

# Zoonotic potential of *Enterocytozoon bieneusi* in pre-weaned Korean native calves

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## Research

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# Abstract

## Background

*Enterocytozoon bieneusi* is the most common microsporidian species that can infect humans and various animals worldwide. To date, there has been limited information on the prevalence and genotypes of *E. bieneusi* infection in cattle in the Republic of Korea. Therefore, this study investigated the prevalence and genotypes of *E. bieneusi* circulating in pre-weaned Korean native calves and determined the age pattern of *E. bieneusi* infection and the relationship between *E. bieneusi* infection and diarrhea.

## Methods

The prevalence of *E. bieneusi* infection in pre-weaned Korean native calves was screened by polymerase chain reaction. PCR-positive products were sequenced to determine the genotype of *E. bieneusi*. A Chi-square analysis used to compare the associations between diarrhea and infection rate of *E. bieneusi* in each age ranges or all ages.

## Results

PCR and sequencing analysis revealed an overall prevalence of *E. bieneusi* of 16.9% (53/314) in pre-weaned calves. The prevalence of *E. bieneusi* was the highest in September (36.2%), followed by March (28.3%). *E. bieneusi* infection ( $\chi^2 = 5.82$ ,  $P = 0.016$ ) was associated with diarrhea in calves. Our results also indicated that *E. bieneusi* infection was statistically associated with calf age ( $\chi^2 = 11.61$ ,  $P = 0.003$ ), and the prevalence of *E. bieneusi* infection was significantly higher in calves aged 21–40 days (odds ratio = 2.90, 95% confidence interval: 1.54–5.45;  $P = 0.001$ ) than in those aged 1–20 days. Interestingly, the association between *E. bieneusi* infection and diarrhea was observed only in calves aged 1–20 days ( $\chi^2 = 5.82$ ,  $P = 0.010$ ). Furthermore, our results indicated that *E. bieneusi* infection in pre-weaned calves may be associated with mild diarrhea rather than severe diarrhea. We identified five genotypes, BEB4 ( $n = 12$ ), BEB8 ( $n = 23$ ), CHN6 ( $n = 1$ ), I ( $n = 1$ ), and J ( $n = 16$ ), and all of which belonged to Group 2. The genotype BEB8 was the most prevalent among all age groups irrespective of diarrhea. In contrast, the genotype I was identified only in one calf aged 10 days with diarrhea. Except for CHN6, the other four genotypes were mostly observed in cattle, and all exhibited zoonotic potential.

## Conclusions

To our knowledge, this is the first report of the presence of the genotypes BEB4 and CHN6 in pre-weaned Korean native calves. Zoonotic *E. bieneusi* infection was prevalent in pre-weaned Korean native calves, indicating that cattle may play an important role as a reservoir host for *E. bieneusi* transmission to humans.

# Background

Microsporidia are obligate intracellular protozoan-like fungi that infect a wide range of invertebrates and vertebrates including humans [1]. Among approximately 17 human-pathogenic microsporidian species, *Enterocytozoon bieneusi* is the most common [2]. *E. bieneusi* usually causes gastrointestinal illnesses such as wasting syndrome and chronic diarrhea in immunocompromised patients (AIDS or organ transplant recipients, patients with cancer); however, it also leads to asymptomatic and symptomatic infections in immunocompetent individuals [3, 4, 5, 6, 7]. *E. bieneusi* is primarily transmitted through the fecal-oral route, and *E. bieneusi* spores from seemingly healthy animals, humans, and contaminated water or food could be potential sources of infection [8]. Despite the clinical and public health importance of *E. bieneusi*, the implication of *E. bieneusi* has not been emphasized because of the low incidence rate of *E. bieneusi* infection in most of the countries.

