Genetic diversity in Enterocytozoon bieneusi from dogs and cats in China: host specificity and public health implications

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Abstract

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 dogs including 20.5% of stray dogs and 11.7% of pet dogs, and in 11.5% of pet cats. Higher infection rates were recorded in the > 2 years and 1 to 2 years age groups in dogs and cats, respectively. All together, 24 genotypes, including 11 known and 13 new ones, were detected in 65 infected animals. In 54 positive dogs, 18 genotypes, nine known (PtEbIX, O, D, CM1, EbpA, Peru8, Type IV, EbpC and PigEBITS5) and nine new (CD1 to CD9), were found. In contrast, eight genotypes, four known (D, BEB6, I and PtEblX) and four new (CC1 to CC4), were identified in 11 infected cats. The dominant genotype in dogs was PtEbIX (26/54). Phylogenetic analysis revealed that eight known genotypes (D, Peru8, Type IV, CM1, EbpC, PigEBITS5, O and EbpA) and seven new ones (CD1 to CD4 and CC2 to CC4) were the members of zoonotic group 1, whereas genotypes CD7, CD8 and CD9 together with PtEblX 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. This study concludes that zoonotic genotypes are common in both animals along with host specific genotypes in dogs.

Key words: *Enterocytozoon bieneusi*; ITS; Dogs and cats; Zoonotic potential; Host specificity.
Introduction

Microsporidia, eukaryotic obligate intracellular pathogens, are considered to be highly diverged and specialized parasites, formerly classified as protozoa (1) and recently included in the Kingdom Fungi 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 one 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 analysis of the internal transcribed spacer (ITS) of the ribosomal RNA gene 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 continuously 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 both 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 zoonotic
transmission of such diseases, remain scarce. One study by Zhang and his colleagues reported two new genotypes of *E. bieneusi* in dogs in China (17). Likewise, studies in dogs and cats are also few in other parts of the world. In the 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 the organism at the genotypes 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 1988. Prior to fecal specimen collection, appropriate permission was obtained from 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), one site each in Sichuan province (n=40), Shaanxi province (n=30) and Chongqing city (n=34) in China. Ninety six pet cat fecal specimens were collected from four places (Zhengzhou, Xinxiang, Xuchang, and Jiaozuo) in Henan Province. The dogs and cats were aged between < 6 months to 8 years. The age was obtained only from pet animals. Of the animals, 231 dogs were male and 117...
were female while 35 cats were male and 61 were female (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 animals or from the grounds after defecation. The specimens were gathered during the period between January 2013 to February 2014.

The specimens were kept cool during shipment and after arrival at 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 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 with distilled water by centrifugation to remove the potassium dichromate. Genomic DNA was extracted using the E.Z.N.A.R® Stool DNA kit (Omega Biotek Inc., Norcross, USA) according to manufacturer-recommended protocols. The extracted DNA was stored at −20 °C until used in PCR analysis.

For the detection of *E. bieneusi* in the DNA from each specimen, a 390 bp fragment including the entire ITS (243 bp) as well as portions of the flanking large and small subunits of the RNA gene (21) was amplified by a nested PCR using the primers EBITS3 (5'-GGTCATAGGGATGAAGAG-3') and EBITS4 (5'-TTCGAGTTTCTTCGCCTC-3') in
primary PCR and primers EBITS1 (5’-GCTCTGAATATCTATGGCT-3’) and EBITS2.4 (5’-ATCGCCGACGGATCCAAGTG-3’) in secondary PCR. The primary PCR consisted of 35 cycles of 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, secondary PCR consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s with identical conditions of initial denaturation and final extension to primary PCR (22). Each specimen was analyzed twice by using 2 µl of extracted DNA per PCR performed in an Applied Biosystems® 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). rTaq amplification enzyme (Takara Biotechnology Co. Ltd., Dalian, China) was used for PCR amplification. To neutralize PCR inhibitors, 400 ng/µl of non-acetylated 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™ staining.

All amplified products were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) after being purified by Montage PCR filters (Millipore, Bedford, MA). The nucleotide sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if 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 in naming *E. bieneusi* ITS genotypes (23).

