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Genetic Diversity in *Enterocytozoon bieneusi* Isolates from Dogs and Cats in China: Host Specificity and Public Health Implications

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To explore the genetic diversity, host specificity, and zoonotic potential of *Enterocytozoon bieneusi*, feces from 348 stray and pet dogs and 96 pet cats from different locations in China were examined by internal transcribed spacer (ITS)-based PCR. *E. bieneusi* was detected in 15.5% of the dogs, including 20.5% of stray dogs and 11.7% of pet dogs, and in 11.5% of the pet cats. Higher infection rates were recorded in the >2-year and the 1- to 2-year age groups in dogs and cats, respectively. Altogether, 24 genotypes, including 11 known and 13 new, were detected in 65 infected animals. In 54 positive dogs, 18 genotypes, 9 known (PtEbIX, O, D, CM1, EbpA, Peru8, type IV, EbpC, and PigEBITS5) and 9 new (CD1 to CD9), were found. In contrast, 8 genotypes, 4 known (D, BEB6, I, and PtEbIX) and 4 new (CC1 to CC4), were identified in 11 infected cats. The dominant genotype in dogs was PtEbIX (26/54). Phylogenetic analysis revealed that 8 known genotypes (D, Peru8, type IV, CM1, EbpC, PigEBITS5, O, and EbpA) and 7 new genotypes (CD1 to CD4 and CC2 to CC4) were the members of zoonotic group 1, whereas genotypes CD7, CD8, and CD9 together with PtEbIX belonged to the dog-specific group, and genotypes CD6 and CC1 were placed in group 2 with BEB6 and I. Conversely, genotype CD5 clustered with CM4 without belonging to any previous groups. We conclude that zoonotic genotypes are common in dogs and cats, as are host-specific genotypes in dogs.

Microsporidia, obligate eukaryotic intracellular pathogens, are considered to be highly diverged and specialized parasites, formerly classified as protozoa (1) and recently included in the fungus kingdom without further subdivision (2). They infect a wide variety of vertebrate and invertebrate hosts (3). Among the human-infecting microsporidian species, *Enterocytozoon bieneusi* is the most frequently diagnosed in AIDS patients with chronic diarrhea, organ transplant recipients, children, the elderly, and patients with malignant diseases and diabetes (4, 5). In addition, *E. bieneusi* has been reported in various wild, domestic, and companion mammals and birds worldwide (4, 6). Thus, microsporidiosis by *E. bieneusi* is regarded as a zoonosis, although the range of animal hosts and their involvement in transmission are poorly understood.

Recent molecular approaches based on sequence and phylogenetic analyses of the internal transcribed spacer (ITS) of ribosomal DNA (rDNA) enable us to assess the host specificity and public health significance of the organism (6, 7). There are now at least 204 reported ITS genotypes of *E. bieneusi*, and new genotypes have been identified in various animals, humans, and water bodies (6, 8–10). In phylogenetic analysis, these genotypes form some unique groups. Group 1 is found in humans and animals, while groups 2 to 8 are found mostly in specific hosts and wastewater (7, 8, 11).

Recently, zoonotic *E. bieneusi* genotypes have been reported in AIDS patients, children, nonhuman primates, pigs, and urban wastewater in China (8, 10–16). However, studies in companion animals, such as dogs and cats, which are considered high-risk hosts for the zoonotic transmission of such diseases, remain scarce. One study by Zhang and colleagues reported two new genotypes of *E. bieneusi* in dogs in China (17). Likewise, studies in dogs and cats have been few in other parts of the world. In these few studies, both host-specific and zoonotic genotypes of *E. bieneusi* have been reported in dogs and cats (18–20).

The purpose of the present study was to examine the occurrence and genetic diversity of *E. bieneusi* in dogs and cats in some parts of China and to assess the host specificity and zoonotic potential of this organism at the genotype level.

MATERIALS AND METHODS

Ethical approval. The research protocol was reviewed and approved by the research ethics committee of the Henan Agricultural University, and the present work was conducted in accordance with the Chinese Laboratory Animal Administration Act of 1988. Prior to fecal specimen collection, appropriate permissions were obtained from the owners of the animals whenever possible.

