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Genetic Polymorphism and Zoonotic Potential of *Enterocytozoon bieneusi* from Nonhuman Primates in China

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***Enterocytozoon bieneusi* is an important zoonotic pathogen. To assess the human-infective potential of *E. bieneusi* in nonhuman primates (NHPs), we examined the prevalence and genotype distribution of *E. bieneusi* in 23 NHP species by PCR and sequence analysis of the ribosomal internal transcribed spacer (ITS). A total of 1,386 fecal specimens from NHPs from five provinces in China were examined, and *E. bieneusi* was detected in 158 (11.4%) specimens from five NHP species, including cynomolgus monkey (67.7%), rhesus macaque (8.8%), Japanese macaque (33.3%), white-headed langur (13.6%), and golden snub-nosed monkey (3.5%) ($P < 0.0001$). The infection rates were 70.2%, 21.5%, 8.5%, 7.5%, and 5.6% in Guangdong, Yunnan, Guangxi, Henan, and Sichuan Provinces, respectively ($P < 0.0001$). The prevalence was significantly higher in captive (13.7%) than in free-range (5.0%) animals ($P < 0.0001$). Altogether, 16 ITS genotypes were observed, including nine known genotypes (IV, D, Henan V, Peru8, PigEBITS7, EbpC, Peru11, BEB6, and I) and seven new genotypes (CM1 to CM7). The common genotypes included CM1, IV, and D, which were detected in 43, 31, and 30 specimens, respectively. Phylogenetic analysis revealed that seven known genotypes (but not BEB6 and I) and four new genotypes (CM1, CM2, CM3, and CM6) belonged to the previously described group 1 with zoonotic potential. Genotypes CM5 and CM7 clustered with group 2, whereas genotype CM4 did not belong to any of the previously proposed groups. It was concluded that humans and NHPs residing in the same geographical location shared the same *E. bieneusi* genotypes, indicating a potential role of these animals in the zoonotic transmission of *E. bieneusi*.**

The microsporidia constitute a phylum (Microspora) that represents a group of about 1,300 species belonging to 160 genera of obligatory eukaryotic intracellular parasites capable of infecting nearly all animal phyla. Microsporidia are category B pathogens in the NIAID Priority Pathogens List (1–4). *Enterocytozoon bieneusi*, the most common species among approximately 14 human-pathogenic microsporidian species, is responsible for diarrhea and enteric diseases in various mammals and birds (5). It is one of the major causes of opportunistic infections in immunocompromised persons (3, 6) and has commonly been reported in children with diarrhea or malnutrition (7) and in other immunocompetent individuals (8). Although the infective stage of *E. bieneusi*, i.e., the environmentally resistant spore, has commonly been detected in many different hosts and water, little is known regarding the environmental source of microsporidia for the human population (4, 9, 10). Various animals, including nonhuman primates (NHPs), can serve as potential reservoirs of human infection (4, 11, 12).

Thus far, >100 *E. bieneusi* genotypes have been described from various hosts and water, based on DNA sequence analysis of the ribosomal internal transcribed spacer (ITS) (13). Phylogenetic analysis of ITS sequences revealed the presence of several genotypic groups: a large group (group 1) of closely related genotypes found in both humans and many animals, groups (groups 2 to 5) of host-adapted genotypes associated with specific animal hosts with no major public health importance, a small group (group 7) found in Nigerian AIDS patients, and a group found in urban wastewater (group 6) in China (5, 14).

The role of NHPs in the transmission of *E. bieneusi* has not yet been investigated fully due to the paucity of genetic studies of such host species. Three recent studies, in Guizhou and Guangxi, China, and in Kenya, reported infection rates of 28.2%, 18.5%, and 12.3% for *E. bieneusi* in free-range macaques, cynomolgus monkeys, and baboons, respectively. Both human-pathogenic and new ITS genotypes were identified (11, 12, 15).