Statistical analysis

Differences of infection rates were compared using the chi-square test implemented in the software QuickCales (GraphPad 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 the 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% (54/348) were found 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 city (Table 1). This difference in infection rates among sampling sites was not statistically significant (*P*=0.332). The infection rate in stray dogs (20.5%, 31/151) was significantly higher than in pet dogs (11.7%, 23/197) (*P*=0.037). Of the 96 pet cat fecal specimens, 11 (11.5%) were
positive for *E. bieneusi*, with the infection rate varying from 0 to 25.0% among four sampling sites in Henan province (Table 1). The difference in infection rates in cats among the four locations was statistically significant (*P*=0.01).

The infection rates based on age and gender of dogs and cats are shown in Table 2. In dogs, the highest infection rate (13.9%, 16/115) was recorded in the >2 years age group while the lowest (5.3%, 1/19) was in the 6 to 12 months age group. The difference in infection rates among age groups of dogs was 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). However, the difference was not statistically significant (*P*=0.533). In cats, the infection rate ranged from 0 to 13.0% in different age groups, although the variation was not statistically significant (*P*=0.953). The difference in infection rates (14.3% versus 9.8%) between male and female cats was also not significant (*P*=0.535).

**ITS genotypes in dogs and cats**

In 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 ones (named as CD1 to CD9 and CC1 to CC4), were found in 65 positive specimens from dogs and cats. In 54 positive dog specimens, 18 genotypes were observed, nine of which were known (PtEbIX, O, D, CM1, EbpA, Peru8, Type IV, EbpC and PigEBITS5) and nine were new ones (CD1 to CD9). In contrast, eight genotypes were identified in 11 positive cat specimens, belonging to four known genotypes D, BEB6, I and PtEbIX, and four new ones (CC1 to CC4).
In dogs, the dominant genotype, PtEbIX was observed in 26 of the 54 positive specimens (48.2%). Genotypes O and CD8 were found in four specimens each while genotype D was found in three specimens. Genotypes EbpA, CM1 and CD7 were detected in two specimens each whereas the remaining ones were seen in one specimen each. In cats, the more common genotypes, D and BEB6, were seen in three and two specimens, respectively while the other genotypes were observed in one specimen each. The distribution of the genotypes based on geographical source, type, age and gender of 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) compared to established genotypes CM1 (KF305581), LW1 (JX000571), EbpD (JQ029735), CM4 (KF543866), PtEbIX (AB359947), BEB6 (KF543869) and D (KF305583), respectively. Genotypes CD6 and CC4 had two SNPs compared to genotypes BEB6 (KF543869) and CHN4 (HM992511). Genotypes CD1 and CC3 had four and three SNPs compared to genotypes Henan-V (KF305585) and D (KF305583), respectively. In contrast, genotypes CD8 and CD9 had four and five SNPs, respectively compared to 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 EpbA) and seven new ones (CD1 to CD4 and CC2 to CC4) were the
members of zoonotic group 1. However, a good 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 (KF543866, 8) without belonging to any of the previously determined groups. Hence, they together were located between groups 3 and 5 (Fig. 1).

Discussion

*Enterocytozoon bieneusi* was initially considered as a human-specific parasite, especially in AIDS patients. However, it has been recently reported in a broad range of domestic, wild and companion animals. Thus far, many *E. bieneusi* genotypes of zoonotic potentials have been determined in various animal hosts including dogs and cats on the basis of ITS sequence analysis. 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% dogs including 20.5% stray dogs and 11.7% pet dogs. Similarly, 11.5% pet cats were found to be infected with *E. bieneusi*. These results are in agreement with 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 both 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, genotypes PtEbIX, D and Type IV were previously found in dogs and cats in Portugal, Colombia, Japan, Switzerland, Thailand, Germany (18-20, 26-29). However, nine of the known genotypes, including O, D, EbpA, Peru8, Type IV, EbpC, I, PigEBITS5 and BEB6 (reported as SH5 in children by Wang and others, 15), have been reported in both humans and other animals worldwide (6, 8, 10, 12-16, 18, 24, 30, 31). Thus, dogs and cats might play a role in zoonotic transmission of *E. bieneusi* genotypes. Nevertheless, the genotype PtEbIX is considered to be dog specific with worldwide distribution (18). The remaining known genotype CM1 has recently been reported in nonhuman primates in China (8).

