

学位論文要旨

Molecular epidemiological study of *Enterocytozoon bieneusi* infection in dogs, in Japan

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 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 ( $\leq 3$  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.  $\geq$ 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  $\geq$ 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 to 100% 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. bienersi* 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 *E. bienersi* in family pet dogs, pet shop puppies, and breeding kennel dogs, in Japan. The results suggest that *E. bienersi* 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. bienersi* infection. It is also demonstrated that there is no correlation between *E. bienersi* infection and fecal condition in dogs. The risk of zoonotic transmission of *E. bienersi* 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.