China is a major supplier of nonhuman primates for biomedical research. Many colonies of cynomolgus monkeys (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) have been established in China since the 1950s. NHPs are also commonly maintained in zoos and zoological gardens. In addition, China is rich in wild NHP resources in its forest and nature reserves. Therefore, there is a high possibility of interspecies transmission of *E. bieneusi* among humans, NHPs, and other animals. The present study was conducted to detect and characterize *E. bieneusi* in NHPs to better understand the public health potential of parasites from these animals.

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MATERIALS AND METHODS

Ethics statement. This study was conducted in accordance with the Chinese Laboratory Animal Administration Act of 1988. The research protocol was reviewed and approved by the Research Ethics Committee of Henan Agricultural University. Specimens were obtained from feces defecated by the animals after acquiring the permission of owners of the animals or properties.

Sampling. A total of 1,386 fresh fecal specimens were collected from 23 NHP species housed in zoos, breeding facilities, research laboratories, and nature reserves in Henan, Guangxi, Sichuan, Yunnan, and Guangdong Provinces in China between 2009 and 2013 (Table 1). Upon arrival at Henan Agricultural University, feces from each container was transferred into a 50-ml centrifuge tube, and water was added to the tube. The specimen was sieved through a 7.62-cm-diameter sieve with a pore size of 45 μm and concentrated by centrifugation. The concentrated fecal material was stored in a 2.5% potassium dichromate solution at 4°C prior to DNA extraction.

DNA extraction and molecular detection. The stored fecal specimens were washed three times by centrifugation with distilled water. Genomic DNA was extracted from the specimens by using an EZNAR Stool DNA kit (Omega Biotek Inc.) according to manufacturer-recommended procedures. The extracted DNA was stored at −20°C before it was used for PCR analysis. DNA from each specimen was analyzed for *E. bieneusi* by a nested PCR targeting an approximately 392-bp region of the partial 18S rRNA gene, the complete internal transcribed spacer (ITS), and the partial 5.8S rRNA gene (16). The KOD-Plus-Neo amplification enzyme (Toyobo Co. Ltd., Osaka, Japan) was used for PCR amplification. To neutralize 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.

Nucleotide sequencing and phylogenetic analysis. Positive secondary PCR products were purified by use of Montage PCR filters (Millipore, Bedford, MA) and sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). The accuracy of nucleotide sequences was confirmed by bidirectional sequencing and by sequencing of a new PCR product if necessary. The sequences obtained were aligned with reference sequences downloaded from GenBank by use of the program ClustalX 1.83 (http://www.clustal.org/) to determine genotypes. The genotypes from this study were compared with known *E. bieneusi* ITS genotypes by 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 (13).

Statistical analysis. Infection rates were compared by the use of the chi-square test, and a difference was considered significant when the *P* value was <0.05. The analysis was done using QuickCalcs software (GraphPad Software Inc., La Jolla, CA).

Nucleotide sequence accession numbers. Representative nucleotide sequences from this study were deposited in GenBank under accession numbers KF305581 to KF305589 and KF543866 to KF543872.

RESULTS

Prevalence. *Enterocytozoon bieneusi* was detected in 158 (11.4%) of the 1,386 NHPs sampled. It was found in five NHP species, including cynomolgus monkey (67.7%), rhesus macaque (8.8%), Japanese macaque (33.3%), white-headed langur (13.6%), and golden snub-nosed monkey (3.5%), among 23 NHP species enrolled in this study (Table 1). The differences in infection rates among NHP species were highly significant ($P < 0.0001$). NHPs in Guangdong had a higher infection rate (70.2%) than those in Yunnan (21.5%), Guangxi (8.5%), Henan (7.5%), and Sichuan (5.6%), and the differences were statistically significant ($P < 0.0001$). The prevalence of infection was significantly higher in captive NHPs (13.7%) than in free-range animals (5.0%) ($P < 0.0001$). In captive animals, the infection rate at research laboratories (26.5%) was higher than those at monkey farms (13.0%) and zoos (7.4%) ($P < 0.0001$). The associations between geographical locations of nonhuman primates and their habitat types are shown in Table 2. The specimens collected from a monkey farm in Guangdong and a research laboratory in Yunnan had higher infection rates of *E. bieneusi*, whereas the free-range animals from Henan and Sichuan had lower infection rates.