Ten of the established genotypes including D, Type IV, EbpC, O, Peru8, I, BEB6, CM1, EbpA and PtEbIX, observed in this study 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 findings of a recent study in Thailand (18). Although, it is postulated that genotype PtEbIX is strictly dog specific (19), we have detected this genotype in one cat specimen.
Phylogenetic analysis shows that seven of the 13 new *E. bieneusi* genotypes such as CD1 to CD4 and CC2 to CC4, belong to the so-called zoonotic group 1 (7). Of them, genotypes CD1, CC2 and CC3 are related to genotypes Henan-V (14) and D, with one to four nucleotide differences, forming subgroup 1a. Likewise, genotypes CD2 and CC4 have one to two nucleotide substitutions comparative to 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 1d.

Among the new host-specific genotypes, genotypes CD7 to CD9 have one to five nucleotide differences compared to the dog specific genotype PtEbIX and thus cluster together at the base of the phylogenetic tree. The genotypes CD6 and CC1 are related to genotype BEB6 with one to two nucleotide substitutions and are placed in the so-called cattle specific group 2. It should be noted that two previously identified genotypes in dogs (CHN5 and CHN6) in China by Zhang and others (17), are also clustered in group 2 in phylogenetic analysis. Furthermore, two nonhuman primate genotypes (CM5 and CM7) have been found to be the members of this group in a recent study (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 the group 2 are not cattle specific (8). The remaining new genotype CD5 is most related (one 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 on the presence of new genotype groups of *E. bieneusi* (8, 9, 32).
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 same or genetically related genotypes in dogs, cats, other animals, urban wastewater and humans in the same geographic area indicates 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*.

Acknowledgements

This study was supported in part by the State Key Program of National Natural Science Foundation of China (No. 31330079), Innovation Scientists and Technicians Troop Construction Projects of Henan Province (No. 134200510012), the International Cooperation and Exchange Projects of the National Natural Science Foundation of China (No. 31110103901), the Key National Science and Technology Specific Projects (No. 2012ZX10004220),
References


Table 1. Occurrence and genotype distribution of *E. bieneusi* in dogs and cats in China.

<table>
<thead>
<tr>
<th>Host</th>
<th>Host location</th>
<th>No. of specimens examined</th>
<th>No. (%) of positive specimens</th>
<th>No. of positive/no. of specimens examined (%)</th>
<th>ITS genotypes (n)</th>
</tr>
</thead>
<tbody>
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<td>Dog</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zhengzhou</td>
<td>200</td>
<td>32 (16.0)</td>
<td>21/111 (18.9)</td>
<td>PtEbIX (10), O (4), EbpA (2), PigEBIT5S (1), D (1), CD3 (1), CD4 (1), CD5 (1)</td>
</tr>
<tr>
<td></td>
<td>Jiaozuo</td>
<td>44</td>
<td>2 (4.6)</td>
<td>11/89 (12.4)</td>
<td>PtEbIX (3), CM1 (2), Peru8 (1), Type IV (1), CD2 (1), CD6 (1), CD7 (2)</td>
</tr>
<tr>
<td></td>
<td>Sub total</td>
<td>244</td>
<td>34 (13.9)</td>
<td>21/111 (18.9)</td>
<td>PtEbIX (10), O (4), EbpA (2), PigEBIT5S (1), D (1), CD3 (1), CD4 (1), CD5 (1)</td>
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<tr>
<td></td>
<td>Chengdu, Sichuan</td>
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<td>10 (25.0)</td>
<td>10/40 (25.0)</td>
<td>PtEbIX (9), CD1 (1)</td>
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<tr>
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<td>Xian, Shaanxi</td>
<td>30</td>
<td>6 (20.0)</td>
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<td>PtEbIX (2), EpbC (1), CD8 (2), CD9 (1)</td>
</tr>
<tr>
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<td>Chongqing</td>
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<td>4 (11.8)</td>
<td>4/34 (11.8)</td>
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<td></td>
<td>Total for dog</td>
<td>348</td>
<td>54 (15.5)</td>
<td>31/151 (20.5)</td>
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<td>Cat</td>
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<td>10 (25.0)</td>
<td>10/40 (25.0)</td>
<td>D (3), BEB6 (1), I (1), PtEbIX (1),</td>
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<td>Location</td>
<td>Count</td>
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<td>Percentage</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Xinxiang</td>
<td>24</td>
<td>1 (4.2)</td>
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<tr>
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<td>0 / 30</td>
<td></td>
<td></td>
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<tr>
<td>Jiaozuo</td>
<td>2</td>
<td>0</td>
<td>0 / 2</td>
<td></td>
<td></td>
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<tr>
<td><strong>Total for cat</strong></td>
<td><strong>96</strong></td>
<td><strong>11 / 96 (11.5)</strong></td>
<td><strong>11 / 96 (11.5)</strong></td>
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<td></td>
</tr>
</tbody>
</table>