Sources and collection of specimens. A total of 348 dog fecal specimens, including 151 from stray animals and 197 from pet animals, were collected at two sites in Henan Province ($n = 244$) and one site each in Sichuan Province ($n = 40$), Shaanxi Province ($n = 30$), and Chongqing ($n = 34$) in China. Ninety-six pet cat fecal specimens were collected from four cities (Zhengzhou, Xinxiang, Xuchang, and Jiaozuo) in Henan Province. The dogs and cats were between <6 months and 8 years of age. The age was obtained from the pet animals only. Of the animals, 231 dogs were male and 117 were female, while 35 cats were male and 61 were female

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TABLE 1 Occurrence and genotype distributions of *E. bieneusi* in dogs and cats in China

Host and geographic locations	No. of specimens examined	No. (%) of positive specimens	No. of positive specimens/no. of specimens examined (%) and ITS genotypes (n^a) in:	
			Stray animals	Pet animals
Dog				
Henan Province				
Zhengzhou	200	32 (16.0)	21/111 (18.9); PtEbIX (10), O (4), EbpA (2), PigEBITS5 (1), D (1), CD3 (1), CD4 (1), CD5 (1)	11/89 (12.4); PtEbIX (3), CM1 (2), Peru8 (1), type IV (1), CD2 (1), CD6 (1), CD7 (2)
Jiaozuo	44	2 (4.6)		2/44 (4.6); D (2)
Subtotal	244	34 (13.9)	21/111 (18.9); PtEbIX (10), O (4), EbpA (2), PigEBITS5 (1), D (1), CD3 (1), CD4 (1), CD5 (1)	13/133 (9.8); PtEbIX (3), CM1 (2), D (2), Peru8 (1), type IV (1), CD2 (1), CD6 (1), CD7 (2)
Chengdu, Sichuan Province	40	10 (25.0)	10/40 (25.0); PtEbIX (9), CD1 (1)	
Xian, Shaanxi Province	30	6 (20.0)		6/30 (20.0); PtEbIX (2), EbpC (1), CD8 (2), CD9 (1)
Chongqing	34	4 (11.8)		4/34 (11.8); PtEbIX (2), CD8 (2)
Total	348	54 (15.5)	31/151 (20.5); PtEbIX (19), O (4), EbpA (2), PigEBITS5 (1), D (1), CD1 (1), CD3 (1), CD4 (1), CD5 (1)	23/197 (11.7); PtEbIX (7), CM1 (2), D (2), Peru8 (1), EbpC (1), type IV (1), CD2 (1), CD6 (1), CD7 (2), CD8 (4), CD9 (1)
Cat				
Henan Province				
Zhengzhou	40	10 (25.0)		10/40 (25.0); D (3), BEB6 (1), I (1), PtEbIX (1), CC1 (1), CC2 (1), CC3 (1), CC4 (1)
Xinxiang	24	1 (4.2)		1/24 (4.2); BEB6 (1)
Xuchang	30	0		0/30
Jiaozuo	2	0		0/2
Total	96	11/96 (11.5)		11/96 (11.5) D (3), BEB6 (2), I (1), PtEbIX (1), CC1 (1), CC2 (1), CC3 (1), CC4 (1)

[a] *n*, number of specimens.

(Tables 1 and 2). The animals were apparently healthy. The pet animals were fed by their owners and allowed to roam to some extent. The fecal specimens were collected either from the rectum of the animals or from the grounds after defecation. The specimens were gathered between January 2013 and February 2014.

The specimens were kept cool during shipment. After arrival at the Laboratory of Veterinary Parasitology, Henan Agricultural University, feces from each container were transferred in water into a 50-ml centrifuge tube. The specimens were sieved through a 7.62-cm-diameter sieve with a pore size of 45 μm and were concentrated by centrifugation. The concentrated fecal specimens were then stored in 2.5% potassium dichromate solution at 4°C until DNA extraction.

DNA extraction, PCR amplification, and nucleotide sequencing. The stored fecal specimens were washed three times by centrifugation with distilled water to remove the potassium dichromate. Genomic DNA was extracted using the E.Z.N.A. stool DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to manufacturer-recommended protocols. The extracted DNA was stored at −20°C until used in the PCR analysis.

