Parasites Hosts Dis 2024;62(4):484-489
https//doi.org/10.3347/PHD.24072
pISSN 2982-5164 • eISSN 2982-6799

Brief Communication

Genotypic analysis of zoonotic *Enterocytozoon bieneusi* in wild deer in Korea

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Received: 14 October 2024
Accepted: 4 November 2024

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Citation
Noh G, Lee H, Lee SH, Seo MG, Kim KT, Lee J, Nazim K, Park SJ, Rhee MH, Kwak D. Genotypic analysis of zoonotic *Enterocytozoon bieneusi* in wild deer in Korea. Parasites Hosts Dis 2024;62(4):484-489.

Abstract

Enterocytozoon bieneusi is an important microsporidian protozoa that causes intestinal disorders in humans. We collected 191 fecal samples from roadkill deer carcasses, among which 13 (6.8%) showed positive reaction for *E. bieneusi* by polymerase chain reaction assay. Phylogenetic analysis revealed 6 distinct genotypes, 1 of which was novel. All genotypes belonged to Group 1, which has low host specificity, indicating possible transmission through sylvatic cycle. *E. bieneusi* infection was predominant in female deer ($P<0.05$).

Keywords: *Enterocytozoon bieneusi*, deer, genotyping, zoonosis

Enterocytozoon bieneusi, previously classified in the kingdom Fungi, is a zoonotic microsporidia currently grouped into Cryptomycota, a sister group to fungi [1,2]. This opportunistic pathogen causes intestinal disorders, such as chronic diarrhea and intestinal malabsorption, in immunocompromised or immunocompetent individuals, while most infections are asymptomatic [3,4].

Polymerase chain reaction (PCR)-based sequencing analysis of the polymorphic internal transcribed spacer (ITS) region of *E. bieneusi* has resulted in the identification of over 500 *E. bieneusi* genotypes in a wide range of host animals, including mammals and birds [2,5]. To date, 11 major groups have been established using phylogenetic analysis: zoonotic genotypes Groups 1 and 2, and host-adapted genotypes Groups 3–11. Thus, *E. bieneusi* is a critical public health concern [6]. *E. bieneusi* transmission mainly occurs via the fecal–oral route, beginning with the intake of environmentally-resistant spores that invade host cells via a polar tube that penetrates the cell. There are also reports of airborne and hospital-associated transmission [7-12].

Because of its zoonotic potential, *E. bieneusi* has been globally studied. However, compared with other animals, studies investigating the prevalence of *E. bieneusi* infection in deer are scarce, particularly in Korea. Therefore, we investigated the prevalence of *E. bieneusi* infection in deer in Korea, including the Korean water deer (*Hydropotes inermis argyropus*), which is a dominant species in Korea, and roe deer (*Capreolus capreolus*).

We collected 191 fresh fecal samples from Korean water deer or roe deer that were accidentally killed on highways or roadsides from March 2018 to June 2021. When a roadkill

Author contributions
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Conflict of interest
The authors declare no conflict of interest related to this study.

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carcass is found in Korea, it is usually reported to the government office by citizens passing by on the road, leading to immediate collection by a field dispatch unit. Thus, the carcass is kept in a fresh condition. The fecal samples were directly collected per rectum by trained veterinarians, placed in plastic tubes, and delivered to the laboratory for analysis. Data on the collection region, season, sex, weight, and host species were recorded, with missing information marked as "unknown." The samples were classified into three regions according to provincial boundaries: northern (Gangwon, Gyeonggi, Incheon, and Seoul), central (Chungbuk, Chungnam, Gyeongbuk, Jeonbuk, Sejong, and Daejeon), and southern (Gyeongnam, Jeonnam, Jeju, Gwangju, Busan, and Ulsan). The study population was categorized into five groups according to weight: <10 kg, ≥10 to <15 kg, ≥15 to <20 kg, ≥20 kg, and unknown. Statistical analysis was performed using the χ^2 test in SPSS v26.0 (IBM Corporation, Armonk, NY, USA). *P*-values <0.05 were considered statistically significant. Approval number of dead wild animals was not needed.