D (3), BEB6 (2), I (1), PtEbIX (1), CC1 (1), CC2 (1), CC3 (1), CC4 (1)

\( n \), no of specimens
Table 2. Occurrence and genotype distribution of *E. bieneusi* in dogs and cats by age and gender.

<table>
<thead>
<tr>
<th>Host</th>
<th>Characteristics</th>
<th>No. of positive/no. of specimens tested (%)</th>
<th>ITS genotypes (n)</th>
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<tr>
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</tr>
<tr>
<td>Dog</td>
<td>Age groups</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 6 months</td>
<td>1/14 (7.1)</td>
<td>D (1)</td>
</tr>
<tr>
<td></td>
<td>6-12 months</td>
<td>1/19 (5.3)</td>
<td>PtEbIX (1)</td>
</tr>
<tr>
<td></td>
<td>1-2 years</td>
<td>5/49 (10.2)</td>
<td>PtEbIX (2), CM1 (1), D (1), CD8 (1)</td>
</tr>
<tr>
<td></td>
<td>&gt; 2 years</td>
<td>16/115 (13.9)</td>
<td>PtEbIX (4), CM1 (1), Peru8 (1), EbpC (1), Type IV (1), CD2 (1), CD6 (1), CD7 (2), CD8 (3), CD9 (1)</td>
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<td></td>
<td>Gender</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>Male</td>
<td>38/231 (16.5)</td>
<td>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),</td>
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<tr>
<td></td>
<td>Female</td>
<td>16/117 (13.7)</td>
<td>PtEbIX (6), O (1), EbpA (2), Type IV (1), D (1), CD1 (1), CD4 (1), CD8 (2), CD9 (1)</td>
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<tr>
<td>Cat</td>
<td>Age groups</td>
<td></td>
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<tr>
<td></td>
<td>&lt; 6 months</td>
<td>0/2</td>
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</tr>
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<td></td>
<td>6-12 months</td>
<td>1/11 (9.1)</td>
<td>D (1)</td>
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<td>1-2 years</td>
<td>3/23 (13.0)</td>
<td>PtEbIX (1), I (1), CC1 (1)</td>
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<td>&gt; 2 years</td>
<td>7/60 (11.7)</td>
<td>D (2), BEB6 (2), CC2 (1), CC3 (1), CC4 (1)</td>
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<td>Gender</td>
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<td>Female</td>
<td>6/61 (9.8)</td>
<td>D (2), BEB6 (1), CC2 (1), CC3 (1), CC4 (1)</td>
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</table>

* Age groups applicable only for pet dogs. n, no of specimens.
**Figure caption**

Figure 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 greater than 50% from 1,000 replicates are shown on nodes. Each sequence from GenBank is identified by the accession number, host origin, and the genotype designation. NHP represents nonhuman primates. The group terminology for the clusters is based on Thellier and Breton (7). The genotypes found in this study are in bold. The observed known genotypes are indicated by ‘open box’ while the novel genotypes of dogs and cats are indicated by ‘filled triangle’ and ‘filled diamond’, respectively.