Genotypes and their distribution. A total of 16 *E. bieneusi* ITS genotypes were observed in this study. They consisted of nine known genotypes (IV, D, Henan V, Peru8, PigEBITS7, EbpC, Peru11, BEB6, and I) and seven new ones (CM1 to CM7). The most common genotypes included CM1, IV, and D, which were observed in 43, 31, and 30 specimens, respectively. Genotypes Henan V, Peru8, CM4, CM2, and Peru11 were found in 10, 9, 8, 4, and 3 specimens, respectively, whereas genotypes PigEBITS7, EbpC, and BEB6 were observed in 5 specimens each. Other genotypes were seen in one specimen each (Table 1).

The largest number (13) of genotypes was found in rhesus macaques, and the dominant genotypes were CM1 ($n = 25$), D ($n = 17$), and IV ($n = 15$). Other genotypes included Henan V ($n = 7$), CM4 ($n = 7$), BEB6 ($n = 5$), EbpC ($n = 5$), Peru8 ($n = 4$), PigEBITS7 ($n = 4$), I ($n = 1$), CM5 ($n = 1$), CM6 ($n = 1$), and CM7 ($n = 1$). The most frequent genotypes in cynomolgus monkeys were IV and CM1, which were seen in 16 and 14 specimens, respectively, whereas genotypes Peru8, CM2, D, Peru11, CM3, and Henan V were seen in 1 to 3 specimens. White-headed langurs were infected mostly with genotype D ($n = 11$), followed by CM1 ($n = 4$), Peru8 ($n = 2$), Peru11 ($n = 1$), PigEBITS7 ($n = 1$), and CM2 ($n = 1$). Japanese macaques and golden snub-nosed monkeys were infected only with genotypes Henan V ($n = 3$) and CM4 ($n = 1$), respectively.

The dominant genotypes in Guangdong specimens were IV ($n = 15$) and CM1 ($n = 14$), whereas other genotypes, such as Peru8, CM2, D, Peru11, and CM3, were detected in 1 to 3 specimens. The frequently observed genotypes in Guangxi were D ($n = 14$) and CM1 ($n = 12$); other, less common ones included Peru8 ($n = 2$), IV ($n = 1$), CM2 ($n = 1$), and Peru11 ($n = 1$). Genotypes Henan V ($n = 10$), D ($n = 8$), CM4 ($n = 7$), EbpC ($n = 5$), PigEBITS7 ($n = 4$), IV ($n = 1$), I ($n = 1$), CM5 ($n = 1$), CM6 ($n = 1$), and CM7 ($n = 1$) were detected in Henan Province. In contrast, genotypes IV ($n = 13$) and CM1 ($n = 12$) were prevalent in Yunnan NHPs, although Peru8 and D were also seen in 4 and 2 specimens, respectively. On the other hand, Sichuan NHPs were infected with genotypes CM1 ($n = 5$), BEB6 ($n = 5$), D ($n = 4$), IV ($n = 1$), PigEBITS7 ($n = 1$), and CM4 ($n = 1$) (Table 2).

The dominant genotypes in captive NHPs included CM1 ($n = 43$), IV ($n = 31$), and D ($n = 24$), followed by Henan V ($n = 10$), Peru8 ($n = 9$), CM4 ($n = 8$), PigEBITS7 ($n = 5$), CM2 ($n = 4$), Peru11 ($n = 3$), CM3 ($n = 1$), CM5 ($n = 1$), and I ($n = 1$). In comparison, free-range NHPs were infected with genotypes D ($n = 6$), EbpC ($n = 5$), BEB6 ($n = 5$), CM6 ($n = 1$), and CM7 ($n = 1$). In the captive subgroups, the major genotypes were CM1 ($n = 31$), D ($n = 22$), and IV ($n = 17$) at monkey farms, Henan V ($n = 10$) in zoos, and IV (13) and CM1 (12) in research laboratories.

