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Prevalence and genotypes of *Enterocytozoon bieneusi* in weaned beef calves on cow-calf operations in the USA

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Abstract To determine the prevalence and genotype distribution of *Enterocytozoon bieneusi* in weaned beef calves in the USA, fecal samples were collected from 819 calves (6–18 months of age) from 49 operations. Feces were sieved and subjected to density gradient centrifugation to remove fecal debris and to concentrate spores. DNA extracted from each sample was subjected to the polymerase chain reaction (PCR) to amplify the complete internal transcriber spacer (ITS). All PCR-positive specimens were sequenced to determine the genotype(s) present. Overall, *E. bieneusi* was detected in 34.8% of the 819 fecal samples. The highest prevalence was found in the Midwest region (42.7%) followed by the South (35.8%) and the West (23.2%). The prevalence of *E. bieneusi* varied considerably from operation to operation (0–100%). A prevalence of 100% was observed in three operations, one in the Midwest and two in the South; *E. bieneusi* was not found in six operations, three in the South and three in the West. Sequence analysis revealed the presence of six genotypes, four previously reported (I, J, BEB4, and type IV) and two

novel genotypes (BEB8 and BEB9). Mixed infections were identified in five specimens, three contained I and BEB4 and two contained J and BEB4. Most of the positive calves (238 of 285) harbored genotypes with zoonotic potential including I (59), J (108), BEB4 (65), type IV (1), mixed I/BEB4 (3), and mixed J/BEB4 (2).

Microsporidia have been recognized as opportunistic pathogens of immunocompromised patients, but immunocompetent individuals have also been found to be infected (Didier 2005; Sak et al. 2011a). *Enterocytozoon bieneusi* is the species found most frequently in humans, mainly associated with chronic diarrhea and wasting syndrome (van Gool et al. 1995; Sobottka et al. 1998). *E. bieneusi* has also been found in a variety of wild, farm, and companion mammals and in birds (Santín and Fayer 2009a, 2011). It has been reported in surface water suggesting the possibility that domestic and wild animals could be sources (Dowd et al. 1998).

Over 100 genotypes of *E. bieneusi* have been identified based on nucleotide sequence polymorphisms in the 243-bp internal transcriber spacer (ITS) region of the rRNA gene. Some genotypes appear host specific for humans, others specific for animals, and still others appear zoonotic (Santín and Fayer 2011). Twenty-three genotypes have been found in feces from both humans and animals (Table 1). Of those, nine were found in cattle and humans (Table 1). Cattle are considered to be the only hosts for nine other genotypes (BEB3, PtEb XI, BEB7, 4948 FL-2, CEbA, CEbD, CEbF, M, and N) and for BEB6, also identified from goats (Dengjel et al. 2001; Fayer et al. 2007; Feng et al. 2011; Lee 2007, 2008; Lobo et al. 2006b; Santín et al. 2005; Sulaiman et al. 2004). *E. bieneusi* in cattle was first reported in three calves in Germany (Rinder et al. 2000) and shortly thereafter in seven additional calves (Dengjel et al. 2001). Extensive studies of

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Table 1 *Enterocytozoon bienersi* genotypes identified by the internal transcriber spacer (ITS) sequence and reported in humans and other hosts