Genotyping of *E. bieneusi* and assessment of its host specificity and zoonotic potential are dependent on the sequence analysis of the ribosomal internal transcribed spacer (ITS) [9]. Currently, 474 *E. bieneusi* genotypes have been identified in various hosts. By phylogenetic analysis, the genotypes of *E. bieneusi* have been clustered into at least 11 groups (groups 1–11) [10]. Group 1 contains the most genotypes found in humans and is considered to be zoonotic. Groups 2–11 have a narrow host range and are associated with specific hosts (ruminants, nonhuman primates, horses, and dogs) and wastewater [11, 12]. To date, more than 50 *E. bieneusi* genotypes have been identified in cattle, most of which belong to Group 2 [13]. Among them, some genotypes (BEB4, BEB6, I, and J) were detected in humans [14, 15, 16, 17], suggesting that cattle can serve as potential reservoirs of human infection.

According to several studies, BEB4, I, and J are common genotypes of *E. bieneusi* genotypes found in pre-weaned calves worldwide [16, 18, 19, 20]. However, there is limited information available about the infection rates and genotype distribution of *E. bieneusi* in pre-weaned Korean native calves. Therefore, we conducted this study to identify the prevalence and genotypes of *E. bieneusi* circulating among pre-weaned Korean native calves and to determine the age pattern of *E. bieneusi* infection and the relationship between *E. bieneusi* and diarrhea.

## Methods

### Ethics statement

All procedures and possible consequences were explained to farm owners/managers of the surveyed farm. Written informed consent was obtained for the collection of fecal samples from the owners of the Korean native calves. Permission was also obtained from farm owners/managers before the collection of fecal samples.

### Sample Collection

From January to October 2018, a total of 314 fecal samples were collected directly by an experienced veterinarian from the rectum of pre-weaned Korean native calves (aged  $\leq 60$  days) in 10 different farms in the ROK, transported to the Animal Immunology Laboratory of Kyungpook National University in a cooler with ice packs, and stored at 4°C before DNA extraction. The fecal consistency of each calf was categorized as normal or diarrheic according to its physical characteristics.

## Dna Extraction And Pcr Amplification

Genomic DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) from approximately 200 mg of each fecal sample according to the manufacturer's instructions and then stored at -20 °C until used in PCR analysis. *E. bieneusi* was screened based on the ITS region of the rRNA by nested PCR under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min [21]. *C. parvum* and *G. duodenalis* infections were also detected using the 60-kDa glycoprotein (*gp60*) and  $\beta$ -giardin genes [22, 23, 24]. Secondary PCR products were separated by electrophoresis on 1.5% agarose gels and then visualized after staining with ethidium bromide. In this study, only samples showing a good sequencing result were considered to be positive for *E. bieneusi*.

## Sequencing And Phylogenetic Analysis

The secondary PCR products were purified using the AccuPower PCR Purification Kit (Bioneer, Daejeon, ROK) and used for direct sequencing (Macrogen, Daejeon, ROK). To determine the genotype of *E. bieneusi*, the nucleotide sequences obtained in this study were aligned using ClustalX and compared with the reference sequences from the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>). A phylogenetic tree was constructed based on nucleotide alignments using the maximum-likelihood method implemented in the MEGA 7 software and bootstrap analysis was used to evaluate the robustness with 1000 replicates.

## Statistical analysis

Statistical analysis was performed using SPSS Statistics 25 software package for Windows (SPSS Inc., Chicago, IL, USA). Chi-square ( $\chi^2$ ) test was used to compare the associations between diarrhea and infection rate of *E. bieneusi* in each age range or all ages investigated in this study. In addition, the prevalence of *E. bieneusi* for each age range was determined using binary univariate logistic regression models. The odds ratio (OR) and 95% CI were calculated to determine the likelihood of association. A *P* value of  $\leq 0.05$  was considered to be statistically significant.