Phylogeny. Phylogenetic analysis revealed that most of the

TABLE 1 Prevalence of *Enterocytozoon bieneusi* and genotypes in nonhuman primates based on PCR and sequence analysis of the ITS locus

Common name	Scientific name	No. of specimens	No. (%) of positive specimens	Geographical source (province) (*n*)	Habitat (*n*)	ITS genotype(s) (*n*)
Cynomolgus monkey	*Macaca fascicularis*	62	42 (67.7)	Guangdong (57)	Farm (57)	CM1 (14), IV (15), D (2), Peru8 (3), CM2 (3), Peru11 (2), CM3 (1)
				Henan (3)	Zoo (3)	Henan V (1), IV (1)
				Sichuan (2)	Zoo (2)	
Assam macaque	*Macaca assamensis*	6	0	Henan (5)	Zoo (5)	
				Sichuan (1)	Zoo (1)	
Rhesus macaque	*Macaca mulatta*	1,048	92 (8.8)	Guangxi (220)	Farm (220)	CM1 (8), D (3), IV (1)
				Henan (470)	Zoo (40)	Henan V (6)
					Farm (221)	PigEBITS7 (4), CM4 (7), D (2), CM5 (1), I (1)
					Free range (209)	D (6), CM6 (1), CM7 (1), EbpC (5)
				Yunnan (122)	Research lab (117)	CM1 (12), IV (13), D (2), Peru8 (4)
					Zoo (5)	
				Sichuan (236)	Farm (85)	CM1 (5), D (4), IV (1)
					Free range (151)	BEB6 (5)
Japanese macaque	*Macaca fuscata*	9	3 (33.3)	Henan (8)	Zoo (8)	Henan V (3)
				Sichuan (1)	Zoo (1)	
Pig-tailed monkey	*Macaca nemestrina*	1	0	Yunnan (1)	Zoo (1)	
Tibetan macaque	*Macaca thibetana*	1	0	Yunnan (1)	Zoo (1)	
White-headed langur	*Presbytis leucocephalus*	147	20 (13.6)	Guangxi (143)	Farm (143)	D (11), CM1 (4), Peru8 (2), CM2 (1), Peru11 (1)
				Sichuan (4)	Zoo (4)	PigEBITS7 (1)
Olive baboon	*Papio anubis*	12	0	Henan (11)	Zoo (11)	
				Sichuan (1)	Zoo (1)	
Hamadryas baboon	*Papio hamadryas*	6	0	Yunnan (3)	Zoo (3)	
				Sichuan (3)	Zoo (3)	
Gibbon	*Hylobates* sp.	16	0	Henan (6)	Zoo (6)	
				Yunnan (9)	Zoo (9)	
				Sichuan (1)	Zoo (1)	
Golden monkey	*Cercopithecus kandti*	9	0	Henan (5)	Zoo (5)	
				Yunnan (2)	Zoo (2)	
				Sichuan (2)	Zoo (2)	
Tufted capuchin	*Sapajus paella*	7	0	Henan (3)	Zoo (3)	
				Sichuan (4)	Zoo (4)	
Mandrill	*Mandrillus sphinx*	16	0	Henan (5)	Zoo (5)	
				Sichuan (11)	Zoo (11)	
Orangutan	*Pongo* sp.	3	0	Henan (2)	Zoo (2)	
				Yunnan (1)	Zoo (1)	
Golden snub-nosed monkey	*Rhinopithecus roxellana*	29	1 (3.5)	Sichuan (27)	Zoo (7)	
					Farm (20)	CM4 (1)
				Yunnan (2)	Zoo (2)	
Hanuman	*Cercopithecus* sp.	4	0	Sichuan (4)	Zoo (4)	
Green monkey	*Chlorocebus sabaeus*	2	0	Sichuan (2)	Zoo (2)	
Red-faced spider monkey	*Ateles paniscus*	2	0	Sichuan (2)	Zoo (2)	
Squirrel monkey	*Saimiri* sp.	2	0	Sichuan (2)	Zoo (2)	
Red colobus monkey	*Procolobus* sp.	1	0	Yunnan (1)	Zoo (1)	
Gray leaf monkey	*Presbytis hosei*	1	0	Yunnan (1)	Zoo (1)	
Ring-tailed lemur	*Lemur catta*	1	0	Sichuan (1)	Zoo (1)	
Francois's leaf monkey	*Trachypithecus francoisi*	1	0	Yunnan (1)	Zoo (1)	
Total		1,386	158 (11.4)			CM1 (43), IV (31), D (30), Henan V (10), Peru8 (9), CM4 (8), PigEBITS7 (5), EbpC (5), BEB6 (5), CM2 (4), Peru11 (3), CM3 (1), CM5 (1), CM6 (1), CM7 (1), I (1)