<i>E. bienersi</i> genotype (GenBank accession number)	Synonyms (GenBank accession number)	Host	Location	Reference
CAF1 (DQ683746)	PEBE	Humans and pigs	Gabon, Niger, and Korea	Breton et al. (2007), Espern et al. (2007), and Jeong et al. (2007)
EbpC (AF076042)	E (AF135832), WL13 (AY237221), Pen4 (AY371279), and WL17 (AY237225)	Humans, pigs, beavers, otters, muskrats, raccoons, and foxes	Germany, Japan, Peru, Switzerland, Thailand, USA, and Vietnam	Depazes et al. (1996), Breitenmoser et al. (1999), Sulaiman et al. (2003a, b), Bern et al. (2005), Leelayoova et al. (2006, 2009), Cama et al. (2007), Espern et al. (2007), Abe and Kimata (2010), Reetz et al. (2009), and Feng et al. (2011)
Peru 16 (EF014427)		Humans and guinea pigs	Peru	Cama et al. (2007)
Peru10 (AY371285)		Humans and cats	Colombia and Peru	Sulaiman et al. (2003a), Bern et al. (2005), Santin et al. (2006), and Cama et al. (2007)
Type IV (AF242478)	K (AF267141), Pen2 (AY371277), PEBIII (DQ885579), BEB5 (AY331009), BEB5-var (AY331010) ^a , and CMITS1	Humans, cattle, cats, and dogs	Cameroon, Colombia, Gabon, England, France, Germany, Japan, Korea, Malawi, Netherlands, Niger, Peru, Portugal, Uganda, and USA	Liguory et al. (1998, 2001), Dengiel et al. (2001), Sadler et al. (2002), Tumwine et al. (2002), Sulaiman et al. (2003a, 2004), Bern et al. (2005), Sarfati et al. (2006), Santin et al. (2006, 2008), Lobo et al. (2006b), Breton et al. (2007), Cama et al. (2007), Espern et al. (2007), Lee (2008), Abe et al. (2009), and ten Hove et al. (2009)
WL11 (AY237219)	Pen5 (AY371280)	Humans, cats, dogs, and foxes	Colombia, Peru, and USA	Sulaiman et al. (2003a, b), Bern et al. (2005), Santin et al. (2006, 2008), and Cama et al. (2007)
O (AF267145)		Humans and pigs	Germany and Thailand	Dengiel et al. (2001), Leelayoova et al. (2006, 2009), and Reetz et al. (2009)
PigEBITS7 (AF348475)		Humans and pigs	Thailand and USA	Buckholt et al. (2002) and Leelayoova et al. (2006)
Peru6 (AY371281)	PEb I (DQ425107), and PEb VII (DQ885583)	Humans, cattle, birds, and dogs	Peru, Portugal, and USA	Sulaiman et al. (2003a), Bern et al. (2005), Santin et al. (2005), Lobo et al. (2006a, b), and Cama et al. (2007)
WL15 (AY237224)	WL16 (AY237224) and Peru14 (EF014430) ^a	Humans, beavers, foxes, raccoons, and muskrats	Peru and USA	Sulaiman et al. (2003b) and Cama et al. (2007)
D (AF101200)	WL8 (AY237216), PigEBITS9 (AF348477), Peru9 (AY371284), PEb VI (DQ885582), and CEbC (EF139197)	Humans, cattle, pigs, beavers, foxes, muskrats, raccoons, falcons, horses, dogs, mice, and mactaques	Abu Dhabi, Brazil, Colombia, Czech Republic, Gabon, Cameroon, England, Japan, Korea, Malawi, Netherlands, Niger, Peru, Portugal, Russia, Spain, Thailand, USA, Vietnam	Chalifoux et al. (2000), Buckholt et al. (2002), Sadler et al. (2002), Sulaiman et al. (2003a, b), Bern et al. (2005), Santin et al. (2005), Lobo et al. (2006b), Cama et al. (2007), Breton et al. (2007), Leelayoova et al. (2006), Lee (2007, 2008), Espern et al. (2007), Muller et al. (2008), Sak et al. (2008), Abe and Kimata (2010), Saksirisampan et al. (2009), Santin et al. (2010), ten Hove et al. (2009), Feng et al. (2011), Galván et al. (2011), Sak et al. (2011b), and Sokolova et al. (2011)
S6 (FJ439682)		Humans and mice	Czech Republic, Germany, and Malawi	ten Hove et al. (2009) and Sak et al. (2011b)
Peru8 (AY371283)	WL8 (AY237216)	Humans, chickens, and mice	Czech Republic, Germany, Malawi, and Peru	Sulaiman et al. (2003a), Bern et al. (2005), Cama et al. (2007), ten Hove et al. (2009), Feng et al. (2011), and Sak et al. (2011b)
CZ3 (GU198951)		Humans and mice	Czech Republic and Germany	Sak et al. (2011a, b)
C (AF101199)	II (AF242476)	Humans and mice	Czech Republic, France, Germany, Netherlands, and Switzerland	Rinder et al. (1997), Liguory et al. (1998, 2001), Dengiel et al. (2001), ten Hove et al. (2009), and Sak et al. (2011b)
BEB4 (AY331008)	CHN1 (HM992509)	Humans, cattle, and pigs	China, Czech Republic, and USA	Fayer et al. (2007), Santin et al. (2005), Santin and Fayer (2009b), Sulaiman et al. (2004), Sak et al. (2011a), and Zhang et al. (2011)
EbpA (AF076040)	F (AF132833)	Humans, cattle, pigs, and mice	Czech Republic, Germany, Japan, Switzerland, and USA	Breitenmoser et al. (1999), Rinder et al. (2000), Dengiel et al. (2001), Buckholt et al. (2002), Sak et al. (2008, 2011a, b), Abe and Kimata (2010), and Reetz et al. (2009)
PigITS5 (AF348173)	PEbA	Humans, pigs, and mice	Czech Republic, Germany, Japan, Korea, and USA	Buckholt et al. (2002), Jeong et al. (2007), Abe and Kimata (2010), and Sak et al. (2011a, b)
I (AF135836)	BEB2 (AY331006) and CEbE (AF139199)	Humans and cattle	China, Germany, Korea, and USA	Rinder et al. (2000), Dengiel et al. (2001), Fayer et al. (2007), Santin et al. (2005), Santin and Fayer (2009b), Sulaiman et al. (2004), Lee (2007, 2008), and Zhang et al. (2011)