DNA was extracted from the fecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were determined spectrophotometrically using an Infinite 200 PRO NanoQuant plate reader (Tecan, Männedorf, Switzerland) before storage at −20°C until use. The presence of *E. bieneusi* was determined using nested PCR targeting the ITS region, with the primer pair ITSF1 (forward, 5′-GGTCATAGGGATGAAGAG-3′) and ITSR1 (reverse, 5′-TTCGAGTTCTTTCGCGCTC-3′) and the primer pair ITSF2 (forward, 5′-GCTCTGAATATCTATGGCT-3′) and ITSR2 (reverse, 5′-ATCGCCGACG-GATCCAAGTG-3′) used in the first and second PCR rounds, respectively. The expected amplicon size was 389 bp [12]. Each PCR assay was performed in a final reaction volume of 20 μl comprising 1 μl of each primer, 2 μl template DNA, and 16 μl distilled H_2O in the lyophilized AccuPower HotStart PCR Premix (Bioneer, Daejeon, Korea) using a MastercyclerPro thermal cycler (Eppendorf, Hamburg, Germany). The cycling conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; and final extension at 75°C for 5 min. The resultant PCR amplicons were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining. All PCR-positive samples underwent bidirectional sequencing (Macrogen, Daejeon, Korea). The obtained sequences were aligned using BioEdit v7.2.5 (https://thalljiscience.github.io/page2.html, accessed 10 Oct. 2024), revealing 13 consensus sequences, of which 6 were identical and consequently removed. The remaining 7 sequences were submitted to GenBank (accession numbers: OR335887–OR335893) and compared with an *E. bieneusi* reference sequence (DQ885585) in NCBI BLAST. A maximum likelihood phylogenetic tree with 1,000 bootstrap replicates was generated using MEGA 7 software [13].

The *E. bieneusi* infection rate in our study was 6.8% (13/191 samples) (Table 1). *E. bieneusi* infection has been studied in various species, including bats, wild boars, domestic pigs, dairy cattle, primates in zoos, lambs, and other wildlife species [2,5,7,14-20]. Studies on *E. bieneusi* infection in deer have been performed in several countries, mainly in China [16,20]. However, studies on *E. bieneusi* infection in water deer and roe deer are scarce. This study compared the *E. bieneusi* infection rate with other Cervidae species, including sambar deer (*Rusa unicolor*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and

Table 1. Prevalence of *Enterocytozoon bieneusi* in Korean wild deer

Variable		No. positive/No. tested (%)	*P*-value
Region	Northern	0/24	0.054
	Central	3/89 (3.4)	
	Southern	9/68 (13.2)	
	Unknown	1/10 (10.0)	
Season	Spring	4/41 (9.8)	0.583
	Summer	2/38 (5.3)	
	Autumn	0/19	
	Winter	0/0	
	Unknown	7/93 (7.5)	
Sex	Female	5/26 (19.2)	0.008
	Male	0/45	
	Unknown	8/120 (6.7)	
Weight	<10 kg	0/11	0.483
	≥10 to <15 kg	2/36 (5.6)	
	≥15 to <20 kg	3/20 (15.0)	
	≥20 kg	0/2	
	Unknown	8/122 (6.6)	
Species	Korean water deer	11/172 (6.4)	0.239
	Roe deer	2/19 (10.5)	
Total		13/191 (6.8)	

sika deer (*Cervus nippon*). The rate of *E. bieneusi* infection was reported as 4.1% (25/610 samples) in an Australian population including sambar deer, red deer, and fallow deer [17], which was slightly lower than in our study. However, the infection rate of *E. bieneusi* in wild red deer in Spain was reported as much lower (1.5%, 5/329 samples) [20] and the infection rate in sika deer and red deer in China was reported to be much higher (35.9%, 221/615 samples) [16] than in our study. Studies on *E. bieneusi* infection in bats, wild boars, calves, Korean water deer, raccoon dogs, and dairy cattle have been conducted in Korea, and the infection rate in Korean water deer was reported as 53.6% (52/97) [15], considerably higher compared with that in our study. This result demonstrated that there may be various differences depending on the methodology, study region, and sampling time.