TABLE 2 Prevalence and ITS genotype distribution of *Enterocytozoon bieneusi* in NHPs by geographical location in relation to habitat type

Geographical location (province)	No. of positive animals/no. of animals tested (%)				ITS genotypes (no. of specimens)
	Captive				
	Monkey farm	Zoo	Research lab	Free range	
Guangdong	40/57 (70.2)				IV (15), CM1 (14), Peru8 (3), CM2 (3), D (2), Peru11 (2), CM3 (1)
Guangxi	31/363 (8.5)				D (14), CM1 (12), Peru8 (2), IV (1), CM2 (1), Peru11 (1)
Henan	15/221 (6.8)	11/88 (12.5)		13/209 (6.2)	Henan V (10), D (8), CM4 (7), EbpC (5), PigEBITS7 (4), IV (1), I (1), CM5 (1), CM6 (1), CM7 (1)
Yunnan		0/27 (0)	31/117 (26.5)		IV (13), CM1 (12), Peru8 (4), D (2)
Sichuan	11/105 (10.5)	1/48 (2.1)		5/151 (3.3)	CM1 (5), BEB6 (5), D (4), IV (1), PigEBITS7 (1), CM4 (1)

positive animals were infected with genotypes belonging to the previously described human-pathogenic group (16) or the recently renamed group 1 (5, 17) (Fig. 1). Seven established genotypes (IV, D, Henan V, Peru8, PigEBITS7, EbpC, and Peru11) and four new genotypes (CM1, CM2, CM3, and CM6) belonged to this group. Two of the new genotypes had one nucleotide difference (G-to-A substitution) from genotype IV, at positions 104 and 179, and they were named CM3 and CM2, respectively, whereas genotype CM1 had two nucleotide differences (G-to-A substitutions at both positions) (data not shown). Thus, genotypes CM1, CM2, and CM3 formed one group (group 1c) with type IV by phylogenetic analysis. The other new genotype that belonged to group 1, i.e., CM6, had one nucleotide difference (C-to-T substitution) from genotype EbpC, and thus was placed in the same group (group 1d) as EbpC. New genotypes CM5 and CM7 differed from genotype BEB6 at nucleotide positions 196 (G-to-T substitution) and 170 (C-to-T substitution), respectively, and they formed a cluster (group 2) together with BEB6 and genotype I. The remaining new genotype, CM4, did not cluster with any of the known *E. bieneusi* genotype groups (Fig. 1) because of significant genetic differences.

DISCUSSION

The results of the present study show that *E. bieneusi* is a common parasitic pathogen in NHPs, as it was detected in 11.4% of the specimens examined. A similar infection rate (12.3%) for *E. bieneusi* was reported for captive baboons in Kenya (12), while higher infection rates (28.2% and 18.5%) were found in free-range rhesus macaques in a public park in Guizhou, China, and in cynomolgus monkeys in Guangxi, China, respectively (11, 15). The high *E. bieneusi* infection rate in rhesus macaques in the published study by Ye and others (11) might have been due to waterborne transmission, as *E. bieneusi* was also found in lake water of the public park where the study was conducted. In contrast, most of our specimens were from breeding facilities, zoos, and research laboratories under controlled conditions. Thus, in our study, an infection rate of 8.8% was seen in rhesus macaques. However, we did observe a very high *E. bieneusi* infection rate (67.7%) in cynomolgus monkeys, which were mostly from breeding facilities. A higher rate of infection was observed in the specimens from Guangdong Province. All of those specimens were collected from one monkey farm, which might have had a higher cross-transmission rate due to insufficient husbandry practices. In captivity, the higher prevalence of *E. bieneusi* might be because of the constant availability of susceptible animals and crowding.