Table 1 (continued)

<i>E. bieneusi</i> genotype (GenBank accession number)	Synonyms (GenBank accession number)	Host	Location	Reference
J (AF135837)	BEB1 (AY331005), PIEb X (DQ885586), and CEBB (EF139196)	Humans, cattle, and birds	China, Germany, Korea, Portugal, and USA	Rinder et al. (2000), Dengiel et al. (2001), Reetz et al. (2002), Lobo et al. (2006b), Fayer et al. (2007), Santin et al. (2005), Sulaiman et al. (2004), Lee (2007, 2008), Santin and Fayer (2009b), and Zhang et al. (2011)
CHN3 (HM092511)		Humans and cattle	China	Zhang et al. (2011)
CHN4 (HM092512)		Humans and cattle	China	Zhang et al. (2011)
WL12 (AY237220)		Humans, beavers, and others	Brazil and USA	Sulaiman et al. (2003b) and Feng et al. (2011)

^a Unpublished

dairy cattle, from neonates to mature milking cows, in farms along the east coast of the USA have reported infection with several genotypes of *E. bieneusi* (Fayer et al. 2003, 2007; Santin et al. 2004, 2005). A longitudinal study of 30 dairy cattle from birth to 24 months of age on a farm in eastern USA found that 100% of the animals became infected with *E. bieneusi* at some time during the study (Santin and Fayer 2009b). Genotypes I, J, and BEB4 were identified, with genotype I being the dominant genotype, infecting all calves and present in each calf for the longest duration (Santin and Fayer 2009b). Although *E. bieneusi* was detected in dairy and beef cattle in Korea, data did not distinguish dairy and beef breeds (Lee 2007).

The absence of data on the prevalence *E. bieneusi* in beef cattle led to the present study which was conducted to determine the occurrence of *E. bieneusi* in beef cattle using fecal specimens obtained from cow-calf operations throughout the USA and to determine which genotypes were present.

Materials and methods

Cow-calf operation location and sample collection

The US Department of Agriculture (USDA) National Animal Health Monitoring System conducted the Beef 2007–2008 study on cow-calf operations in 24 states (USDA 2008). Fecal samples for *E. bieneusi* testing were available from operations in 20 of the 24 states from three regions (Midwest—Iowa, Kansas, Missouri, Nebraska, North Dakota, and South Dakota; South—Alabama, Georgia, Louisiana, Mississippi, Oklahoma, Tennessee, Texas, and Virginia; and West—California, Colorado, Idaho, New Mexico, Oregon, and Wyoming). Participating producers each collected up to 20 fecal samples directly from the rectum of 6- to 18-month-old weaned calves or from fresh fecal pats on the ground from calves to assess parasite burden.

