High Prevalence of *Enterocytozoon bieneusi* in Asymptomatic Pigs and Assessment of Zoonotic Risk at the Genotype Level

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Enterocytozoon bieneusi is an emerging and clinically significant enteric parasite infecting humans and animals and can cause life-threatening diarrhea in immunocompromised people. Pigs are considered to be one of the main reservoir hosts of *E. bieneusi* based on their high prevalence rates and zoonotic genotypes in pigs. As an opportunistic pathogen, *E. bieneusi* infection of pigs can be inapparent, which leads to neglect in detecting this parasite in pigs and assessing the epidemiological role of pigs in the transmission of human microsporidiosis. In the present study, 95 healthy pigs aged 2 or 3 months were randomly selected from three areas in Heilongjiang Province, China. *E. bieneusi* isolates were identified and genotyped based on the small-subunit (SSU) rRNA and internal transcribed spacer (ITS) regions of the rRNA gene by PCR and sequencing. A high prevalence of *E. bieneusi* was observed, 83.2% (79/95) at the SSU rRNA locus versus 89.5% (85/95) at the ITS locus. Ten ITS genotypes were obtained, comprising six known genotypes—EbpA ($n = 30$), D ($n = 19$), H ($n = 18$), O ($n = 11$), CS-1 ($n = 1$), and LW1 ($n = 1$)—and four novel genotypes named HLJ-I to HLJ-IV; 70.6% (60/85) of *E. bieneusi* genotypes were zoonotic (genotypes EbpA, D, and O). The findings of a high prevalence of *E. bieneusi* in pigs and a large percentage of zoonotic genotypes indicate that pigs may play a role in the transmission of *E. bieneusi* to humans and may become an important source of water contamination in our investigated areas.

Microsporidia are a diverse group of obligate intracellular eukaryotic fungi that have the ability to infect humans and animals. They are considered to be one of the emerging opportunistic pathogens. To date, there are about 1,300 species in 160 genera formally described, and approximately 14 species in 8 genera have been found in humans (1). Molecular epidemiological data have confirmed that *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem* are the four major microsporidial species infecting humans (2). Among them, *E. bieneusi* shows the highest frequency in cases of human microsporidiosis, particularly in immunocompromised persons, such as HIV-infected patients and transplant recipients (3–6). Infections with *E. bieneusi* in HIV-negative, immunocompetent, and otherwise healthy individuals, as well as travelers, have also been reported (5). Clinical symptoms of microsporidiosis caused by *E. bieneusi* are variable, ranging from asymptomatic infection to acute or chronic diarrhea, depending on the health status of the hosts, the infective doses, and the virulence of the genotypes (5). Most importantly, the disease can cause increased mortality through life-threatening diarrhea in immunocompromised patients (7).

Microsporidial spores, the infective stage, have been found to survive in different environments, such as bodies of water, soil, and food products (8–10). The spores of *E. bieneusi* have been isolated from a drinking source watershed, recreational bathing water, and wastewater treatment plants (11–14). Studies to trace sources of contamination and to elucidate transmission routes of *E. bieneusi* have been conducted worldwide through genotyping *E. bieneusi* isolates from different hosts. The internal transcribed spacer (ITS) region of the rRNA gene is most commonly used in genotyping *E. bieneusi* isolates. To date, besides humans, it has been isolated from pigs, wild boars, macaques, dogs, cats, cattle, llamas, horses, gorillas, baboons, raccoons, muskrats, beavers, foxes, otters, chickens, pigeons, parrots, sparrows, aquatic birds,

and falcons (15–19). The finding of the same ITS genotypes of *E. bieneusi* in humans and animals supported the possibility of zoonotic transmission. Pigs are considered to be one of the most important reservoir hosts of *E. bieneusi*, and molecular epidemiological surveys have been conducted in 11 countries since the first report of *E. bieneusi* in pigs in Switzerland (20). Genotyping data for *E. bieneusi* isolates from pigs, including wild boars, have identified 34 genotypes, with 13 of them found in humans (Table 1) (17, 21–31). Therefore, genotyping of *E. bieneusi* isolates from pigs will provide useful information to assess the risk of zoonotic transmission.

In China, to date, eight molecular epidemiological studies of *E. bieneusi* have been conducted for 13 provinces, and it has been isolated in humans, pigs, cattle, dogs, and macaques, as well as in urban wastewater from wastewater plants (14, 27, 31–35) (Tables 2 and 3). The finding of the same ITS genotypes of *E. bieneusi* in China, eight in pigs and humans and four in pigs, wastewater, and humans, have raised public health concerns (Tables 2 and 3) (14, 27, 31–36), as the findings imply the potential for cross-genotype transmission from pigs to humans and the risk of exposure to environmental pathogens. Considering that *E. bieneusi* has been detected in healthy pigs (30), it is important to carry out a molecular epidemiological investigation of *E. bieneusi* in asymptomatic pigs, since inapparent infection may lead to less attention being paid to detecting zoonotic pathogens in these animals. Thus, pigs

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TABLE 1 *E. bieneusi* genotypes in natural infection of pigs and wild boars worldwide

Country ^a	Host	No. positive ^b /no. examined (%)	Genotype ^c (no.)	Zoonotic genotype ^c (no.)	% zoonotic genotypes	Reference
United States	Pig	64/202 (31.7)	D (3); F (2); Pig1 to Pig9	D (3); F (2); Pig5; Pig7; Pig9	Nonspecific	23
Austria	Wild boar	6/44 (13.6)	Henan-I (3); EbpC (2); D (1)	Henan-I (3); EbpC (2); D (1)	100	17
China	Pig	10/61 (16.4)	CHN1 (4); CHN7 (4); CHN8 (1); CHN9 (1); CHN10 (2)	CHN1 (4)	33.3	31
	Pig	51/113 (45.1)	D (1); EbpA (5); EbpC (12); Henan-I (1); Henan-III (1); Henan-IV (4); EbpD (1); H/EbpC (1); O (6); CS-1 (7); CS-2 (1); CS-3 (1); CS-4 (2); CS-5 (1); CS-6 (1); CS-7 (1); CS-8 (5)	D (1); EbpA (5); EbpC (12); Henan-I (1); Henan-III (1); Henan-IV (4); O (6); EbpD (1)	60.8	27
Czech Republic	Pig	74/79 (93.7)	F (70); D (2); Peru9 (2)	F (70); D (2); Peru9 (2)	100	30
	Wild boar	20/231 (8.7)	EpbA (10); D (5); G (1); WildBoar3 (1); WildBoar4 (2); EbpC (1)	EpbA (10); D (5); EbpC (1)	80.0	17
Germany	Pig	4/6 (66.7)	G (1); H (1); F (3)	F (3)	75.0	29
	Pig	5/50 (10.0)	F (3); O (1); G (1); H (1)	F (3); O (1)	80.0	24
	Pig	12/34 (32.3)	O (3); E (1); F (1); E1 (1); F1 (1); Bfrmr2 (1); Bfrmr5 (1)	O (3); E (1); F (1); Bfrmr2 (1)	50.0	28
Japan	Pig	10/30 (33.3)	F (1); H (4); PigEBITS5 (1); D (1); E (2); mixed infection (1)	F (1); D (1); E (2)	40.0	21
South Korea	Pig	67/472 (14.2)	EBITS5 (4); EBITS3 (2); EBITS4 (2); H (1); CAF1 (1)	CAF1 (1); EBITS5 (4)	50.0	25
Poland	Wild boar	10/129 (7.6)	EbpC (3); EbpA (2); WildBoar2 (1); WildBoar3 (1); WildBoar5 (2); WildBoar6 (1)	EbpC (3); EbpA (2)	50.0	17
Slovak Republic	Wild boar	2/56 (3.6)	WildBoar1 (1); D (1)	D (1)	50.0	17
Switzerland	Pig	38/109 (34.9)	EbpA (12); EbpB (6); EbpC (7); EbpD (3)	EbpA (12); EbpC (7)	67.9	22
Thailand	Pig	42/268 (15.7)	E (12); O (8); H (1)	E (12); O (8)	95.2	26