## Results

### Prevalence of *E. bieneusi*

The overall prevalence of *E. bieneusi* was found to be 16.9% (53/314) in pre-weaned Korean native calves regardless of diarrhea. Among 10 different farms examined, *E. bieneusi* was detected in 6 farms (Table 1). We compared the infection rate of *E. bieneusi* according to the month. As shown in Table 2, the prevalence of *E. bieneusi* was the highest in September (36.2%), followed by March (28.3%) and October (14.7%); however, *E. bieneusi* infection was not detected in July and August. When *E. bieneusi* infection was compared according to the fecal consistency, there were 11.9% and 22.1% infection rate in both diarrheic and normal feces, respectively. *E. bieneusi* infection was associated with diarrhea ( $\chi^2 = 5.82$ ,  $P = 0.016$ ; Table 3). Co-infection with *E. bieneusi* and *Cryptosporidium parvum* was not detected; however, co-infection with *E. bieneusi* and *Giardia duodenalis* was observed in diarrheic (6.3%, 10/314) and normal feces (1.9%, 3/314). Although there was no statistical significance, the risk of diarrhea was increased by 3.36-fold during co-infection with *E. bieneusi* and *G. duodenalis* (95% confidence interval (CI): 0.91–12.43;  $P = 0.056$ ; Table 4). *E. bieneusi*-positive samples were compared according to the age group of the calves. As shown in Table 5, the prevalence of *E. bieneusi* was the highest in calves aged 21–40 days, followed by those aged 41–60 days and 1–20 days ( $\chi^2 = 11.61$ ,  $P = 0.003$ ). The risk of being positive to *E. bieneusi* was 2.9-fold higher in calves aged 21–40 days (95% CI: 1.54–5.45;  $P = 0.001$ ) than in those aged 1–20 days. The association between *E. bieneusi* infection and diarrhea according to the age group was analyzed by chi-square test. *E. bieneusi* infection was found to be associated with diarrhea only in calves aged 1–20 days ( $\chi^2 = 6.61$ ,  $P = 0.010$ ; Table 6).

Table 1  
Prevalence and genotypes of *E. bieneusi* in pre-weaned Korean native calves

Region	Specimens	No. of positive samples	ITS genotype (No.)
Anseong	39	4	BEB8 ( $n = 2$ ), J ( $n = 2$ )
Geochang	78	22	BEB4 ( $n = 3$ ), BEB8 ( $n = 8$ ), J ( $n = 11$ )
Gimje	71	1	I ( $n = 1$ )
Gyeongju	6	0	–
Jeongeup	1	0	–
Mungyeong	82	21	BEB4 ( $n = 9$ ), BEB8 ( $n = 9$ ), CHN6 ( $n = 1$ ), J ( $n = 2$ )
Naju	1	0	–
Sangju	2	1	J ( $n = 1$ )
Yecheon	1	0	–
Youngju	33	4	BEB8 ( $n = 4$ )
Total	314	53	BEB4 ( $n = 12$ ), BEB8 ( $n = 23$ ), CHN6 ( $n = 1$ ), I ( $n = 1$ ), J ( $n = 16$ )

Table 2  
Prevalence of *E. bieneusi* in pre-weaned Korean native calves according to the month

Month	No. of examined calves	No. of positive calves	Positive rate (%)
January	2	0	0%
March	53	15	28.3%
April	68	6	8.8%
May	27	3	11.1%
June	23	1	4.3%
July	12	0	0%
August	7	0	0%
September	47	17	36.2%
October	75	11	14.7%
Total	314	53	16.9%

Table 3  
Association between diarrhea and presence of *E. bieneusi* in pre-weaned Korean native calves

Variables	No. (%) of <i>E. bieneusi</i> -positive samples	No. (%) of <i>E. bieneusi</i> -negative samples	Total	$\chi^2$ ( <i>P</i> value)	OR (95% CI)
Fecal consistency					
Diarrhea	19 (11.9%)	141 (88.1%)	160 (100)	5.82 (0.016)	0.48 (0.26–0.88)
Non-diarrhea	34 (22.1%)	120 (77.9%)	154 (100)		

Table 4

Detection rates of all pathogen species in pre-weaned Korean native calves according to the diarrhea status