For the detection of *E. bieneusi* in the DNA from each specimen, a 390-bp fragment, including the entire ITS (243 bp) and portions of the flanking large and small subunits of the rDNA (21), was amplified by a nested PCR using the primers EBITS3 (5′-GGTCATAGGGATGAAGAG-3′) and EBITS4 (5′-TTCGAGTTCTTTCGCGCTC-3′) in the primary PCR and the primers EBITS1 (5′-GCTCTGAATATCTATGGCT-3′) and EBITS2.4 (5′-ATCGCCGACGGATCCAAGTG-3′) in the secondary PCR. The primary PCR consisted of 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s, with an initial denaturation (94°C for 5 min) and a final extension (72°C for 10 min). In contrast, the secondary PCR consisted of 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, with conditions for the initial denaturation and final extension identical to those of the primary PCR (22). Each specimen was analyzed twice by using 2 μl of extracted DNA per PCR performed in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The rTaq amplification enzyme (TaKaRa Biotechnology Co., Ltd., Dalian, China) was used for PCR amplification. To neutralize the PCR inhibitors, 400 ng/μl of nonacetylated bovine serum albumin (Solarbio Co., Ltd., Beijing, China) was used in the primary PCR. The secondary PCR products were examined by agarose gel electrophoresis and visualized after GelRed (Biotium, Inc., Hayward, CA) staining.

All amplified products were sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) after being purified by Montage PCR filters (Millipore, Bedford, MA). Nucleotide sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product, when necessary.

Molecular analysis. To determine genotypes, the obtained sequences were aligned with reference sequences downloaded from GenBank using the program ClustalX 1.83 (http://www.clustal.org/). The genotypes from this study were compared with known *E. bieneusi* ITS genotypes using a neighbor-joining analysis of the aligned *E. bieneusi* sequences implemented in the program Mega 5 (http://www.megasoftware.net/). Bootstrap analysis was used to assess the robustness of clusters using 1,000 replicates. The established nomenclature system was used for naming the *E. bieneusi* ITS genotypes (23).

Statistical analysis. Differences in infection rates were compared using the chi-square test implemented in the software QuickCalcs (Graph-

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TABLE 2 Occurrence and genotype distributions of *E. bieneusi* in dogs and cats by age and gender

Host and characteristics	No. positive/ no. of specimens tested (%)	ITS genotypes (n^b)
Dog		
Age group[a]		
<6 mo	1/14 (7.1)	D (1)
6–12 mo	1/19 (5.3)	PtEbIX (1)
1–2 yr	5/49 (10.2)	PtEbIX (2), CM1 (1), D (1), CD8 (1)
>2 yr	16/115 (13.9)	PtEbIX (4), CM1 (1), Peru8 (1), EbpC (1), type IV (1), CD2 (1), CD6 (1), CD7 (2), CD8 (3), CD9 (1)
Gender		
Male	38/231 (16.5)	PtEbIX (21), O (3), PigEBITS5 (1), Peru8 (1), D (2), CM1 (2), EbpC (1), CD2 (1), CD3 (1), CD5 (1), CD6 (1), CD7 (1), CD8 (2)
Female	16/117 (13.7)	PtEbIX (6), O (1), EbpA (2), type IV (1), D (1), CD1 (1), CD4 (1), CD8 (2), CD9 (1)
Cat		
Age group		
<6 mo	0/2	
6–12 mo	1/11 (9.1)	D (1)
1–2 yr	3/23 (13.0)	PtEbIX (1), I (1), CC1 (1)
>2 yr	7/60 (11.7)	D (2), BEB6 (2), CC2 (1), CC3 (1), CC4 (1)
Gender		
Male	5/35 (14.3)	D (1), PtEbIX (1), BEB6 (1), I (1), CC1 (1)
Female	6/61 (9.8)	D (2), BEB6 (1), CC2 (1), CC3 (1), CC4 (1)

[a] Age groups applicable for pet dogs only.
[b] *n*, number of specimens.

Pad Software, Inc., La Jolla, CA). A difference was considered significant when the *P* value was <0.05.

Nucleotide sequence accession numbers. Representative nucleotide sequences from this study were deposited in GenBank under accession numbers KJ668719 to KJ668742.