^a The numbers of genotypes are not consistent with the numbers of positives in some countries because not all isolates were genotyped successfully for Germany, Switzerland, and Thailand; there were mixed-genotype infections in China; and only some of the isolates from South Korea were genotyped.

^b The numbers of positives are from PCR results.

^c The names of genotypes are from publications.

with no clinical signs may have more time and opportunity to continually shed human-infective spores through feces into the environment to spread the disease. In Heilongjiang Province in northeastern China, pigs are one of the major economic animals. The aims of the present study were to determine the prevalence of natural infection of *E. bieneusi* in asymptomatic pigs in three areas of Heilongjiang Province, to analyze genetic characterizations of *E. bieneusi* isolates, and to assess the risk of zoonotic transmission at a genotype level by comparing the ITS gene sequences of *E. bieneusi* obtained with those from GenBank.

MATERIALS AND METHODS

Ethics statement. Before beginning work on the present study, we contacted the farm owners and obtained their permission to have their animals involved. During specimen collection, all work with animals followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and was approved by the Animal Ethical Committee of Harbin Medical University.

Fecal-specimen collection. Between May 2012 and September 2013, approximately 10 g of fecal specimens was collected from each of 95 pigs in three areas of Heilongjiang Province (30 from Wuchang, 29 from Yanshou, and 36 from Mingshui). Farms were selected based only on the owners' willingness to participate and the accessibility of animals for sampling. All the fecal specimens were collected from the ground immediately after defecation by using a sterile disposable latex glove and then placed in labeled sterile tubes individually. The ages of pigs ranged from 2 to 3 months. At the time of sampling, all the pigs were in good health. All the

specimens were transported to our laboratory in a cooler with ice packs within 24 h. After the fecal specimens were filtered and concentrated, they were all stored in 2.5% potassium dichromate at 4°C prior to being used in molecular biological characterizations.

DNA extraction. The potassium dichromate was washed off the fecal specimens with distilled water by centrifugation at $1,500 \times g$ for 10 min four times at room temperature. Genomic DNA was extracted from 200 mg of each processed specimen using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended procedures. The eluted DNA was stored at -20°C until further use in PCR analysis.

PCR amplification. An *E. bieneusi*-specific nested PCR was performed to amplify an approximately 607-bp nucleotide fragment of the small-subunit (SSU) rRNA gene. Two pairs of primers (C1 and C2, and EBIEF1 and EBIER1) were used in the first and second PCR amplifications, respectively. In fact, primers C1 and C2 were complementary to bases 1 to 18 and 1152 to 1170 of the *E. bieneusi* SSU rRNA sequence (GenBank accession no. [L16868](#)), respectively, while primers EBIEF1 and EBIER1, highly specific for diagnosis of *E. bieneusi*, were complementary to bases 295 to 315 and 881 to 901 of the above SSU rRNA sequence, respectively ([37, 38](#)). The cycle parameters for the two PCR amplifications were as follows: 30 cycles at 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s, followed by a final extension step at 72°C for 5 min, and 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, followed by a final extension step at 72°C for 5 min.

An approximately 390-bp nucleotide fragment of the rRNA gene of *E. bieneusi* containing 76 bp of the 3' end of the SSU rRNA gene, 243 bp of the internal gene, and 70 bp of the 5' region of the large-subunit (LSU) rRNA gene was amplified to identify and genotype the *E. bieneusi* isolates

TABLE 2 Prevalence and distribution of *E. bieneusi* genotypes in animals and environments by geographical location in China