Pathogen	Positive in diarrhea samples ( <i>n</i> = 160)	Positive in non-diarrhea samples ( <i>n</i> = 154)	$\chi^2$ ( <i>P</i> value)	OR (95% CI)
<i>Cryptosporidium parvum</i>	11 (6.9%)	14 (9.1%)	0.53 (0.468)	0.74 (0.32–1.68)
<i>Giardia duodenalis</i>	23 (14.4%)	18 (11.7%)	0.50 (0.480)	1.27 (0.66–2.46)
<i>Enterocytozoon bieneusi</i>	19 (11.9%)	34 (22.1%)	5.82 (0.016)	0.48 (0.26–0.88)
<i>E. bieneusi</i> + <i>G. duodenalis</i>	10 (6.3%)	3 (1.9%)	3.66 (0.056)	3.36 (0.91–12.43)

Table 5

Distribution of *E. bieneusi* in pre-weaned Korean native calves according to age

Age (Days)	Frequency of <i>E. bieneusi</i> positivity (%)	$\chi^2$ ( <i>P</i> value)	<i>P</i> value	OR	95% CI
1–20 (Ref.)	19/173 (11.0%)	11.61 (0.003)	–	1.00	–
21–40	30/114 (26.3%)		0.001	2.90	1.54–5.45
41–60	4/17 (14.8%)		0.563	1.41	0.44–4.51

Table 6

Association between diarrhea and presence of *E. bieneusi* in pre-weaned Korean native calves according to age

Age (Days)	Fecal consistency	Frequency of <i>E. bieneusi</i> positivity (%)	$\chi^2$ ( <i>P</i> value)	OR	95% CI
1-20	Diarrhea	6/102 (5.9%)	6.61 (0.010)	0.28	0.10-0.77
	Non-diarrhea	13/71 (18.3%)			
21-40	Diarrhea	10/44 (22.7%)	0.48 (0.490)	0.74	0.31-1.76
	Non-diarrhea	20/70 (28.6%)			
41-60	Diarrhea	3/14 (21.4%)	1.01 (0.596)	3.27	0.30-36.31
	Non-diarrhea	1/13 (7.7%)			

## Genotypes of *E. bieneusi*

To determine the genotypes of *E. bieneusi* detected in pre-weaned Korean native calves, a total of 53 ITS-positive samples were sequenced. Five distinct genotypes, BEB4 ( $n = 12$ ), BEB8 ( $n = 23$ ), CHN6 ( $n = 1$ ), I ( $n = 1$ ), and J ( $n = 16$ ), were identified. Among them, BEB8 was found to be the most prevalent genotype in pre-weaned calves regardless of diarrhea. In contrast, the genotypes CHN6 and I were found only in one calf aged 16 days and 10 days with diarrhea, respectively. In particular, the genotypes BEB8 and J were detected in all age groups. BEB4 was found only in calves aged up to 40 days and only in two farms. The diversity of the identified genotypes was decreased with age (Table 7). As shown in Table 1, the distribution of *E. bieneusi* genotypes on the farms was different. Three farms (Gimje, Sangju, and Youngju) had only one genotype, whereas the others had two to three genotypes (Table 1). Of the 53 ITS-positive samples, 16 sequences were included in the phylogenetic tree, and all these isolates belonged to Group 2 (Fig. 1). Except for CHN6, the other four genotypes have been mostly observed in cattle. The genotype CHN6 identified in this study showed 98.5% homology with an isolate found in feces of humans in China (Fig. 1). To the best of our knowledge, this is the first report of the presence of the genotypes BEB4 and CHN6 in pre-weaned Korean native calves.