RESULTS

Occurrence of *E. bieneusi* in dogs and cats. In the PCR amplification of *E. bieneusi* in 348 dog fecal specimens, 15.5% (54) were found to be positive, including 13.9% (34/244) in Henan Province, 25.0% (10/40) in Sichuan Province, 20.0% (6/30) in Shaanxi Province, and 11.8% (4/34) in Chongqing (Table 1). The differences in infection rates among the sampling sites were not statistically significant ($P = 0.332$). The infection rate in stray dogs (20.5% [31/151]) was significantly higher than that in pet dogs (11.7% [23/197]) ($P = 0.037$). Of the 96 pet cat fecal specimens, 11.5% (11) were positive for *E. bieneusi*, with the infection rates varying from 0% to 25.0% among the four sampling sites in Henan Province (Table 1). The differences in infection rates in cats among the four locations were statistically significant ($P = 0.01$).

The infection rates based on the age and gender of the dogs and cats are shown in Table 2. In dogs, the highest infection rate (13.9% [16/115]) was recorded in the >2-year-old group, while the lowest rate (5.3% [1/19]) was in the 6- to 12-month-old group. The differences in infection rates among the different age groups of dogs were not statistically significant ($P = 0.681$). The infection rate in male dogs (16.5% [38/231]) was higher than that in female dogs (13.7% [16/117]), but the difference was not statistically significant ($P = 0.533$). In cats, the infection rates ranged from 0% to 13.0% in the different age groups, although the variation was not statistically significant ($P = 0.953$). The difference in the infection rates between male and female cats (14.3% versus 9.8%, respectively) was also not significant ($P = 0.535$).

ITS genotypes in dogs and cats. In the nucleotide sequence analysis, a total of 24 *E. bieneusi* ITS genotypes, including 11 known (PtEbIX, O, D, CM1, EbpA, Peru8, type IV, EbpC, PigEBITS5, BEB6, and I) and 13 new (CD1 to CD9 and CC1 to CC4) genotypes, were found in 65 positive specimens from dogs and cats. In 54 positive dog specimens, 18 genotypes were observed, 9 of which were known (PtEbIX, O, D, CM1, EbpA, Peru8, type IV, EbpC, and PigEBITS5) and 9 of which were new (CD1 to CD9). In contrast, in 11 cat specimens that tested positive, 8 genotypes belonging to 4 known genotypes (D, BEB6, I, and PtEbIX) and 4 new genotypes (CC1 to CC4) were identified.

In dogs, the dominant genotype, PtEbIX, was observed in 26 of the 54 positive specimens (48.2%). Genotypes O and CD8 were found in 4 specimens each, while genotype D was found in 3 specimens. Genotypes EbpA, CM1, and CD7 were detected in 2 specimens each, whereas the remaining genotypes were seen in 1 specimen each. In cats, the more common genotypes, D and BEB6, were seen in 3 and 2 specimens, respectively, while the other genotypes were observed in 1 specimen each. The distributions of the genotypes based on the geographic source, type, age, and gender of the dogs and cats are shown in Tables 1 and 2.

Genetic relationships. The new genotypes CD2, CD3, CD4, CD5, CD7, CC1, and CC2 had one single nucleotide polymorphism (SNP) comparable to those of established genotypes CM1 (GenBank accession number KF305581), LW1 (JX000571), EbpD (JQ029735), CM4 (KF543866), PtEbIX (AB359947), BEB6 (KF543869), and D (KF305583), respectively. Genotypes CD6 and CC4 had two SNPs comparable to those of genotypes BEB6 (KF543869) and CHN4 (HM992511), respectively. Genotypes CD1 and CC3 had four and three SNPs comparable to those of genotypes Henan-V (KF305585) and D (KF305583), respectively. In contrast, genotypes CD8 and CD9 had four and five SNPs, respectively, comparable to that of genotype PtEbIX (AB359947).