Sample processing, PCR, gene sequencing, and phylogenetic analysis

Specimens received at the Environmental Microbial and Food Safety Laboratory were examined for the presence of *E. bieneusi* after spores were concentrated from feces by sieving and CsCl density gradient centrifugation to remove fecal debris and concentrate spores as described (Fayer et al. 2007). Each 15 g sample of feces was suspended in 35-ml dH₂O and passed through a sieve with a 45- μ m pore size. The filtered suspension was transferred to a 50-ml conical tube, the volume adjusted to 50 ml with dH₂O, and the tube was centrifuged at 1,800 \times g for 15 min. The pellet was resuspended in 25 ml dH₂O; 25 ml CsCl (1.4 specific gravity)

was added, and the contents mixed by vortexing. The tube was centrifuged at $300\times g$ for 20 min, and 4 ml of supernatant was aspirated from the surface. The aspirate was washed twice with dH₂O, and the final pellet was suspended in 500 μ l of dH₂O for subsequent DNA processing.

DNA was extracted from each specimen using a DNeasy Tissue Kit (Qiagen, Valencia, California). The slightly modified protocol, described below, employed reagents from the manufacturer. Fifty microliters of processed specimen was suspended in 180 μ l of ATL buffer, thoroughly mixed, 20 μ l of proteinase K (20 mg/ml) was added, and the suspension was mixed again. Following overnight incubation at 55°C, 200 μ l of AL buffer was added. The remaining protocol followed manufacturer's instructions except, to recover more DNA, the nucleic acid was eluted in 100 μ l of AE buffer.

The polymerase chain reaction (PCR) amplification was performed using a set of nested primers specific for *E. bienersi* that amplified the ITS and portions of the flanking large and small subunits of the rDNA (~400 bp). The outer primers were EBITS3 (5' GGTCATAGGGATGAAGAG 3') and EBITS4 (5' TTCGAGTTCTTTCGCGCTC 3'), and the inner primers were EBITS1 (5' GCTCTGAATATCTATGGCT 3') and EBITS2.4 (5' ATCGCCGACGGATCCAAGTG 3') (Buckholt et al. 2002). The reaction mixture (50 μ L) contained 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH=9), 0.2 mM dNTPs, 50 pmol of each primer, 2.5 U of Taq (Qiogene Inc., Carlsbad, California), and 2.5 μ L of BSA (0.1 g/10 mL). After denaturation at 94°C for 3 min, the first PCR samples were subjected to 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 40 s), followed by a final extension at 72°C for 10 min. Conditions for the secondary PCR were identical to the primary PCR except only 30 cycles were carried out with an annealing temperature of 55°C. These reactions produced fragments of 435 and 390 bp, respectively. Negative and positive controls were included in all sets of PCRs, as well as amplifying the negative control from the first PCR in the second reaction to check for low level contamination. The PCR products were subjected to electrophoresis in a 1% agarose gel and visualized by staining the gel with ethidium bromide.

All PCR-positive samples were directly sequenced with the inner set of primers used for the secondary PCR. PCR products were purified using EXO-SAP enzyme (USB Corporation, Cleveland, Ohio). Purified products were sequenced at 10 μ L reactions using Big Dye™ chemistries, and an ABI3130 sequence analyzer (Applied Biosystems, Foster City, California). Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Inc., Madison, Wisconsin). The sequences were compared with sequences in the GenBank database by BLAST analysis. Nucleotide sequences obtained in the present study were deposited in the GenBank database under accession numbers JQ044398 and JQ044399.

Phylogenetic analyses

Sequences from all *E. bienersi* genotypes identified in cattle and humans were aligned using the ClustalW algorithm using the MEGA version 4 (Tamura et al. 2007). ClustalW determines that when a gap is inserted it can be removed only by editing, so final alignment adjustments were made manually to remove artificial gaps. Phylogenetic and molecular evolutionary analyses were made using MEGA 4 (Tamura et al. 2007). Phylogenetic inference was by the neighbor-joining method of Saitou and Nei (1987). Genetic distance was calculated with the Kimura two-parameter model.