We also analyzed the risk factors predisposing to *E. bieneusi* infection, such as locality, season, sex, weight, and host species. Female deer had a significantly higher infection rate than males (19.2% vs. 0%, $P=0.008$), although the sex of eight of the infected deer was unknown. Deer thrives in the southern region had a higher infection rate (13.2%) and in the spring season (9.7%), but not statistically significant ($P=0.054$ and 0.583, respectively). Other studies have not reported significant differences in the infection prevalence between females and males [16,18,19]. In Chinese deer, the prevalence was 30.1% in females and 42.1% in males [16]; in Ethiopian lambs, the prevalence was 11.0% in females and 9.0% in males [19]; and in Korean wild boars, the prevalence was 3.0% in females and 2.6% in males [18]. Thus, further studies with a larger sample size are necessary to clarify this association.

Regarding weight and species, the infection rate was high in the ≥15 to <20 kg group (15.0%) and in roe deer (10.5%), albeit not statistically significant ($P=0.483$ and $P=0.239$, respectively).

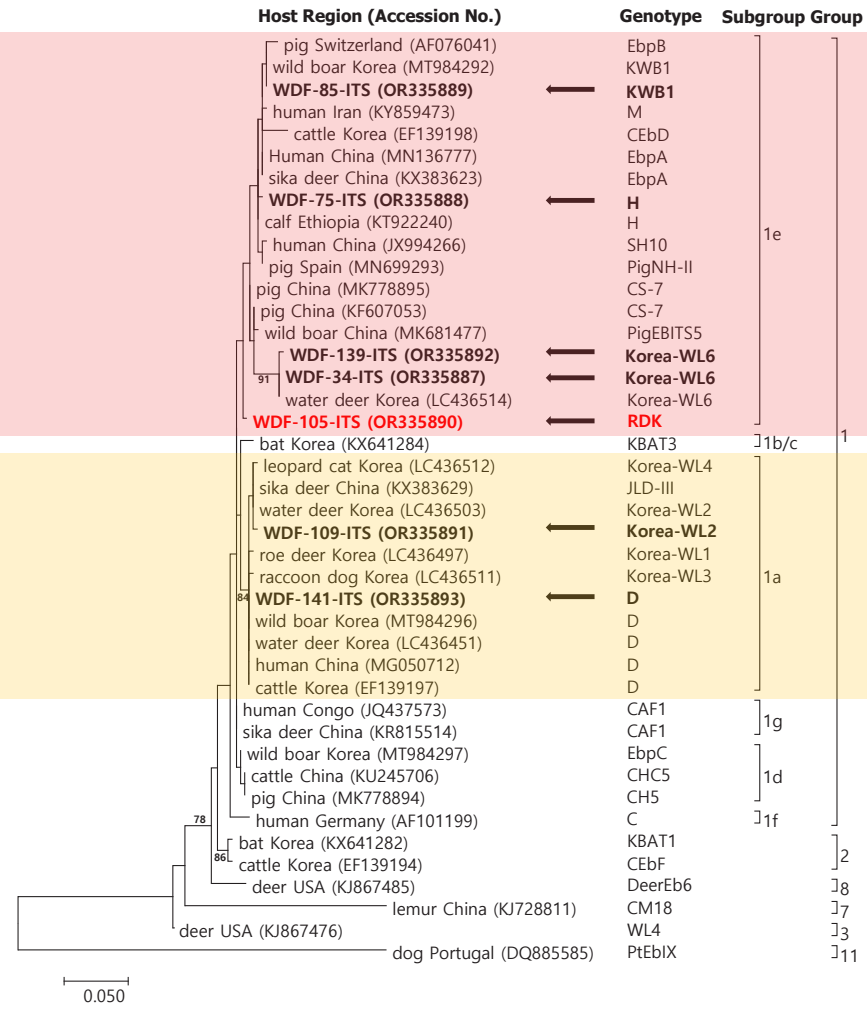

Fig. 1. Phylogenetic analysis of *Enterocytozoon bieneusi* infection in deer in Korea. MEGA 7 software was used to generate a maximum likelihood phylogenetic tree, and 1,000 bootstrap replicates were performed. The genotypes identified in our study are indicated in bold, and the novel genotype RDK is indicated in red. The genotypes (group and subgroup) are indicated on the right.