Of the 16 *E. bieneusi* genotypes found in this study, 8 (IV, D, PigEBITS7, EbpC, Peru11, I, Peru8, and Henan V) have been reported in humans (4, 18, 19). Thus, at least some of the genotypes in NHPs have zoonotic potential, and NHPs could be reservoirs of human microsporidiosis.

All nine established *E. bieneusi* genotypes seen in this study have been reported in China, including the following: D, found in AIDS patients, children, cynomolgus monkeys, and urban wastewater; IV, found in HIV-positive and -negative patients, rhesus macaques, and urban wastewater; EbpC, found in HIV-positive and -negative patients, children, rhesus macaques, and urban wastewater; Peru11, found in HIV-negative patients, children, rhesus macaques, cynomolgus monkeys, and urban wastewater; Peru8 and PigEBITS7, found in AIDS patients and urban wastewater; I, found in cattle and children; Henan V, found in AIDS patients; and BEB6, found in urban wastewater (11, 14, 15, 19, 20, 21). In the present study, genotypes IV and D were found in the specimens from all five provinces. Likewise, Li and associates (14) also reported genotypes D and IV as the dominant ones in wastewater samples from four major cities in China. Genotype Henan V, previously reported in AIDS patients in Henan (19), was detected only in the specimens from Henan Province in this study, indicating a more limited geographical distribution and the likely occurrence of interspecies transmission of this genotype. The observation of cattle genotypes BEB6 and I only in free-range monkeys in Sichuan Province implies their possible cattle origin. However, these two genotypes have also been found in humans and urban wastewater in China (14, 20).

Four of the seven new *E. bieneusi* genotypes, CM1 to CM3 and CM6, are genetically related to the genotypes in so-called group 1 *E. bieneusi* (5) by phylogenetic analysis and thus have zoonotic potential. Among these, genotypes CM1 to CM3 are closely related to genotype IV, with one or two nucleotide differences, and are placed in group 1c, whereas genotype CM6 has one nucleotide substitution relative to genotype EbpC and is placed in group 1d (5). The genetic similarity of these new genotypes to the most prevalent zoonotic genotypes, types IV and EbpC in AIDS patients in China, suggests that they are potential human pathogens (19). Two of the new genotypes (CM5 and CM7) have only one nucleotide difference from genotype BEB6 and form a cluster with the genotypes of cattle-specific group 2 (5). These two new genotypes

