100% similarity. The present study found genotype CD7, which has been recognized as a dog-specific genotype, in dogs for the first time in Japan. The data suggests that *E. bieneusi* is at low level in pet shop puppies, in Japan. Unfortunately, the information of sanitary management in each pet shop could not evaluate. However, the results suggest that the major factor influencing the higher *E. bieneusi* infection in pet shops was insufficient sanitation control. The role of pet shop puppies as reservoir for *E. bieneusi* transmission to humans is likely to be low in Japan.

Chapter 3: Molecular determination and genotyping of *E. bieneusi* in breeding kennel dogs, in Japan. A total of 314 fresh fecal samples were randomly collected from breeding kennel dogs

(from 2-months-old to 11-years-old and divided into two groups: <1-year-old vs. ≥1-year-old). The breeding kennels consisted five breeding kennels (BK-1 to BK-5) located in five different prefectures, in Japan. The present study is the first one reporting the molecular prevalence of *E. bieneusi* in breeding kennel dogs, in Japan. Overall, prevalence of *E. bieneusi* infection was 11.8% and was found 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 dogs (22.5%) revealed a higher tendency than that of ≥1-year-old dogs (10.2%). Moreover, two facilities of BK-4 (19.4%) and BK-5 (20.3%) recorded higher prevalence than other facilities. No significant differences were observed in fecal condition. A sequencing analysis demonstrated that 33 of 37 PCR positive samples shared 99 to 100% similarity with the sequences of *E. bieneusi* genotype PtEb IX (accession number KJ668719) retrieved from the GenBank database and the remaining 4 isolates from two breeding kennels (BK-2 and BK-5) corresponded to genotype CD7 (accession number KJ668734) with 99 to100% similarity. The above results suggest that the prevalence of *E. bieneusi* infection is affected by the condition of the facility rather than the ages of dogs. There is a potential for infection from environment contamination. The reinfection and/or the reactivation of microsporidiosis according to the concentration 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 immunosuppressive status, are likely to be major cause of high levels of infection in some facilities. The role of breeding kennel dogs as reservoirs for *E. bieneusi* transmission to humans is likely to be low in Japan, because the genotypes of PtEb IX and CD7 are recognized as dog-specific genotypes.

Conclusion: The present study is the first large scale report investigating the molecular prevalence and characterization of *Enterocytozoon bieneusi* in family pet dogs, pet shop puppies,

and breeding kennel dogs, in Japan. The results suggest that *E. bieneusi* infection is common in domestic dogs but is relatively low level. Exceptionally, in dogs kept in breeding kennel, the dogs of <1-year-old and the appointed facilities maintain high levels of *E. bieneusi* infection. It is also demonstrated that there is no correlation between *E. bieneusi* infection and fecal condition in dogs. The risk of zoonotic transmission of *E. bieneusi* from dogs to humans is likely to be low in Japan, because all sequenced isolates here were identified as dog-specific genotypes such as PtEb IX and CD7.

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Table 1 ***E. bieneusi*** **genotypes reported in dogs**

Host	Genotype	Geographic distribution	Reference
Dog	D*	Portugal	Lobo et al. (2006)
		Poland	Piekarska et al. (2017)
	Type IV*	Colombia	Santin et al. (2008)
	WL11*	Colombia	Santin et al. (2008)
	Peru6*	Portugel	Lobo et al. (2006)
	PtEb IX	Colombia	Santin et al. (2008)
		Japan	Abe et al. (2009)
		China	Karim et al. (2014)
			Xu et al. (2016)
		Switzerland	Mathis et al. (1990)
		Portugal	Lobo et al. (2006)
		Poland	Piekarska et al. (2017)
	CD1	China	Karim et al. (2014)
	CD2	China	Karim et al. (2014)
	CD3	China	Karim et al. (2014)
	CD4	China	Karim et al. (2014)
	CD5	China	Karim et al. (2014)
	CD6	China	Karim et al. (2014)
	CD7	China	Karim et al. (2014)
	CD8	China	Karim et al. (2014)
	CD9	China	Karim et al. (2014)
	CM1	China	Karim et al. (2014)
	EbpA	China	Karim et al. (2014)
	EbpC	China	Karim et al. (2014)
	O	China	Karim et al. (2014)
	Peru8	China	Karim et al. (2014)
	PigEBITS5	China	Karim et al. (2014)
	CHN5	China	Zhang et al. (2011)
	CHN6	China	Zhang et al. (2011)