Province ^a	Host	No. positive/no. examined (%)	Genotypes ^b (no.)	Zoonotic genotype(s) ^b (no.)	% zoonotic genotypes	Reference
Guangdong	Monkey	40/57 (70.2)	IV (15); CM1 (14); Peru8 (3); CM2 (3); D (2); Peru11 (2); CM3 (1)	IV (15); Peru8 (3); D (2); Peru11 (2)	55.0	32
Guangxi	Laboratory macaque	38/205 (18.5)	D (16); Peru11 (2); WL15 (1); Macaque3 (15); Macaque4 (2); noisy sequences (2)	D (16); Peru11 (2); WL15 (1)	50.0	35
	Nonhuman primate	31/363 (8.5)	IV (1); CM1 (12); Peru8 (2); CM2 (1); D (14); Peru11 (1)	IV (1); Peru8 (2); D (14); Peru11 (1)	58.1	32
Guizhou	Macaque	116/411 (28.2)	Peru11 (69); WL15 (28); EbpC (4); Type IV (6); Macaque1 (1); Macaque2 (1); unknown (7)	Peru11 (69); WL15 (28); EbpC (4); Type IV (6)	92.2	34
	Lake water	13/23 (56.5)	Peru11 (4); WL15 (5); EbpC (1); Type IV (2); LW1 (1)	Peru11 (4); WL15 (5); EbpC (1); Type IV (2)	92.3	34
	Nonhuman primate	39/518 (7.5)	Henan V (10); D (8); CM4 (7); EbpC (5); PigEBITS7 (4); IV (1); I (1); CM5 (1); CM6 (1); CM7 (1)	Henan V (10); D (8); EbpC (5); PigEBITS7 (4); IV (1); I (1)	74.4	32
Heilongjiang	Pig	39/86 (45.3)	D (1); EbpA (3); EbpC (10); EbpD (1); Henan-IV (4); O (3); CS-1 (6); CS2 (1); CS3 (1); CS4 (2); CS5 (1); CS6 (1); CS7 (1); CS8 (4)	D (1); EbpA (3); EbpC (10); Henan-IV (4); O (3); EbpD (1)	56.4	27
Inner Mongolia Jilin	Pig	2/2 (100)	EbpC (1); O (1)	EbpC (1); O (1)	100	27
	Pig	10/25 (40.0)	H/EbpC (1); Henan-I (1); O (2); EbpC (1); CS-1 (1); EbpA (2); Henan-III (1); CS-8 (1)	Henan-I (1); O (2); EbpC (1); EbpA (2); Henan-III (1)	70.0	27
	Pig	10/61 (16.4)	CHN1 (4); CHN7 (4); CHN8 (1); CHN9 (1); CHN10 (2)	CHN1 (4)	33.3	31
	Dog	2/26 (7.8)	CHN5 (1); CHN6 (1)		0.0	31
	Cattle	35/93 (37.6)	I (8); J (9); CHN1 (10); CHN3 (14); CHN4 (2)	I (8); J (9); CHN1 (10); CHN3 (14); CHN4 (2)	100	31
Jiangsu	Wastewater	54/87 (62.1)	Peru6 (1); Peru8 (1); Peru11 (2); D (50); WL12 (1); WL14 (2)	Peru6 (1); Peru8 (1); Peru11 (2); D (50); WL12 (1); WL14 (2)	100	14
Shandong	Wastewater	100/109 (91.7)	TypeIV (5); EbpC (1); EbpD (1); Peru6 (3); D (71); PtEbIV (1); BEB6 (1); PigEBITS7 (1); WW1 (4); WW2 (1); WW3 (15); WW4 (3); WW5 (1); WL4 (1); WW6 (4); WW7 (1); PtEbIX (4); WW8 (4); WW9 (1)	Type IV (5); EbpC (1); EbpD (1); Peru6 (3); D (71); BEB6 (1); PigEBITS7 (1)	67.5	14
Sichuan	Nonhuman primate	17/304 (5.6)	CM1 (5); BEB6 (5); D (4); IV (1); PigEBITS7 (1); CM4 (1)	BEB6 (5); D (4); IV (1); PigEBITS7 (1)	64.7	32
Shanghai	Wastewater	85/90 (94.5)	TypeIV (4); EbpC (2); Peru8 (1); Peru11 (2); D (78); PigEBITS7 (6); PigEBITS8 (1)	Type IV (4); EbpC (2); Peru8 (1); Peru11 (2); D (78); PigEBITS7 (6)	98.9	14
Wuhan	Wastewater	99/100 (99.0)	TypeIV (3); EbpC (5); Peru6 (1); Peru8 (11); Peru11 (6); C (1); D (80); EbpA (2); BEB6 (1); WW3 (5); WL4 (1); PtEbIX (3)	Type IV (3); EbpC (5); Peru6 (1); Peru8 (11); Peru11 (6); C (1); D (80); EbpA (2); BEB6 (1)	92.4	14
Yunnan	Nonhuman primate	31/144 (21.5)	IV (13); CM1 (12); Peru8 (4); D (2)	IV (13); Peru8 (4); D (2)	61.3	32

^a The numbers of genotypes are not consistent with the numbers of positives in Jilin, Jiangsu, and Shandong Provinces due to mixed-genotype infections.

^b The names of genotypes are from publications.

using two pairs of primers, EBIT3S3 and EBIT3S4, and EBIT3S1 and EBIT3S2.4 for the first and the second amplifications, respectively (23). The two sets of cycling parameters were 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s and 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, with both of them having a final extension step at 72°C for 10 min. A negative control with no DNA was included in all PCR tests.

All secondary PCR products mentioned above were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide before sequencing. If DNA preparations of *E. bieneusi* were positive at one gene

locus and negative at the other, they were subjected to two more repeated PCR amplifications accordingly at the locus negative for *E. bieneusi*.

Nucleotide sequencing and data analysis. All secondary PCR products of both genes were directly sequenced using the secondary PCR primers after being purified on an ABI Prism 3730 XL DNA Analyzer by Sinogeno-max Biotechnology Co., Ltd. (Beijing, China), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product, if necessary, for some DNA preparations.

TABLE 3 Genotypes of *E. bieneusi* in humans by geographical location in China

Province	Host	No. positive/no. examined (%)	Genotypes (<i>n</i>)	Reference
Henan	HIV-positive patient	39/683 (5.7)	EbpC (18); D (7); Type IV (6); PigEBITS7 (1); EbpD (1); Peru8 (1); Henan-I to Henan-V (1 each)	33
	HIV-negative patient	29/683 (4.2)	EbpC (21); D (5); Type IV (1); Peru11 (1); unknown (1)	33
Jilin ^a	Child	9/40 (25.5)	I (3); J (3); CHN1 (5); CHN2 (2); CHN3 (4); CHN4 (3)	31
Shanghai	Hospitalized child	24/573 (4.2)	Peru11 (6); EbpC (1); EbpA (2); D (1); SH1 (1); SH2 (3); SH3 to SH12 (1 each)	36

^a The number of genotypes is not consistent with the number of positives due to mixed-genotype infections.

E. bieneusi was identified and genotyped based on the SSU rRNA and ITS genes by comparing the obtained sequences with published sequences from GenBank using the Basic Local Alignment Search Tool (BLAST) and ClustalX 1.83. The genotypes of *E. bieneusi* isolates obtained in the present study were given the first published name if they had 100% homology with sequences in GenBank, while the others were given new names by adding numbers after an abbreviation of Heilongjiang Province (HLJ) according to the order of their appearance. All the genotypes in our study were named based on only 243 bp of the ITS gene region.

Phylogenetic analysis. To better present the diversity of genotypes and the relationship of newly obtained isolates to known ones, a neighbor-joining tree was constructed using the software Mega 5 (<http://www.megasoftware.net/>), based on the evolutionary distances calculated by a Kimura 2-parameter model. The reliability of these trees was assessed using bootstrap analysis with 1,000 replicates.

Nucleotide sequence accession numbers. The nucleotide sequences of new ITS genotypes obtained in this study were deposited in the GenBank database under the following accession numbers: [KJ475401](#) to [KJ475404](#).