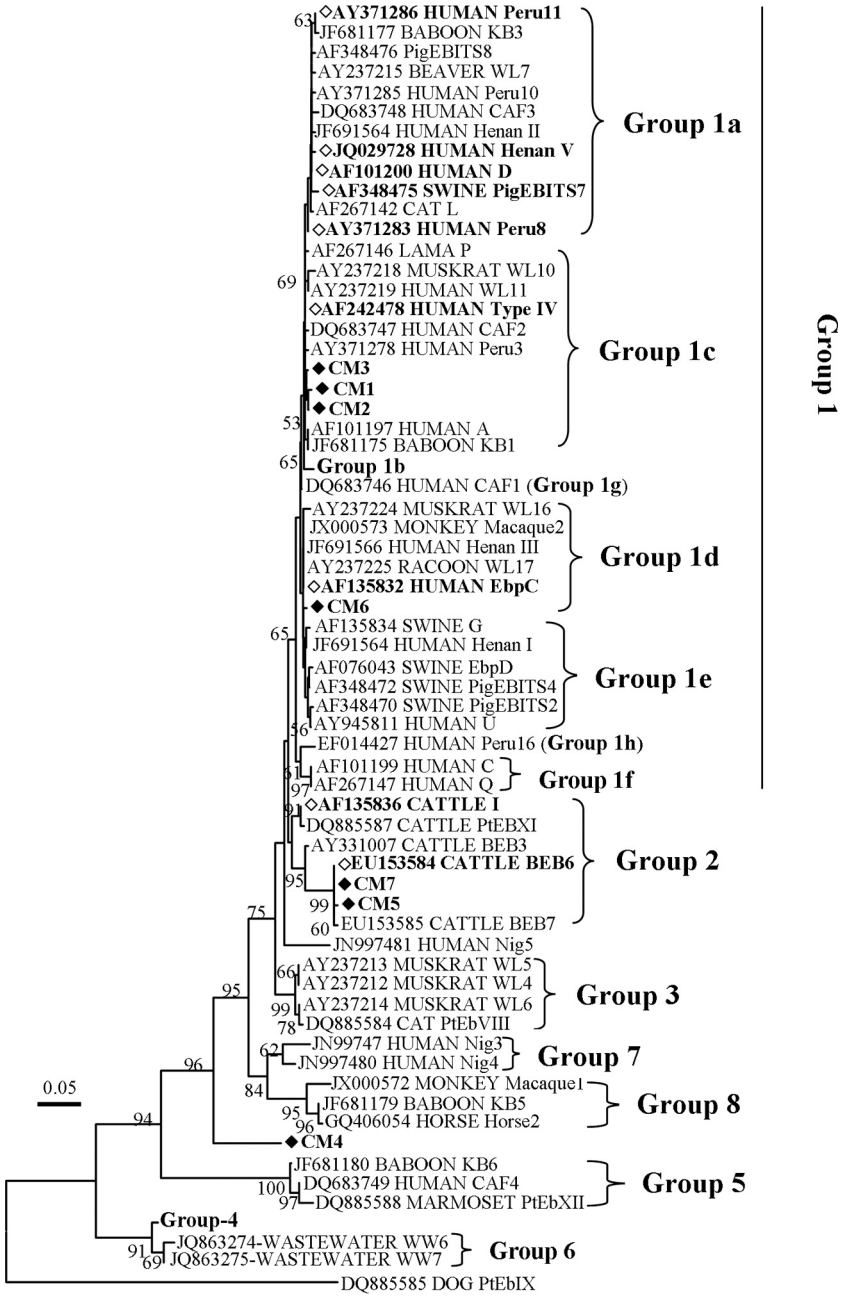

FIG 1 Phylogenetic relationships of *E. bieneusi* genotypes identified in this study and other genotypes previously deposited in GenBank, as inferred by a neighbor-joining analysis of ITS sequences based on genetic distances calculated by the Kimura two-parameter model. Bootstrap values of >50% from 1,000 replicates are shown on nodes. Each sequence from GenBank is identified by its accession number, host origin, and genotype designation. The group terminology for the clusters is based on the work of Sulaiman et al. (16), Thellier and Breton (5), and Li et al. (14). Several unique ITS sequences from monkeys, baboons, and horses are designated group 8 sequences. Known and new genotypes identified in this study are indicated by open and filled diamonds, respectively.

are probably present in nature, since they were observed in only one specimen each. Nevertheless, this and the previous observation of related genotypes in humans suggest that group 2 genotypes are not bovine specific. The remaining new genotype, CM4, is placed outside any of the groups proposed before (14). It lies between the primate-specific genotypic group 5 (14) and the newly proposed group 8 (this study), containing macaque 1 (11), baboon genotype KB5 (12), and horse 2 (22).

In conclusion, NHPs are commonly infected with zoonotic genotypes of *E. bieneusi*. The finding of the same dominant *E. bieneusi* genotypes in both NHPs and humans living in the same geographical locations clearly points out the likely occurrence of cross-species transmission of *E. bieneusi*. In light of this study, efforts should be taken to reduce contact between NHPs and susceptible human populations and contamination of drinking water sources by these reservoir hosts, thus minimizing zoonotic transmission of microsporidiosis. Special care should also be taken by animal attendants, animal care specialists, veterinarians, scien-

tists, and visitors in captive areas such as monkey farms, zoos, and research laboratories to minimize the anthroponotic and zoonotic transmission of parasites, including *E. bieneusi*.

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