Results

Feces from 819 calves from 49 cow-calf operations were examined for *E. bienersi* by PCR (Table 2). At least one positive calf was found in 43 (87.8%) of the cow-calf operations. Overall, 285 of 819 calves (34.8%) were found infected. The highest prevalence was found in the Midwest region (42.7%) followed by the South (35.8%) and the West (23.2%). The prevalence of *E. bienersi* varied considerably from operation to operation (0–100%). A prevalence of 100% was observed in three operations, one in the Midwest region and two in the South; *E. bienersi* was not identified in six operations, three in the South and three in the West.

All 285 PCR positive specimens were sequenced to determine the genotype(s) and sequences were compared with those in the GenBank database by BLAST analysis. In Table 2, genotypes of *E. bienersi* identified in each cow-calf operation are presented. The sequence analysis revealed the presence of six different genotypes (Table 2). Four of those genotypes, I, J, BEB4, and type IV, were previously described in cattle. Two new genotypes, BEB8 and BEB9, were identified for the first time in this study. Of the 285 positive specimens, the most prevalent genotype was J (in 108 specimens), followed by BEB4 (in 65 specimens), I (in 59 specimens), and BEB8 (in 41 specimens). Genotypes BEB9 and type IV were identified in only six and one specimen, respectively. Mixed infections were identified in five specimens, three with I and BEB4 and two with J and BEB4.

A phylogenetic analysis was carried out on all *E. bienersi* genotypes identified in humans and cattle and available in GenBank (Fig. 1). The two new genotypes identified in this study, BEB8 and BEB9, were placed in the cluster with genotypes that are most commonly isolated from cattle.