RESULTS

Prevalence of *E. bieneusi* in pigs. A total of 95 pig fecal specimens were subjected to two nested-PCR protocols for the presence of *E. bieneusi*, based on the SSU rRNA and ITS genes. *E. bieneusi* was found in all three areas in Heilongjiang Province. The average prevalence of *E. bieneusi* in pigs was 83.2% (79/95) at the SSU rRNA locus versus 89.5% (85/95) at the ITS locus, without a statistically significant difference in prevalence at the two loci ($\chi^2 = 1.604$; $P > 0.05$) (Table 4). The prevalence rates of *E. bieneusi* in pigs were different in the three areas, either at the SSU rRNA locus or at the ITS locus. In Wuchang, *E. bieneusi* was detected in 90% of 30 fecal samples from pigs at the two loci. In the other two areas, although differences in prevalence were observed at the two loci, there were no statistically significant differences by a χ^2 test, accounting for 79.3% (23/29) at the SSU rRNA locus versus 93.1% (27/29) at the ITS locus in Yanshou ($\chi^2 = 2.06$; $P > 0.05$) and

80.6% (29/36) at the SSU rRNA locus versus 86.1% (31/36) at the ITS locus in Mingshui ($\chi^2 = 0.50$; $P > 0.05$).

Genetic characterizations at the SSU rRNA locus. Sequence analysis of 79 PCR products of the SSU rRNA gene of *E. bieneusi* showed one base difference (G/A) at nucleotide position 511. For convenience of description, they were named type 511^G ([KJ475400](#)) and type 511^A ([KJ475399](#)) (Table 4). Type 511^G, which was identical to those from cattle in North America ([AY257180](#)), showed absolute predominance in Wuchang (85.19%; 23/27) and in Yanshou (91.3%; 21/23), while type 511^A, which was identical to those from humans in Mexico ([AY298728](#)) and in Poland ([JN107808](#)), sheep in China ([JQ769381](#)), and falcons in the United Arab Emirates ([DQ793212](#)), showed uniqueness in Mingshui based on a prevalence of 100% for the type.

Genotypes of *E. bieneusi* in pigs. By sequence analysis of 85 pig-derived *E. bieneusi* isolates, a total of 11 polymorphic sites were detected within the 243 bp of the ITS gene (Table 5). Homology analysis of ITS gene sequences of *E. bieneusi* revealed the presence of 10 genotypes, comprising 6 known genotypes—EbpA ($n = 30$), D ($n = 19$), H ($n = 8$), O ($n = 11$), CS-1 ($n = 1$), and LW1 ($n = 1$)—and 4 novel genotypes named HLJ-I to HLJ-IV ([KJ475401](#) to [KJ475404](#)). Among them, genotype EbpA had the highest prevalence (35.3%; 30/85) and the widest distribution, accounting for the genotype being detected in all three investigated areas. Meanwhile, we also noticed the different genotypes and percentages of zoonotic genotypes of *E. bieneusi* in pigs in the three investigated areas: 33.3% (9/27), 77.8% (21/27), and 96.8% (30/31) of *E. bieneusi* isolates belonged to zoonotic genotypes in Wuchang, Yanshou, and Mingshui, respectively, based on the fact that genotypes EbpA, D, and O have been found in humans. On average, 70.6% (60/85) of *E. bieneusi* isolates were zoonotic in our investigated areas (Table 4). The percentages of zoonotic genotypes shown in Tables 1 to 4 are proportions of zoonotic genotypes sharing all the *E. bieneusi* genotypes identified.

TABLE 4 Prevalence and genotype distribution of pig-derived *E. bieneusi* isolates in three areas in Heilongjiang Province

Collection site	No. examined	SSU rRNA		ITS		Zoonotic genotype(s) (no.)	% zoonotic genotypes
		No. positive (%)	Type(s) (no.)	No. positive (%)	Genotypes (no.)		
Wuchang	30	27 (90.0)	511 ^G (23); 511 ^A (4)	27 (90.0)	H (16); HLJ-I (2); O (7); EbpA (2)	O (7); EbpA (2)	33.3
Yanshou	29	23 (79.3)	511 ^G (21); 511 ^A (2)	27 (93.1)	H (2); HLJ-II (1); CS-1 (1); LW1 (1); HLJ-III (1); EbpA (21)	EbpA (21)	77.8
Mingshui	36	29 (80.6)	511 ^A (29)	31 (86.1)	HLJ-IV (1); D (19); EbpA (7); O (4)	D (19); O (4); EbpA (7)	96.8
Total	95	79 (83.2)	511 ^G (44); 511 ^A (35)	85 (89.5)	H (18); HLJ-I (2); HLJ-II (1); HLJ-III (1); HLJ-IV (1); CS-1 (1); O (11); LW1 (1); EbpA (30); D (19)	O (11); EbpA (30); D (19)	70.6

TABLE 5 Variation at 11 polymorphic sites within ITS gene sequences of pig-derived *E. bieneusi* isolates

Genotype (no.)	GenBank accession no.	Nucleotide at position:										
		31	81	93	95	113	117	118	136	141	143	196
Known ^a												
EbpA (30)	AF076040	A	T	T	T	T	G	A	A	T	G	G
D (19)	JF776168	G	C	C	G	C	T	G	G	T	A	A
H (18)	AF135835	G	T	T	T	T	G	G	A	T	G	G
O (11)	AF267145	A	T	T	T	T	G	G	A	T	G	G
CS-1 (1)	KF607047	A	T	T	T	T	G	G	G	C	A	A
LW1 (1)	JX000571	A	C	T	G	T	G	G	G	C	A	A
Novel												
HLJ-I (2)	KJ475402	G	T	T	T	T	G	G	A	T	G	A
HLJ-II (1)	KJ475403	G	T	T	G	T	G	G	G	T	A	A
HLJ-III (1)	KJ475404	G	C	T	G	T	G	A	G	C	A	A
HLJ-IV (1)	KJ475401	G	C	T	G	T	T	A	G	T	A	A

^a Accession numbers of known genotypes, indicating that pig-derived *E. bieneusi* isolates have 100% homology with the sequences from GenBank.

Phylogenetic analysis. In a phylogenetic analysis of the ITS sequences, all 10 genotypes of *E. bieneusi* obtained in the present study, comprising 6 known and 4 novel genotypes, were clustered into one group, which was previously described as the human-pathogenic group or “group 1” and renamed recently (2) (Fig. 1).

DISCUSSION

In the present study, differences in the prevalence of *E. bieneusi* were observed in pigs at the two loci, 83.2% (79/95) at the SSU rRNA locus and 89.5% (85/95) at the ITS locus. However, no matter which gene was amplified, the prevalence of *E. bieneusi* in pigs was higher than that in any other study worldwide except one conducted in the Czech Republic, where up to 94% (74/79) of pigs were found to be infected with *E. bieneusi* (30). The prevalence rates of *E. bieneusi* in pigs are reported to be from 3.6% to 94.0% (Table 1). In fact, the differences in prevalences might be related to the sensitivity and specificity of detection methods, the health status of hosts, the experimental design, the overall sample size, livestock practices, and so on.