Phylogenetic analysis of the observed *E. bieneusi* ITS genotypes with reference genotypes revealed that most of the genotypes in this study belonged to the previously designated zoonotic group 1 (7, 24). Eight known genotypes (D, Peru8, type IV, CM1, EbpC, PigEBITS5, O, and EbpA) and seven new genotypes (CD1 to CD4 and CC2 to CC4) were members of zoonotic group 1. However, a large number of animals, especially dogs, were infected with host-specific genotypes. The known genotype PtEbIX, along with new genotypes CD7, CD8, and CD9, clustered within a dog-specific group. Conversely, known genotypes BEB6 and I together with new genotypes CD6 and CC1 were placed in group 2, having so-called cattle host specificity. However, the new genotype CD5 clustered with the nonhuman primate genotype CM4 (GenBank accession number KF543866) (8) without belonging to any of the

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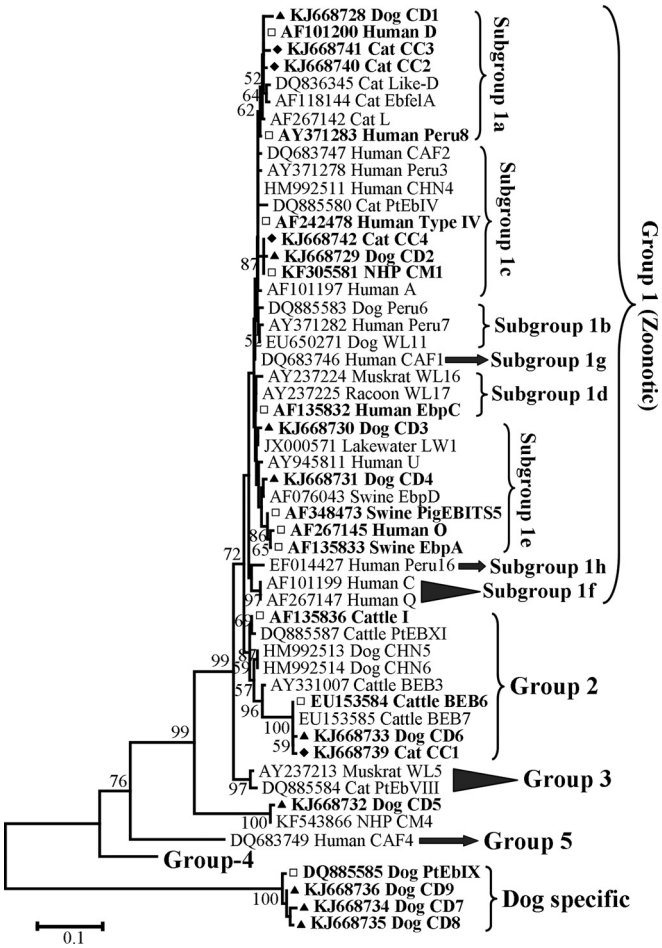

FIG 1 Phylogenetic relationship of *E. bieneusi* genotypes identified in this study and other genotypes previously deposited in GenBank as inferred by a neighbor-joining analysis of ITS sequences based on genetic distances calculated by the Kimura 2-parameter model. Bootstrap values of >50% from 1,000 replicates are shown on nodes. Each sequence from GenBank is identified by its accession number, host origin, and genotype designation. NHP, nonhuman primates. Group terminology for the clusters is based on that of Thellier and Breton (7). Genotypes found in this study are in bold type. Observed known genotypes are indicated by open boxes, while novel genotypes of dogs and cats are indicated by filled triangles and filled diamonds, respectively.

previously determined groups. Hence, they were located together between groups 3 and 5 (Fig. 1).

DISCUSSION

Enterocytozoon bieneusi was initially considered a human-specific parasite, especially in patients with AIDS. However, it was recently reported in a broad range of domestic, wild, and companion animals. Thus far, many *E. bieneusi* genotypes of zoonotic potential have been determined in various animal hosts, including dogs and cats, on the basis of ITS sequence analyses. However, the reservoir hosts and routes of transmission remain poorly understood (6).

In the present study, *E. bieneusi* was found to be a common parasite in dogs and cats. It was detected in 15.5% of dogs, including 20.5% of stray dogs and 11.7% of pet dogs. Similarly, 11.5% of pet cats were found to be infected with *E. bieneusi*. These results are in agreement with the findings of previous studies where the reported infection rates of *E. bieneusi* ranged from 7.8% to 15.0% in dogs (17, 19, 25) and from 5.0% to 31.3% in cats (18, 20, 26, 27). Surprisingly, in these two types of animals, the males were more likely to be infected with *E. bieneusi*, as initially reported in dogs by Santin and associates (19).