Table 7  
Genotype distribution of *E. bieneusi* according to age in pre-weaned Korean native calves

	BEB4	BEB8	CHN6	I	J	Total
1-20	4	8	1	1	5	22
21-40	8	12	-	-	10	30
41-60	-	3	-	-	1	4
Total	12	23	1	1	16	53

## Discussion

The present study showed that the infection rate of *E. bieneusi* in pre-weaned Korean native calves was 16.9%, which is similar to that reported in other studies for cattle in the Republic of Korea (ROK) and several countries [16, 25, 26, 27, 28]. The prevalence of *E. bieneusi* in pre-weaned calves varied from 0 to 50% depending on the farm. This variation could be attributed to nutrition, herd management practices, health of the animal, and hygiene. Interestingly, *E. bieneusi* infection appears to be closely related to seasonal differences. According to our results, *E. bieneusi* infection tended to occur primarily in September (36.2%) and March (28.3%); in contrast, the incidence rate of *E. bieneusi* was rather low in warmer seasons with no infection in July and August. This result was different from that of a previous study that reported a higher prevalence in warmer seasons in the ROK [25]. The differences between the two studies may be explained by the difference in the number of samples collected each month and the age of the calf. However, our results are fairly consistent with those a study conducted in China, which reported a higher prevalence in spring [29]. Although the results are inconclusive, the transmission of *E. bieneusi* may be related to seasonal variations. Further studies are required to investigate the association between *E. bieneusi* infection and seasonal variations.

In this study, *E. bieneusi* infection was associated with diarrhea according to chi-square analysis; however, the infection rate was not high in diarrheic feces. Although *E. bieneusi* was detected in diarrheic feces, it is unlikely that *E. bieneusi* is associated with diarrhea in pre-weaned calves. Interestingly, among the three pathogens examined, the infection rate of *E. bieneusi* was the highest in pre-weaned Korean native calves. This may have been overlooked in diagnostic tests due to uncertainty regarding the role of *E. bieneusi* as a pathogen in calf diarrhea. Furthermore, it is possible that the significance of *E. bieneusi* has not been prominently recognized in fields. Results of the present study showed that co-infection with *E. bieneusi* and *G. duodenalis* was not statistically significant ( $P=0.056$ ); however, it was 3.36-fold more likely to cause diarrhea (95% CI: 0.91–12.43) compared with *E. bieneusi* single infection in calves. In this study, the number of positive samples co-infected with two pathogens was small; thus, these results did not demonstrate an association between diarrhea and co-infection in pre-weaned calves. Although it remains unclear, such co-infection may increase the severity and duration of diarrhea in calves. More

epidemiological investigations are required to determine whether the occurrence of diarrhea is more common in calves co-infected with *E. bieneusi* and *G. duodenalis*.

The prevalence of *E. bieneusi* in calves was significantly associated with the age of the calf ( $P=0.003$ ). The infection rate of *E. bieneusi* was the highest in calves aged 21–40 days, followed by calves aged 41–60 days and 1–20 days. In comparison with calves aged  $\leq 20$  days, the risk of *E. bieneusi* infection was 2.9-fold higher in calves aged 21–40 days ( $P=0.001$ ; Table 5). A possible explanation is that the immune status of calves in this age group may be more susceptible to *E. bieneusi* infection due to the loss of maternal antibodies [30]. To date, several studies have demonstrated the age-related prevalence pattern of *E. bieneusi* infection [18, 26, 27, 31]. However, in contrast to our findings, the prevalence of *E. bieneusi* has been found to increase with age [18, 32, 33]. In the ROK, there are limited studies on *E. bieneusi* infection in cattle; thus, it is not possible to compare the prevalence of *E. bieneusi* according to the age group. In addition, there is no information on the transmission route of *E. bieneusi* on the farms examined; however, *E. bieneusi* infection may be related to the hygiene status of farms rather than the calf age. Therefore, to prevent *E. bieneusi* infection, the farming management system should be improved, which could include no contact with contaminated food and water, cleaning, and disinfection.