It is well known that molecular methods have the advantage of increased detection sensitivity compared to morphological and immunological techniques. However, they are also affected by the genetic structure of the gene fragments amplified. In fact, even at the same gene locus, the amplification rates may be different due to different primers used, as extensive genetic variations have been confirmed within *E. bieneusi* species (28). The health status of hosts, as a major opportunistic pathogenic factor for *E. bieneusi* infection, is closely associated with host age. In Thailand, a significantly higher prevalence of *E. bieneusi* was found in pigs aged 2 to 3.9 months than in pigs in other age groups (26). Recently, Li et al. reported that the infection rates of nursery pigs (21/33; 63.6%) were significantly higher than those of preweaned (25/61; 41.0%; $P < 0.05$) and growing (5/19; 26.3%; $P < 0.01$) pigs (27). However, in the Czech Republic, prevalence rates of *E. bieneusi* among all age categories of pigs were similar to each other (30). In the present study, the high prevalence of *E. bieneusi* might be attributable to the fact that all the fecal specimens were collected from postweaned piglets aged 2 to 3 months. Pigs in this age group are generally considered to have reduced immunity, as the animals lose maternal immunity while their own immunity still needs to develop. Meanwhile, a high intensity of pigs on farms, as well as

environments with poor sanitation, in the investigated areas might increase the opportunity for cross-transmission of *E. bieneusi* infection between different individuals. To date, the highest prevalence (94%) of *E. bieneusi* in pigs was actually detected within a closed, intensive pig farm in the Czech Republic, verifying that livestock practices might influence the prevalence of *E. bieneusi* in animals (30). The true prevalence might even be higher than those actually detected or reported, since shedding of spores was reported to be intermittent or sometimes even below the detection level of PCR (22).

By analyzing the SSU rRNA gene sequences of *E. bieneusi* obtained in the present study, two types, type 511^G ($n = 44$) and type 511^A ($n = 35$), were observed, with a base variation between them (Table 4). Although the SSU rRNA gene is not the gene used for genotyping *E. bieneusi* because of a low polymorphic level, or a relatively conservative nature, the result of homology analysis indicated that type 511^A was identical to human-derived *E. bieneusi* isolates, suggesting the possibility of zoonotic transmission of pig-derived *E. bieneusi*.

Currently, the ITS gene is considered the standard method for genotyping *E. bieneusi* because of a high degree of genetic diversity within the species; it has also been proposed that only 243 bp of the ITS region should be used as a genotyping tool (39). Sequence analysis of the ITS genes of 85 *E. bieneusi* isolates identified 10 genotypes in the present study, comprising 6 known genotypes (EbpA, D, H, O, CS-1, and LW1) and 4 novel genotypes (HLJ-I to HLJ-IV). Three known genotypes (EbpA, D, and O) have been described in humans (19). To date, 13 genotypes of *E. bieneusi* from pigs and wild boars, CAF1, D (Pig9), EbpA (F), EbpC (E), O, Bfrmr2, CHN1, Pig5, Pig7, Henan-I, Henan-III, Henan-IV, and EbpD, have been identified in humans, and zoonotic genotypes are found in all the epidemiological studies of natural infection of *E. bieneusi* in these animals (Table 1). These findings suggested that pigs may play an important role in the epidemiology of *E. bieneusi* as a reservoir host. The percentages of zoonotic genotypes were observed to vary in different countries or different areas within a country, ranging from 33.3% to 100% (Table 1). We also noticed that all the *E. bieneusi* isolates were identified as zoonotic in two studies conducted in Austria and the Czech Republic (17, 30). However, the risk that pigs pose to humans might be greater in the Czech Republic than in Austria, as a higher occurrence of *E.*

FIG 1 Phylogenetic relationships of *E. bienersi* genotypes identified in the study and other known genotypes deposited in GenBank, as inferred by a neighbor-joining analysis of ITS sequences based on genetic distance by the Kimura two-parameter model. The numbers on the branches are percent bootstrapping values from 1,000 replicates. Each sequence is identified by its accession number, host origin, and genotype designation. The group terminology for the clusters is based on the work of Guo et al. (13) and Karim et al. (32). The solid and open circles indicate novel and known genotypes identified in this study, respectively.

bieneusi in pigs was seen in the Czech Republic than in Austria (17, 30). Thus, in the assessment of the zoonotic risk of *E. bienersi* genotypes of animal origin in a specific farm or area, it is scientific and reasonable to consider the occurrence of the parasite together with the percentage of zoonotic genotypes. Four novel genotypes of *E. bienersi* obtained in the present study were clustered into the human-pathogenic group, or “group 1,” by phylogenetic analysis of the ITS sequences, supporting a zoonotic potential (Fig. 1).

Among the six known genotypes identified in the present

study, genotypes H and CS-1 have been found only in pigs (21, 25–27, 29). In spite of this, the conclusion that pigs are a specific host of genotypes H and CS-1 cannot currently be drawn because of the lack of epidemiological data from human and animal microsporidiosis. The fact that genotype LW1 was detected only in pigs showed that the contamination source for genotype LW1 in lake water might be local pigs (34). In the present study, 70.6% of 85 *E. bienersi* isolates in pigs have been described in humans (Table 4). A high prevalence of *E. bienersi* and a large percentage of

zoonotic genotypes in pigs in the investigated areas suggested that the animals posed a serious threat to the local inhabitants.

To date, genotype EbpA has been detected in only a few human cases of microsporidiosis, 10 in the Czech Republic (40) and 1 in Nigeria (41). In a recent study conducted in Shanghai, China, genotype EbpA of *E. bienewsi* was isolated in two children (36). In the present study, genotype EbpA could be detected in all three investigated areas and had the highest prevalence (35.3%; 30/85) and the largest percentage of zoonotic genotypes (50%; 30/60) in pigs. According to previous molecular epidemiological data on *E. bienewsi*, summarized in Table 1, genotype EbpA has been found in 7 out of 11 countries reporting *E. bienewsi* infection in pigs, including wild boars, showing the widest distribution (Table 1). The genotype has also been isolated from cattle, horses, mice, and birds (5, 42). Genotype D took the second place in pigs both in prevalence (22.35%; 19/85) of *E. bienewsi* and in the percentage (31.7%; 19/60) of zoonotic genotypes. This genotype has also been identified in pigs, as well as wild boars, in six countries (Table 1). Genotype D was more frequently detected in humans than other genotypes of *E. bienewsi*. To date, human microsporidiosis caused by genotype D has been found in America, Europe, Asia, and Africa (43–46). It has been commonly detected both in HIV-positive patients and in HIV-negative individuals (3). A similar result has been seen in Henan, China (33). Genotype D has also been isolated from children in Shanghai, China (36). In a study conducted in two renal transplant recipients with diarrhea in Spain, genotype D was the only genotype (44). A large variety of animals have been reported to be infected with genotype D. Besides pigs, the genotype has been isolated in cattle, cats, horses, dogs, and some wild animals, including macaques, muskrats, raccoons, beavers, foxes, and gorillas, as well as birds, such as pigeons and falcons, appearing to have a wide host range (18, 19, 47, 48). The above findings suggest that future studies should focus on epidemiological investigations of *E. bienewsi* in the reservoir hosts mentioned above. The molecular data will be useful to elucidate the transmission dynamics of microsporidiosis in our areas.