Phylogenic analysis revealed 6 distinct *E. bieneusi* genotypes: Korea-WL2 (OR335891), Korea-WL6 (OR335887 and OR335892), H (OR335888), KWB1 (OR335889), D (OR335893), and RDK (OR335890). RDK (OR335890) was a novel genotype with 3 nucleotide substitutions at positions 200 (T/A), 207 (A/G), and 260 (A/G) compared with the reference genotype PigHN-II sequence (MN699293). Among the 7 samples positive for *E. bieneusi*, 1 sample was identical to genotype Korea-WL2 (subgroup 1a, identified in raccoon dogs and Korean water deer) and two were identical to genotype Korea-WL6 (subgroup 1e, identified in Korean water deer) [15]; 1 sample each was identical to genotype H (subgroup 1e), KWB1 (subgroup 1e, identified in wild boar), and D (subgroup 1a, identified in wild boar) [18], respectively. Finally, 1 sample was classified as the novel RDK genotype, which belonged to subgroup 1e. All 6 genotypes identified in our study belonged to

Group 1 (Fig. 1), the largest genetic group of *E. bieneusi*. This group is a significant public health concern because of its low host specificity [5,6].

Of the 191 fecal samples tested in our study, 13 (6.8%) were infected with *E. bieneusi*. There are few studies on *E. bieneusi* infection in roe deer. Only 1 study on Korean water deer has been reported [15]. Although we found a significant association between sex and *E. bieneusi* infection, further studies are necessary to verify this relationship. We identified 6 distinct *E. bieneusi* genotypes, all belonging to Group 1 and 1 of which was novel. *E. bieneusi* is genetically diverse and infects a wide range of host species, including humans. Korea has large populations of Korean water deer and roe deer. Given its zoonotic potential and wide geographic distribution, *E. bieneusi* infection poses a significant public health concern in wild life. Further research on the infection rates and the pathogenicity of the novel RDK genotype is warranted.