*Zoonotic genotypes

Table 2 Molecular determination of *E. bieneusi* in family pet dogs, in Japan

Category	Examined	Positive	Prevalence (%)	*P* value
Overall				
	597	26	4.4	-
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
Environment condition				
Indoor	547	25	4.6	-
Outdoor	50	1	2.0	NS
Dog source				
Private owner	95	3	3.2	<0.05
Pet shop	467	18	3.9	<0.05
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	-
Kyushu	25	1	4.0	NS
Okinawa	52	0	0	<0.05

NS: Not significant

Table 3 Molecular determination of *E. bieneusi* in pet shop puppies, in Japan

Category	Examined	Positive	Prevalence (%)	*P* value
Overall				
	621	38	6.1	-
Fecal condition				
Formed	550	32	5.8	NS
Soft	53	6	11.3	-
Diarrhea	18	0	0	NS
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	-

NS: Not significant

Table 4 Molecular determination of *E. bieneusi* in breeding kennel, in Japan

Category	Examined	Positive	Prevalence (%)	*P* value
Overall				
	314	37	11.8	-
Age				
<1-year-old	40	9	22.5	-
≥1-year-old	274	28	10.2	NS
Fecal condition				
Formed	271	36	13.3	-
Soft	41	1	2.4	NS
Diarrhea	2	0	0	NS
Breeding kennels				
BK-1	48	1	2.1*	<0.01
BK-2	87	4	4.6*	<0.01
BK-3	25	1	4.0	NS
BK-4	31	6	19.4	NS
BK-5	123	25	20.3	-

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

NS: Not significant

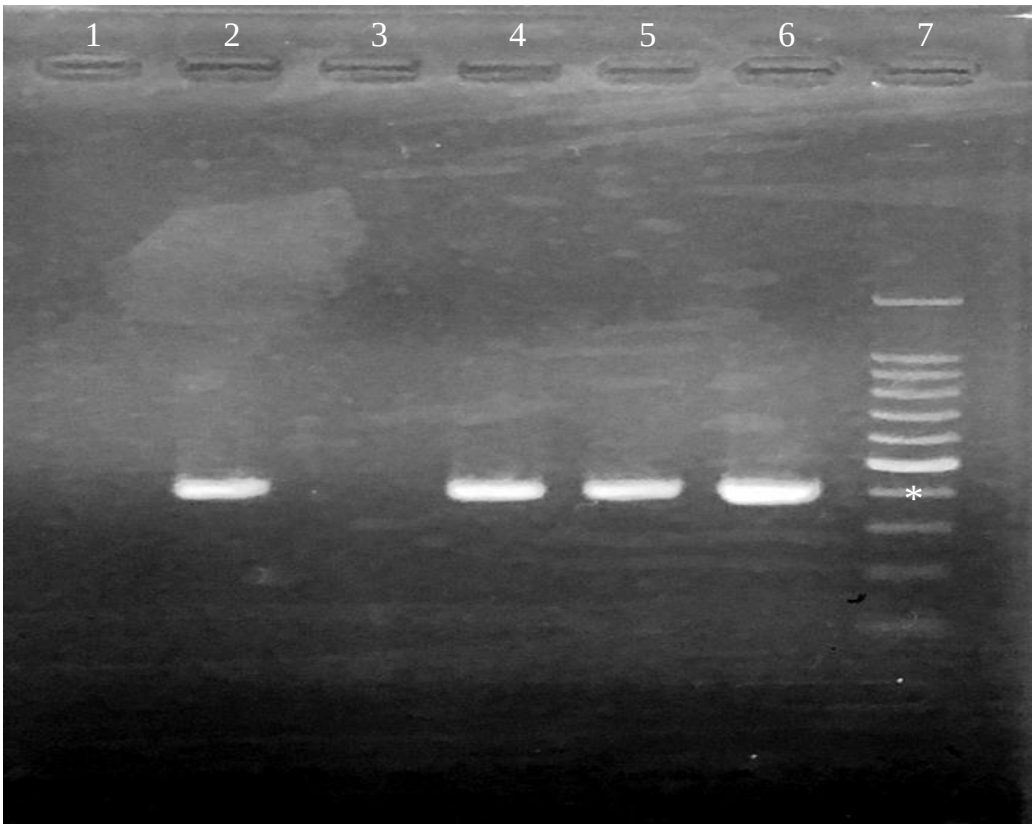

Fig. 1 PCR products on 1.5% agarose gel. Lane 1: *E. bieneusi* negative control, Lane 2: *E. bieneusi* positive control, Lane 3: *E. bieneusi* negative sample, Lane 4-6: *E. bieneusi* positive samples, Lane 7: 100 bp DNA ladder. *Indicates approximately 390 bp.