Genotype O was reported only in an HIV-seropositive patient in Thailand (49). The finding of genotype O in pigs in the present and previous studies supports the potential for zoonotic transmission (24, 26, 28). Genotype O seems to have strict host specificity, as it is found only in pigs and humans. The true host range of genotype O needs to be confirmed by subsequent molecular data from epidemiological studies of microsporidiosis.

The clinical symptoms of microsporidiosis caused by *E. bienewsi* mainly range from asymptomatic infection to acute or chronic diarrhea (50). No association was seen between the occurrence of diarrhea and *E. bienewsi* infections in pigs in the Czech Republic (30). However, in a study of the occurrence of *E. bienewsi* in pigs with diarrhea conducted in South Korea, *E. bienewsi* was detected only in the younger group of piglets with diarrhea, particularly those aged less than 1 week and between 1 and 2 weeks, whereas no significant difference in the rates of occurrence was observed between diarrheic and nondiarrheic piglets aged more than 4 weeks (25). Although numerous epidemiological studies of microsporidiosis in different hosts revealed that the majority of cases caused by *E. bienewsi* seemed to be asymptomatic, the negative effects on the growth and development of humans and animals need to be explored in the future.

In general, *E. bienewsi* seems to be adapted to pigs, based on the fact that the majority of pigs infected with *E. bienewsi* appear

asymptomatic. Thus, pig microsporidiosis caused by *E. bienewsi* might be paid less attention than it should be in assessing the epidemiological role of pigs as a reservoir host of *E. bienewsi*. In fact, pigs have been reported to be infected with *E. bienewsi* at an early age and excrete spores all their lives (30). Moreover, unscientific practices, such as using untreated pig manure as fertilizer directly on open crop or tillage land, result in environmental contamination, and a large number of spores in animal slurry can also enter streams and rivers from pasture runoff to cause wider geographical spread of *E. bienewsi* spores. Understanding the molecular epidemiology of *E. bienewsi* in different hosts is an important step in adequately controlling *E. bienewsi* infection in humans due to lack of an effective vaccine and available drugs. Of course, although sequence analysis of the ITS gene allows the differentiation of genotypes within *E. bienewsi*, as well as zoonotic assessment of *E. bienewsi* isolates of animal origin, new gene markers will be powerful aids to understanding the population genetic structure of *E. bienewsi* and to elucidating its significance in the epidemiology of microsporidiosis, since we could not determine whether the isolates of *E. bienewsi* with the same ITS gene sequences are genetically identical. Meanwhile, the fact that PCR preferentially amplifies only the major genotypes in fecal samples may explain why cases of mixed infection by different genotypes of *E. bienewsi* in pigs are rarely available. Subsequent studies will focus on multilocus sequence typing (MLST) of *E. bienewsi* and molecular detection of other microsporidial species in pigs. All the studies will be helpful in solving the public health problems pigs cause as a reservoir host of *E. bienewsi*.

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REFERENCES

- Keeling P. 2009. Five questions about microsporidia. *PLoS Pathog.* 5:e1000489. <http://dx.doi.org/10.1371/journal.ppat.1000489>.
- Thellier M, Breton J. 2008. *Enterocytozoon bienewsi* in human and animals, focus on laboratory identification and molecular epidemiology. *Parasite* 15:349–58. <http://dx.doi.org/10.1051/parasite/2008153349>.
- Akinbo FO, Okaka CE, Omeregbe R, Adamu H, Xiao L. 2013. Unusual *Enterocytozoon bienewsi* genotypes and *Cryptosporidium hominis* subtypes in HIV-infected patients on highly active antiretroviral therapy. *Am. J. Trop. Med. Hyg.* 89:157–161. <http://dx.doi.org/10.4269/ajtmh.12-0635>.
- Bednarska M, Bajer A, Welc-Faleciak R, Czubkowski P, Teisseyre M, Graczyk TK, Jankowska I. 2013. The first case of *Enterocytozoon bienewsi* infection in Poland. *Ann. Agric. Environ. Med.* 20:287–288.
- Matos O, Lobo ML, Xiao L. 2012. Epidemiology of *Enterocytozoon bienewsi* infection in humans. *J. Parasitol. Res.* 2012:981424. <http://dx.doi.org/10.1155/2012/981424>.
- Rivero-Rodríguez Z, Hernández Sierra A, Arráiz N, Bracho Mora A, Villalobos Perozo R. 2013. Prevalence of *Encephalitozoon intestinalis* and *Enterocytozoon bienewsi* in HIV positive patients to Maracaibo, Venezuela. *Invest. Clin.* 54:58–67. (In Spanish.)
- Waywa D, Kongkriengdaj S, Chaidatch S, Tiengrim S, Kowadisaiburana B, Chaikachonpat S, Suwanagool S, Chaiprasert A, Curry A, Bailey W, Suputtamongkol Y, Beeching NJ. 2001. Protozoan enteric infection in AIDS related diarrhea in Thailand. *Southeast Asian J. Trop. Med. Public Health* 32:151–155.
- Decraene V, Lebbaad M, Botero-Kleiven S, Gustavsson AM, Löfdahl M. 2012. First reported foodborne outbreak associated with microsporidia, Sweden, October 2009. *Epidemiol. Infect.* 140:519–527. <http://dx.doi.org/10.1017/S095026881100077X>.