References

1. Han B, Pan G, Weiss LM. Microsporidiosis in humans. *Clin Microbiol Rev* 2021;34(4):e0001020. https://doi.org/10.1128/CMR.00010-20
2. Lee SH, Oem JK, Lee SM, Son K, Jo SD, et al. Molecular detection of *Enterocytozoon bieneusi* from bats in South Korea. *Medical Mycol* 2018;56(8):1033-1037. https://doi.org/10.1093/mmy/myx136
3. Mathis A, Weber R, Deplazes P. Zoonotic potential of the Microsporidia. *Clin Microbiol Rev* 2005;18(3):423-445. https://doi.org/10.1128/cmr.18.3.423-445.2005
4. Zhang Y, Koehler AV, Wang T, Haydon SR, Gasser RB. New operational taxonomic units of Enterocytozoon in three marsupial species. *Parasit Vectors* 2018;11:371. https://doi.org/10.1186/s13071-018-2954-x
5. Dashti A, Rivero-Juarez A, Santín M, López-López P, Caballero-Gómez J, et al. *Enterocytozoon bieneusi* (Microsporidia): identification of novel genotypes and evidence of transmission between sympatric wild boars (*Sus scrofa ferus*) and Iberian pig (*Sus scrofa domesticus*) in Southern Spain. *Transbound Emerg Dis* 2020;67:2869-2880. https://doi.org/10.1111/tbed.13658
6. Li W, Feng Y, Xiao L. Diagnosis and molecular typing of *Enterocytozoon bieneusi*: the significant role of domestic animals in transmission of human microsporidiosis. *Res Vet Sci* 2020;133:251-261. https://doi.org/10.1016/j.rvsc.2020.09.030
7. Del Coco VF, Córdoba MA, Bilbao G, de Almeida Castro P, Basualdo JA, et al. First report of *Enterocytozoon bieneusi* from dairy cattle in Argentina. *Vet Parasitol* 2014;199(1-2):112-115. https://doi.org/10.1016/j.vetpar.2013.09.024
8. Kicia M, Sędzimirska M, Sak B, Kváč M, Wesołowska M, et al. Respiratory microsporidiosis caused by *Enterocytozoon bieneusi* in an HIV-negative hematopoietic stem cell transplant recipient. *Int J Infect Dis* 2018;77:26-28. https://doi.org/10.1016/j.ijid.2018.07.021
9. Messaoud M, Abbes S, Gnaien M, Rebai Y, Kallel A, et al. High frequency of *Enterocytozoon bieneusi* genotype WL12 occurrence among immunocompromised patients with intestinal microsporidiosis. *J Fungi* (*Basel*) 2021;7(3):161. https://doi.org/10.3390/jof7030161
10. Stentiford GD, Becnel J, Weiss LM, Keeling PJ, Didier ES, et al. Microsporidia-emergent pathogens in the global food chain. *Trends Parasitol* 2016;32(4):657. https://doi.org/10.1016/j.pt.2015.12.004
11. Wang L, Xiao L, Duan L, Ye J, Guo Y, et al. Concurrent infections of *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Clostridium difficile* in children during a cryptosporidiosis outbreak in a pediatric hospital in China. *PLoS Negl Trop Dis* 2013;7(9):e2437. https://doi.org/10.1371/journal.pntd.0002437
12. Ye J, Xiao L, Li J, Huang W, Amer SE, et al. Occurrence of human-pathogenic *Enterocytozoon bieneusi*, *Giardia duodenalis* and *Cryptosporidium* genotypes in laboratory macaques in Guangxi, China. *Parasitol Int* 2014;63(1):132-137. https://doi.org/10.1016/j.parint.2013.10.007
13. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* 2016;33(7):1870-1874. https://doi.org/10.1093/molbev/msw054
14. Karim MR, Dong H, Li T, Yu F, Li D, et al. Predomination and new genotypes of *Enterocytozoon bieneusi* in captive nonhuman Primates in zoos in China: high genetic diversity and zoonotic significance. *PLoS One* 2015;10(2):e0117991. https://doi.org/10.1371/journal.pone.0117991
15. Amer S, Kim SR, Han JI, Na KJ. Prevalence and genotypes of Enterocytozoon bieneusi in wildlife in Korea: a public health concern. *Parasit Vectors* 2019;12(1):160. https://doi.org/10.1186/s13071-019-3427-6
16. Huang J, Zhang Z, Yang Y, Wang R, Zhao J, et al. New genotypes of *Enterocytozoon bieneusi* isolated from sika deer and red deer in China. *Front Microbiol* 2017;8:879. https://doi.org/10.3389/fmicb.2017.00879
17. Zhang Y, Koehler AV, Wang T, Haydon SR, Gasser RB. First

detection and genetic characterisation of *Enterocytozoon bieneusi* in wild deer in Melbourne's water catchments in Australia. *Parasit Vectors* 2018;11(1):2. https://doi.org/10.1186/s13071-017-2577-7
18. Lee H, Seo MG, Lee SH, Oem JK, Kim SH, et al. Distribution and genotypic analysis of *Enterocytozoon bieneusi* from wild boars in Korea. *Med Mycol* 2021;59(9):934-938. https://doi.org/10.1093/mmy/myab030
19. Wegayehu T, Li J, Karim MR, Zhang L. Molecular characterization and phylogenetic analysis of *Enterocytozoon bieneusi* in lambs in Oromia special Zone, Central Ethiopia. *Front Vet Sci* 2020;7:6. https://doi.org/10.3389/fvets.2020.00006
20. Dashti A, Santín M, Köster PC, Bailo B, Ortega S, et al. Zoonotic *Enterocytozoon bieneusi* genotypes in free-ranging and farmed wild ungulates in Spain. *Med Mycol* 2022;60(9):myac070. https://doi.org/10.1093/mmy/myac070