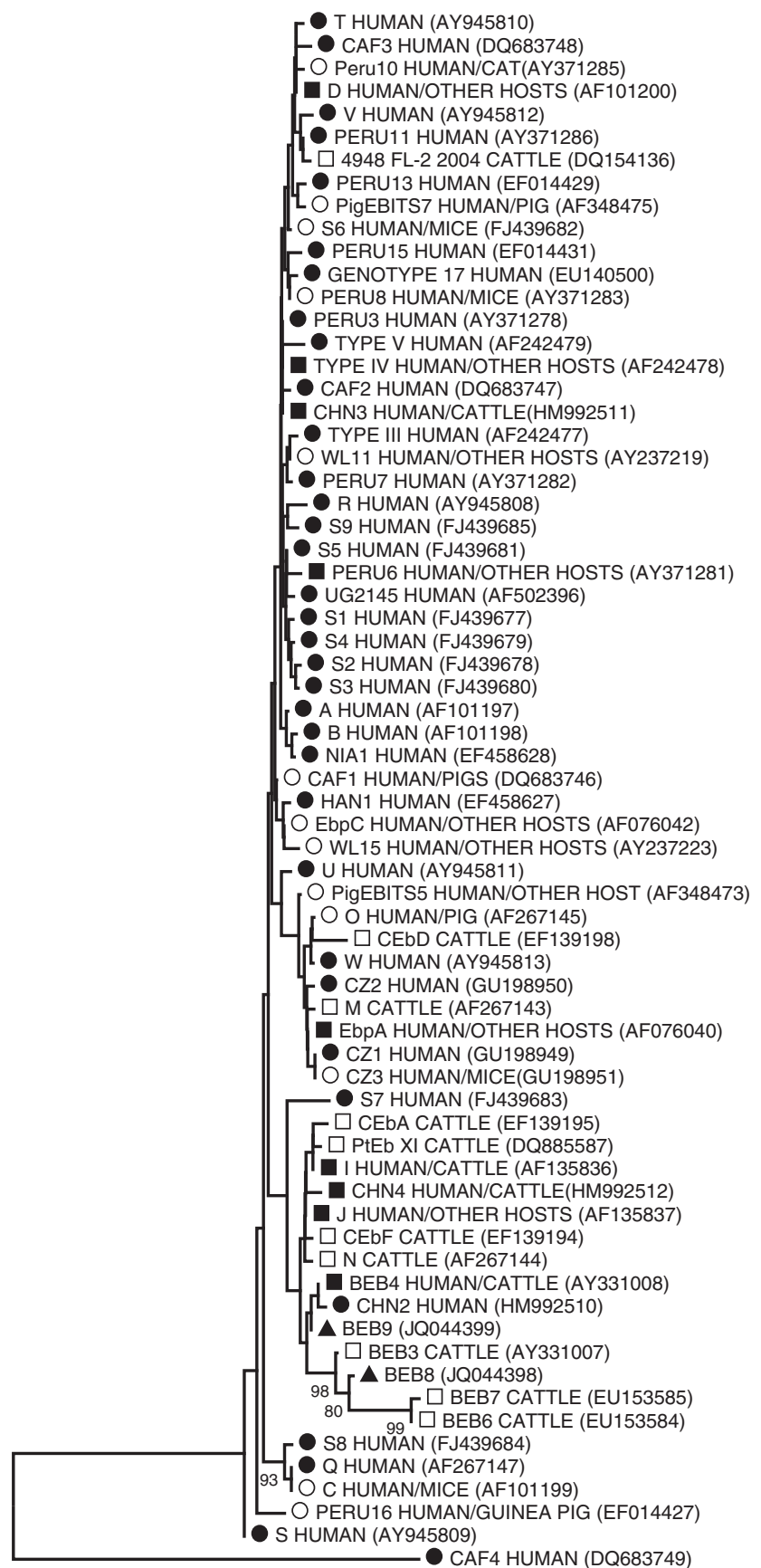
Discussion

E. bienersi genotypes I, J, BEB4, BEB8, BEB9, and type IV were detected by PCR in 285 (34.8%) of 819 weaned beef

Table 2 Numbers of calf samples examined and genotypes of *Enterocytozoon bieneusi* found by region

Region	Number of operations	Number of samples per operation	Number of positives per operation	Prevalence	<i>E. bieneusi</i> genotypes identified
Midwest	20	20	9	45	BEB4 (9)
		20	12	60	J (9), I (1), and BEB4 (2)
		20	13	65	J (3), I (6), and BEB4 (4)
		20	8	40	J (5), I (2), and BEB4 (1)
		20	5	25	J (4) and BEB8 (1)
		20	5	25	J (4) and BEB8 (1)
		20	5	25	J (3) and BEB8 (2)
		20	1	5	J (1)
		20	15	75	I (9), BEBE4 (5), and BEBE4-like (1)
		19	6	31.6	J (2) and I (4)
		18	4	22.2	BEB4 (4)
		17	8	47.1	J (6) and BEB4 (2)
		17	8	47.1	J (3), I (1), BEB4 (4), and
		16	1	6.3	BEB4 (1)
		16	5	31.3	J (5)
		15	11	73.3	BEB4 (1) and BEB8 (10)
		12	11	91.7	I (11)
		11	8	72.7	BEB8 (8)
		8	8	100	BEB4 (1) and BEB8 (7)
8	1	12.5	BEB4 (1)		
South	15	20	9	45	I (2) and BEB4 (7)
		20	7	35	I (1), BEB4 (3), BEB9 (1), and mixed I/BEBE4 (2)
		20	0	0	
		20	6	30	BEB4 (6)
		20	6	30	I (6)
		20	14	70	J (14)
		20	5	25	J (5)
		19	6	31.6	J (5) and BEB4 (1)
		18	12	66.7	I (1), J (6), and BEB4 (5)
		17	5	29.4	J (1) and BEB4 (4)
		14	2	14.3	BEB4 (2)
		10	0	0	
		8	8	100	J (3) and I (5)
3	3	100	J (3)		
3	0	0			
West	14	20	2	10	I (2)
		20	0	0	
		20	4	20	J (3) and BEB4 (1)
		20	1	5	Mixed BEB4/J (1)
		20	3	15	J (1) and I (2)
		20	7	35	I (3) and BEB9 (4)
		20	5	25	J (5)
		20	16	80	J (8), I (1), BEB4 (4), type IV (1), J/BEB4 (1), and I/BEB4 (1)
		20	3	15	I (1) and BEB4 (2)
		20	13	65	J (9), I (1), and BEB4 (3)
		17	3	17.6	BEB4 (3)
		17	1	5.9	BEB4 (1)
		9	0	0	
7	0	0			
Total	49	819	285	34.8	J (108), I (59), BEB4 (65), BEB8 (41), BEB9 (6), type IV (1), mixed I/BEB4 (3), and mixed J/BEB4 (2)