9. Graczyk TK, Kacprzak M, Neczaj E, Tamang L, Graczyk H, Lucy FE, Girouard AS. 2007. Human-virulent microsporidian spores in solid waste landfill leachate and sewage sludge, and effects of sanitization treatments on their inactivation. *Parasitol. Res.* 101:569–575. <http://dx.doi.org/10.1007/s00436-007-0515-x>.
10. Śniński E. 2003. Environmental contamination with protozoan parasite infective stages: biology and risk assessment. *Acta Microbiol. Pol.* 52:97–107.
11. Ben Ayed L, Yang W, Widmer G, Cama V, Ortega Y, Xiao L. 2012. Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bienersi*, *Cyclospora cayentanensis* and *Eimeria* spp. *J. Water Health* 10:431–444. <http://dx.doi.org/10.2166/wh.2012.204>.
12. Cheng HW, Lucy FE, Graczyk TK, Broaders MA, Mastitsky SE. 2011. Municipal wastewater treatment plants as removal systems and environmental sources of human-virulent microsporidian spores. *Parasitol. Res.* 109:595–603. <http://dx.doi.org/10.1007/s00436-011-2291-x>.
13. Guo Y, Alderisio KA, Yang W, Cama V, Feng Y, Xiao L. 2014. Host specificity and source of *Enterocytozoon bienersi* genotypes in a drinking source watershed. *Appl. Environ. Microbiol.* 80:218–225. <http://dx.doi.org/10.1128/AEM.02997-13>.
14. Li N, Xiao L, Wang L, Zhao S, Zhao X, Duan L, Guo M, Liu L, Feng Y. 2012. Molecular surveillance of *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bienersi* by genotyping and subtyping parasites in wastewater. *PLoS Negl. Trop. Dis.* 6:e1809. <http://dx.doi.org/10.1371/journal.pntd.0001809>.
15. Feng Y, Li N, Dearen T, Lobo ML, Matos O, Cama V, Xiao L. 2011. Development of a multilocus sequence typing tool for high-resolution genotyping of *Enterocytozoon bienersi*. *Appl. Environ. Microbiol.* 77:4822–4828. <http://dx.doi.org/10.1128/AEM.02803-10>.
16. Li W, Kiulia NM, Mwenda JM, Nyachio A, Taylor MB, Zhang X, Xiao L. 2011. *Cyclospora papionis*, *Cryptosporidium hominis*, and human-pathogenic *Enterocytozoon bienersi* in captive baboons in Kenya. *J. Clin. Microbiol.* 49:4326–4329. <http://dx.doi.org/10.1128/JCM.05051-11>.
17. Němejc K, Sak B, Květoňová D, Hanzal V, Janiszewski P, Forejtek P, Rajský D, Kotková M, Ravaszová P, McEvoy J, Kváč M. 2014. Prevalence and diversity of *Encephalitozoon* spp. and *Enterocytozoon bienersi* in wild boars (*Sus scrofa*) in Central Europe. *Parasitol. Res.* 113:761–767. <http://dx.doi.org/10.1007/s00436-013-3707-6>.
18. Sak B, Petzelkova KJ, Kvetonova D, Mynarova A, Shutt KA, Pomajbikova K, Kalousova B, Modry D, Benavides J, Todd A, Kvac M. 2013. Long-term monitoring of microsporidia, *Cryptosporidium* and *Giardia* infections in western lowland gorillas (Gorilla gorilla gorilla) at different stages of habituation in Dzanga Sangha protected areas, Central African Republic. *PLoS One* 8:e71840. <http://dx.doi.org/10.1371/journal.pone.0071840>.
19. Santin M, Fayer R. 2011. Microsporidiosis: *Enterocytozoon bienersi* in domesticated and wild animals. *Res. Vet. Sci.* 90:363–371. <http://dx.doi.org/10.1016/j.rvsc.2010.07.014>.
20. Deplazes P, Mathis A, Muler C, Webe R. 1996. Molecular epidemiology of *Encephalitozoon cuniculi* and first detection of *Enterocytozoon bienersi* in faecal samples from pigs. *J. Eukaryot. Microbiol.* 43:93S. <http://dx.doi.org/10.1111/j.1550-7408.1996.tb05018.x>.
21. Abe N, Kimata I. 2010. Molecular survey of *Enterocytozoon bienersi* in a Japanese porcine population. *Vector Borne Zoonotic Dis.* 10:425–427. <http://dx.doi.org/10.1089/vbz.2009.0039>.
22. Breitenmoser AC, Mathis A, Bürgi E, Weber R, Deplazes P. 1999. High prevalence of *Enterocytozoon bienersi* in swine with four genotypes that differ from those identified in humans. *Parasitology* 118:447–453. <http://dx.doi.org/10.1017/S0031182099004229>.
23. Buckholt MA, Lee JH, Tzipori S. 2002. Prevalence of *Enterocytozoon bienersi* in swine: an 18-month survey at a slaughterhouse in Massachusetts. *Appl. Environ. Microbiol.* 68:2595–2599. <http://dx.doi.org/10.1128/AEM.68.5.2595-2599.2002>.
24. Dengjel B, Zahler M, Hermanns W, Heinritz K, Spillmann T, Thomschke A, Löscher T, Gothe R, Rinder H. 2001. Zoonotic potential of *Enterocytozoon bienersi*. *J. Clin. Microbiol.* 39:4495–4499. <http://dx.doi.org/10.1128/JCM.39.12.4495-4499.2001>.
25. Jeong DK, Won GY, Park BK, Hur J, You JY, Kang SJ, Oh IG, Lee YS, Stein BD, Lee JH. 2007. Occurrence and genotypic characteristics of *Enterocytozoon bienersi* in pigs with diarrhea. *Parasitol. Res.* 102:123–128. <http://dx.doi.org/10.1007/s00436-007-0740-3>.
26. Leelayoova S, Piyaaraj P, Subrungruang I, Pagornrat W, Naaglor T, Phumkhan S, Taamasri P, Suwanasri J, Mungthin M. 2009. Genotypic characterization of *Enterocytozoon bienersi* in specimens from pigs and humans in a pig farm community in Central Thailand. *J. Clin. Microbiol.* 47:1572–1574. <http://dx.doi.org/10.1128/JCM.00187-09>.
27. Li W, Diao R, Yang J, Xiao L, Lu Y, Li Y, Song M. 2014. High diversity of human-pathogenic *Enterocytozoon bienersi* genotypes in swine in northeast China. *Parasitol. Res.* 113:1147–1153. <http://dx.doi.org/10.1007/s00436-014-3752-9>.
28. Reetz J, Nöckler K, Reckinger S, Vargas MM, Weiske W, Broglia A. 2009. Identification of *Encephalitozoon cuniculi* genotype III and two novel genotypes of *Enterocytozoon bienersi* in swine. *Parasitol. Int.* 58:285–292. <http://dx.doi.org/10.1016/j.parint.2009.03.002>.
29. Rinder H, Thomschke A, Dengjel B, Gothe R, Löscher T, Zahler M. 2000. Close genotypic relationship between *Enterocytozoon bienersi* from humans and pigs and first detection in cattle. *J. Parasitol.* 86:185–188. [http://dx.doi.org/10.1645/0022-3395\(2000\)086\[0185:CGRBEB\]2.0.CO;2](http://dx.doi.org/10.1645/0022-3395(2000)086[0185:CGRBEB]2.0.CO;2).
30. Sak B, Kváč M, Hanzlíková D, Cama V. 2008. First report of *Enterocytozoon bienersi* infection on a pig farm in the Czech Republic. *Vet. Parasitol.* 153:220–224. <http://dx.doi.org/10.1016/j.vetpar.2008.01.043>.
31. Zhang X, Wang Z, Su Y, Liang X, Sun X, Peng S, Lu H, Jiang N, Yin J, Xiang M, Chen Q. 2011. Identification and genotyping of *Enterocytozoon bienersi* in China. *J. Clin. Microbiol.* 49:2006–2008. <http://dx.doi.org/10.1128/JCM.00372-11>.
32. Karim MR, Wang R, Dong H, Zhang L, Li J, Zhang S, Rume FI, Qi M, Jian F, Sun M, Yang G, Zou F, Ning C, Xiao L. 2014. Genetic polymorphism and zoonotic potential of *Enterocytozoon bienersi* from nonhuman primates in China. *Appl. Environ. Microbiol.* 80:1893–1898. <http://dx.doi.org/10.1128/AEM.03845-13>.
33. Wang L, Zhang H, Zhao X, Zhang L, Zhang G, Guo M, Liu L, Feng Y, Xiao L. 2013. Zoonotic *Cryptosporidium* species and *Enterocytozoon bienersi* genotypes in HIV-positive patients on antiretroviral therapy. *J. Clin. Microbiol.* 51:557–563. <http://dx.doi.org/10.1128/JCM.02758-12>.
34. Ye J, Xiao L, Ma J, Guo M, Liu L, Feng Y. 2012. Anthroponotic enteric parasites in monkeys in public park, China. *Emerg. Infect. Dis.* 18:1640–1643. <http://dx.doi.org/10.3201/eid1810.120653>.
35. Ye J, Xiao L, Li J, Huang W, Amer SE, Guo Y, Roellig D, Feng Y. 2014. Occurrence of human-pathogenic *Enterocytozoon bienersi*, *Giardia duodenalis* and *Cryptosporidium* genotypes in laboratory macaques in Guangxi, China. *Parasitol. Int.* 63:132–137. <http://dx.doi.org/10.1016/j.parint.2013.10.007>.
36. Wang L, Xiao L, Duan L, Ye J, Guo Y, Guo M, Liu L, Feng Y. 2013. Concurrent infections of *Giardia duodenalis*, *Enterocytozoon bienersi*, and *Clostridium difficile* in children during a cryptosporidiosis outbreak in a pediatric hospital in China. *PLoS Negl. Trop. Dis.* 7:e2437. <http://dx.doi.org/10.1371/journal.pntd.0002437>.
37. da Silva AJ, Schwartz DA, Visvesvara GS, de Moura H, Slemenda SB, Pieniazek NJ. 1996. Sensitive PCR diagnosis of infections by *Enterocytozoon bienersi* (microsporidia) using primers based on the region coding for small-subunit rRNA. *J. Clin. Microbiol.* 34:986–987.
38. Raynaud L, Delbac F, Broussolle V, Rabodonirina M, Girault V, Wallon M, Cozon G, Vivares CP, Peyron F. 1998. Identification of *Encephalitozoon intestinalis* in travelers with chronic diarrhea by specific PCR amplification. *J. Clin. Microbiol.* 36:37–40.
39. Santin M, Fayer R. 2009. *Enterocytozoon bienersi* genotype nomenclature based on the internal transcribed spacer sequence. A consensus. *J. Eukaryot. Microbiol.* 56:34–38. <http://dx.doi.org/10.1111/j.1550-7408.2008.00380.x>.
40. Sak B, Brady D, Pelikánová M, Květoňová D, Rost M, Kostka M, Tolarová V, Hůzová Z, Kváč M. 2011. Unapparent microsporidian infection among immunocompetent humans in the Czech Republic. *J. Clin. Microbiol.* 49:1064–1070. <http://dx.doi.org/10.1128/JCM.01147-10>.
41. Akinbo FO, Okaka CE, Omoriege R, Dearen T, Leon ET, Xiao L. 2012. Molecular epidemiologic characterization of *Enterocytozoon bienersi* in HIV-infected persons in Benin City, Nigeria. *Am. J. Trop. Med. Hyg.* 86:441–445. <http://dx.doi.org/10.4269/ajtmh.2012.11-0548>.
42. Del Coco VF, Córdoba MA, Bilbao G, de Almeida Castro P, Basualdo JA, Santin M. 2014. First report of *Enterocytozoon bienersi* from dairy cattle in Argentina. *Vet. Parasitol.* 199:112–115. <http://dx.doi.org/10.1016/j.vetpar.2013.09.024>.
43. Ayinmode AB, Ojuromi OT, Xiao L. 2011. Molecular identification of *Enterocytozoon bienersi* isolates from Nigerian children. *J. Parasitol. Res.* 2011:129542. <http://dx.doi.org/10.1155/2011/129542>.
44. Galván AL, Sánchez AM, Valentín MA, Henriques-Gil N, Izquierdo F,