Among the 11 known *E. bieneusi* genotypes identified in this study, PtEbIX, D, and type IV were previously found in dogs and cats in Portugal, Colombia, Japan, Switzerland, Thailand, and Germany (18–20, 26–29). However, 9 of the known genotypes, including O, D, EbpA, Peru8, type IV, EbpC, I, PigEBITS5, and BEB6 (reported as SH5 in children by Wang et al. [15]), have been reported in humans and in other animals worldwide (6, 8, 10, 12–16, 18, 24, 30, 31). Thus, dogs and cats might play a role in the zoonotic transmission of *E. bieneusi* genotypes. Nevertheless, the genotype PtEbIX is considered to be dog specific and to have worldwide distribution (18). The remaining known genotype, CM1, was recently reported in nonhuman primates in China (8).

Ten of the established genotypes observed in this study, including D, type IV, EbpC, O, Peru8, I, BEB6, CM1, EbpA, and PtEbIX, have been reported in AIDS patients, children, nonhuman primates, cattle, pigs, and urban wastewater in China (8, 10–17). This observation suggests that cross-species transmission of these *E. bieneusi* genotypes occurs commonly in China.

In this study, the major *E. bieneusi* ITS genotype in dogs was PtEbIX, which was also reported as the dominant genotype in dogs in previous studies (19, 26, 29). In contrast, the most common genotype in cats was D, which is supported by the findings of a recent study in Thailand (18). Although, it is postulated that genotype PtEbIX is dog specific (19), we detected this genotype in one cat specimen.

Phylogenetic analysis showed that 7 of the 13 new *E. bieneusi* genotypes (CD1 to CD4 and CC2 to CC4) belong to the so-called zoonotic group 1 (7). Of these, genotypes CD1, CC2, and CC3 are related to genotypes Henan-V (14) and D, with 1 to 4 nucleotide differences, thus forming subgroup 1a. Likewise, genotypes CD2 and CC4 have 1 or 2 nucleotide substitutions comparable to those of genotypes CM1 (8) and CHN4 (17), forming subgroup 1c. The other two genotypes, CD3 and CD4, are related to genotypes LW1 (12) and EbpD, with a single nucleotide substitution, forming subgroup 1e.

Among the new host-specific genotypes, genotypes CD7 to CD9 have 1 to 5 nucleotide differences comparable to those of the dog-specific genotype PtEbIX, and thus they cluster together at the base of the phylogenetic tree. Genotypes CD6 and CC1 are related to genotype BEB6 with 1 or 2 nucleotide substitutions and are placed in the cattle-specific group 2. Note that two genotypes in dogs (CHN5 and CHN6), previously identified in China by Zhang et al. (17), are also clustered in group 2 in the phylogenetic analysis. Furthermore, two nonhuman primate genotypes (CM5 and CM7) were found in a recent study to be in this group (8). Members of this group, such as genotypes BEB4 (reported as CHN1), BEB6 (reported as SH5 in children), I, and J, have also been reported in humans and nonhuman primates in China (8, 15, 17). These observations further suggest that the genotypes of group 2 are not cattle specific (8). The remaining new genotype, CD5, is most related (1 nucleotide substitution) to the nonhuman primate genotype CM4 (8), forming a cluster between group 3 (muskrat genotypes) and group 5 (primate genotypes). This finding supports the suggestion of the presence of new *E. bieneusi* genotype groups (8, 9, 32).

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In conclusion, the present study found that dogs are infected with both potentially zoonotic and dog host-specific genotypes of *E. bieneusi*. In contrast, cats appear to be infected predominantly with zoonotic genotypes. Thus, dogs and cats can serve as potential reservoir hosts for zoonotic *E. bieneusi* genotypes. Furthermore, the presence of the same or genetically related genotypes in dogs, cats, other animals, urban wastewater, and humans in the same geographic area suggests the common occurrence of cross-species transmission of this pathogen. Therefore, studies that include simultaneous sampling of companion animals (dogs and cats) and humans residing in the same location are needed to better understand the zoonotic transmission routes of *E. bieneusi*.

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