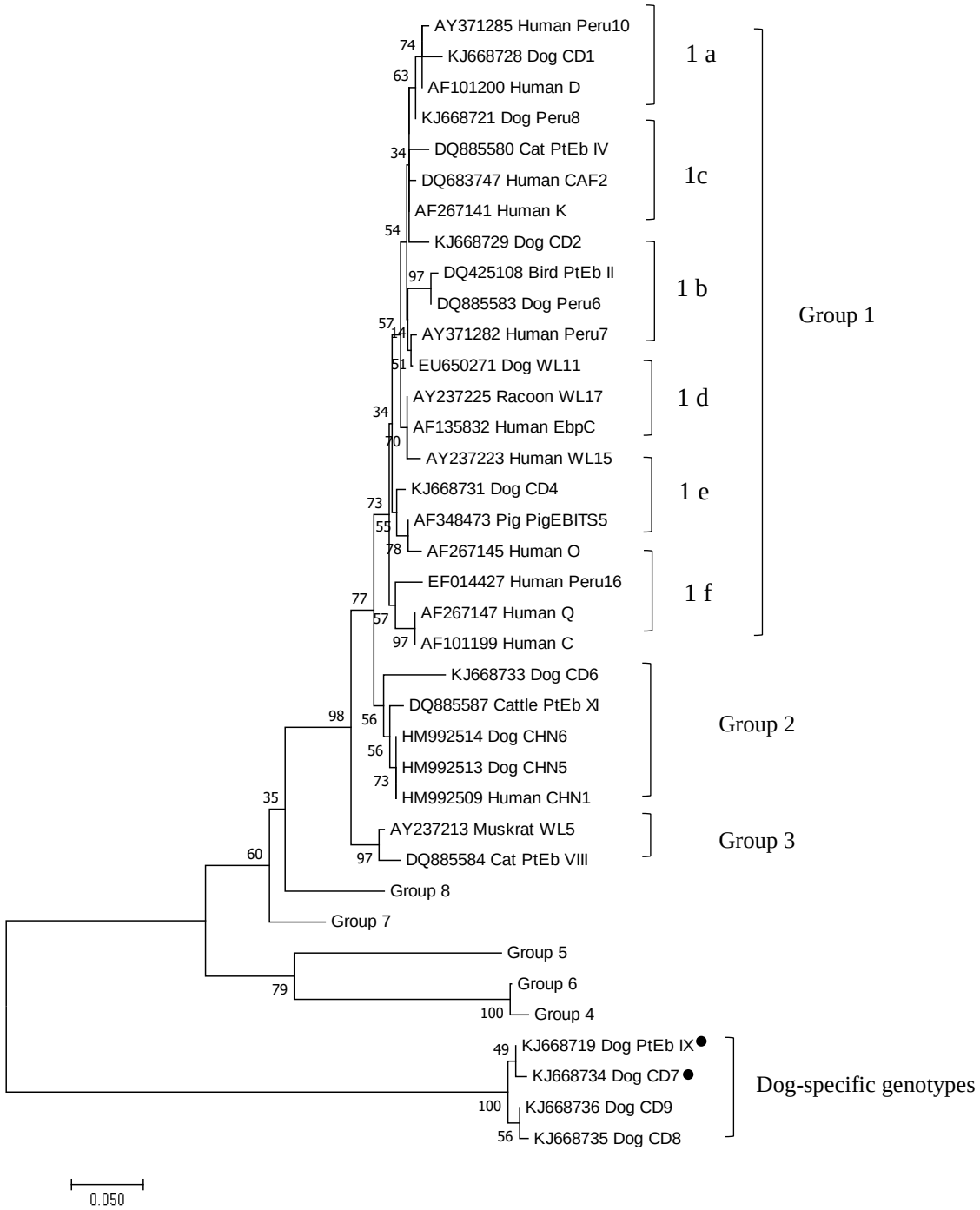

Fig. 2 Phylogenesis relationship of *E. bieneusi* genotypes identified in the present study and know genotypes previously published in GenBank as inferred by a neighbor-joining analysis of ITS sequences base on genetic distances calculated by the kimura 2-parameter model. The numbers on the branches are percent bootstrapping value from 1000 replicates, with more than 50% shown in the tree. Each sequence from GenBank is identified by its accession number, genotype designation, and host origin. *E. bieneusi* genotypes with black circles are genotypes identified in this study.