We also investigated the association between *E. bieneusi* infection and diarrhea according to the age group. Our results revealed that there was a significant correlation between *E. bieneusi* infection and diarrhea in calves aged 1–20 days ( $P=0.010$ ). However, this has been shown to be associated with a low incidence of diarrhea in *E. bieneusi*-infected calves. Based on the results, it is unlikely that *E. bieneusi* is the primary pathogen that causes diarrhea in pre-weaned Korean native calves. Cattle may be a source of environmental contamination by *E. bieneusi*. Therefore, *E. bieneusi* infection in calves should be considered as a zoonotic potential rather than a causative agent of diarrhea.

In the present study, sequence analysis of the ITS gene from 53 *E. bieneusi*-positive isolates identified five known genotypes (BEB4, BEB8, CHN6, I, and J) belonging to Group 2. Unlike previous studies, the genotype BEB8 was the most prevalent in pre-weaned Korean native calves and was found in 45.3% (24/53) of the positive samples. Moreover, this genotype was commonly identified in all age groups regardless of diarrhea. Several studies have reported that the genotype BEB8 can be found in not only cattle [27, 34, 35] but also bats [36], rabbits [37], and monkeys [38], indicating that this genotype might have a potential risk for zoonotic infection in humans. The genotype J, identified in 30.2% (16/53) of *E. bieneusi*-positive samples, was the second most common genotype in all age groups. BEB4 was the third most common genotype and found in calves only up to 40 days. BEB4 has been identified as a zoonotic genotype in cattle in many countries; however, it was first detected in the ROK. Interestingly, the genotype I was detected in only one calf. In contrast, a previous study conducted in the ROK reported the presence of the genotype I in three cattle. This can be attributed to the low incidence of genotype I in cattle in the ROK compared to that in other countries. The present study reported the first identification of the genotype CHN6 in pre-weaned Korean native calves. This isolate showed 98.5% homology with an isolate found in human feces (MN136773) obtained in China. Furthermore, the genotypes BEB4, BEB8, and J were common genotypes in pre-weaned Korean native calves. The differences in the distribution of *E. bieneusi*

in pre-weaned calves according to farms may be attributed to the geographical location and the farm management system. All genotypes identified in this study have a possible zoonotic potential, suggesting that cattle play an important role as a reservoir host in *E. bieneusi* transmission to humans.

## Conclusions

The presence and genotypes of *E. bieneusi* were detected in pre-weaned Korean native calves. *E. bieneusi* infection was associated with diarrhea in calves aged 1–20 days, and the prevalence of *E. bieneusi* was significantly high in calves aged 21–40 days. ITS sequencing identified five genotypes (BEB4, BEB8, CHN6, I, and J), with BEB8 being the most prevalent genotype in pre-weaned Korean native calves. The identification of zoonotic genotypes in pre-weaned calves suggests that these animals could play an important role as reservoir hosts for zoonotic infections.

## Abbreviations

*E. bieneusi*: *Enterocytozoon bieneusi*; CI: confidence interval; *C. parvum*; *Cryptosporidium parvum*; *G. duodenalis*: *Giardia duodenalis*; ITS: internal transcribed spacer; OR: odds ratio; ROK: Republic of Korea

## Declarations

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### Authors' contributions

KSC designed the research and wrote the manuscript. SWH, SUS, and JHR performed the experiments. SHK analyzed the data. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are included in the article.

### Ethics approval and consent to participate

This study did not receive approval from the Institutional Animal Care and Use Committee (IACUC) at Kyungpook National University, because the IACUC at this University evaluates laboratory animals

maintained within indoor facilities and not outdoor animals. A local veterinarian collected all fecal samples and sent them to us.