- Fenoy S, del Aguila C. 2011. First cases of microsporidiosis in transplant recipients in Spain and review of the literature. *J. Clin. Microbiol.* 49: 1301–1306. <http://dx.doi.org/10.1128/JCM.01833-10>.
45. Mori H, Mahittikorn A, Watthanakulpanich D, Komalamisra C, Sukthana Y. 2013. Zoonotic potential of *Enterocytozoon bieneusi* among children in rural communities in Thailand. *Parasite* 20:14. <http://dx.doi.org/10.1051/parasite/2013014>.
 46. Sulaiman IM, Fayer R, Lal AA, Trout JM, Schaefer FW, III, Xiao L. 2003. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human-pathogenic *Enterocytozoon bieneusi*. *Appl. Environ. Microbiol.* 69:4495–4501. <http://dx.doi.org/10.1128/AEM.69.8.4495-4501.2003>.
 47. Mori H, Mahittikorn A, Thammasonthijarern N, Chaisiri K, Rojekitikhun W, Sukthana Y. 2013. Presence of zoonotic *Enterocytozoon bieneusi* in cats in a temple in central Thailand. *Vet. Parasitol.* 197:696–701. <http://dx.doi.org/10.1016/j.vetpar.2013.07.025>.
 48. Pirestani M, Sadraei J, Forouzandeh M. 2013. Molecular characterization and genotyping of human related microsporidia in free-ranging and captive pigeons of Tehran, Iran. *Infect. Genet. Evol.* 20:495–499. <http://dx.doi.org/10.1016/j.meegid.2013.10.007>.
 49. Leelayoova S, Subrungruang I, Suputtamongkol Y, Worapong J, Petmitr PC, Mungthin M. 2006. Identification of genotypes of *Enterocytozoon bieneusi* from stool samples from human immunodeficiency virus-infected patients in Thailand. *J. Clin. Microbiol.* 44:3001–3004. <http://dx.doi.org/10.1128/JCM.00945-06>.
 50. Didier ES, Weiss LM. 2006. Microsporidiosis: current status. *Curr. Opin. Infect. Dis.* 19:485–492. <http://dx.doi.org/10.1097/01.qco.0000244055.46382.23>.