Fig. 1 Phylogenetic relationships among *Enterocytozoon bieneusi* genotypes identified in humans and other hosts, and cattle inferred by a neighbor-joining analysis of the ITS rRNA gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Nucleotide sequences determined in this study are identified with a *filled triangle* before the genotype name. Genotypes identified in humans (*filled circle*); genotypes identified in humans, cattle, and other hosts (*filled square*); genotypes identified in humans and other hosts that do not include cattle (*empty circles*); genotypes identified in cattle (*empty squares*). Bootstrap values of less than 75% are not shown



0.05

calves (6–18 months of age) in the present study. This is the first report of *E. bienersi* genotypes and their prevalence in beef cattle. The first report of *E. bienersi* in cattle was from three calves in a herd of 28 cattle with diarrhea in Germany (Rinder et al. 2000). The first report of *E. bienersi* in cattle in North America found 13 positive dairy calves from six farms in five states out of 413 calves examined on 15 farms in seven eastern states (Fayer et al. 2003). It appeared from further studies at many of the same farms that the prevalence of *E. bienersi* infection in dairy cattle increased as cattle increased in age. Of 452 post-weaned, 3- to 8-month-old calves, 13% were found positive for *E. bienersi* (Santín et al. 2004). Of 571 heifers, 12- to 24 months of age, 23% were positive for *E. bienersi* (Santín et al. 2005). The prevalence of *E. bienersi* infection then appeared to decrease in mature dairy cattle. Of 541 milking cows, 4.4% were positive for *E. bienersi* (Fayer et al. 2007). Of 30 dairy calves followed up from birth to 24 months of age, the same age-related trend in prevalence of infection was observed and eventually all were detected as positive at one point or another with *E. bienersi* (Santín and Fayer 2009b).

In Korea, of 538 fecal samples from cattle, approximately 15% were positive; summer rates were the highest (Lee 2007). The prevalence related to dairy or beef cattle was not reported.

Based on the sequence data from the ITS region, of the six genotypes of *E. bienersi* identified among the 285 PCR positive fecal genotypes I, J, BEB4, and BEB8 were the most prevalent. Except for newly identified BEB8, these results are in agreement with data from dairy cattle in which these genotypes were also the most common (Fayer et al. 2007; Santín and Fayer 2009b; Sulaiman et al. 2004). Genotypes I, J, and BEB4 were thought to be cattle specific, but recently, the host range of these genotypes has increased, having been identified in humans in China and the Czech Republic (Sak et al. 2011a; Zhang et al. 2011). Genotype J has also been identified in chickens in Germany (Reetz et al. 2002).

E. bienersi has been identified in water sources as well as in wild, domestic, and food-producing farm animals, raising concerns of water-borne, food-borne, and zoonotic transmission. The identification of the three most common genotypes of *E. bienersi* of cattle (I, J, and BEB4), now found in humans, changes the paradigm from genotypes thought to be cattle-specific to genotypes with zoonotic (bidirectional) potential. It has been suggested that although *E. bienersi* has broad host specificity, transmission is more restricted, with some genotypes able to circulate within a given host species but only occasionally being transmitted to another host (Henriques-Gil et al. 2010). This appears to fit the pattern for genotypes I, J, and BEB4. Genotype type IV in contrast, was identified not only in one calf in the present study and in a few other cattle from Korea, Portugal, and the USA (Lee 2008; Sulaiman et al. 2004) but also in cats from

Germany, Colombia, Japan, and Portugal (Abe et al. 2009; Dengjel et al. 2001; Lobo et al. 2006b; Santín et al. 2006); in dogs from Colombia (Santín et al. 2008); and in humans from Gabon, Cameroon, Peru, England, Uganda, Niger, France, Malawi, and Netherlands (Bern et al. 2005; Breton et al. 2007; Cama et al. 2007; Espern et al. 2007; Liguory et al. 1998, 2001; Sadler et al. 2002; Sarfati et al. 2006; Sulaiman et al. 2003a; ten Hove et al. 2009; Tumwine et al. 2002).

We report that 34.8% of apparently healthy calves were infected with *E. bienersi*. The identification of genotypes that also have been found in humans highlights the possible role of beef cattle as a source of environmental contamination with genotypes with zoonotic potential.

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