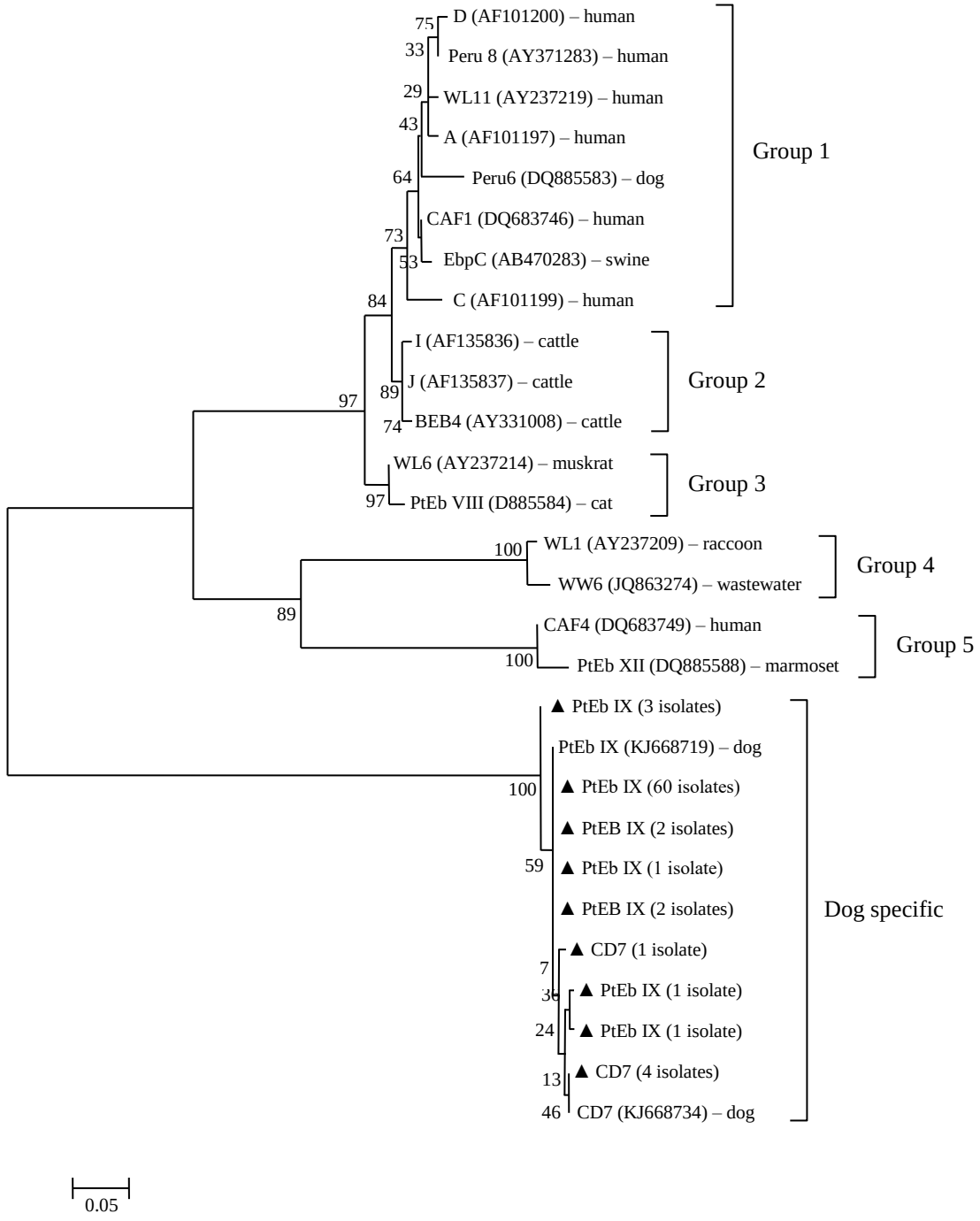

Fig. 3 Phylogenetic analysis of ITS nucleotide sequences of *E. bieneusi* genotypes isolated from pet shop puppies and breeding kennel dogs in the present study.