## Consent for publication

Not applicable

## Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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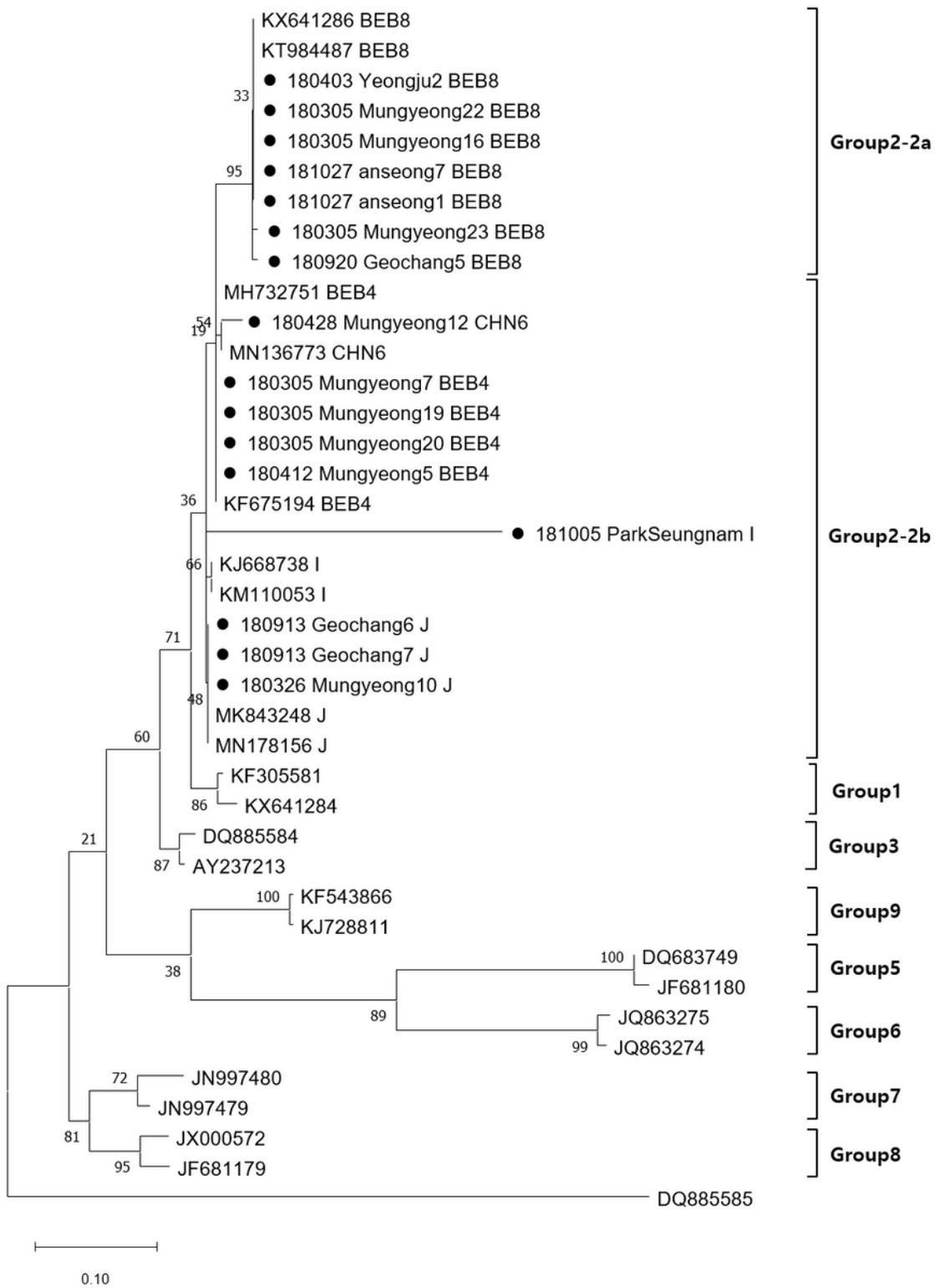
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## Figures



**Figure 1**

Phylogenetic relationships of *Enterocytozoon bienewsi* genotypes identified in this study and other reported genotypes based on the internal transcribed spacer gene sequences. The tree was constructed using the MEGA7 software with the maximum-likelihood method. Numbers at the nodes of the tree indicate bootstrap values as a percentage of 1000 replicates that support each phylogenetic branch. Our isolates identified in this study are marked in bold type with a circle symbol.

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