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Short Communication

First report of *Enterocytozoon bieneusi* from dairy cattle in Argentina

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ABSTRACT

Fecal specimens were obtained from a total of 70 dairy calves less than two months old on 11 municipalities in Buenos Aires, Argentina. After removal of fecal debris by sieving and sucrose flotation, specimens were subjected to PCR to detect the presence of *Enterocytozoon bieneusi*. PCR revealed a 14.3% of prevalence for *E. bieneusi* with 10 positive calves from 7 municipalities. Gene sequence analysis conducted in all samples positives by PCR revealed the presence of six genotypes; four previously reported in cattle as well as humans (D, I, J, and BEB4), one never reported in cattle before but previously reported in humans (EbpC), and one novel genotype (BEB10). These results constitute the first molecular characterization of *E. bieneusi* in Argentina, and suggest a potential risk of zoonotic transmission in this area.

1. Introduction

Microsporidia constitutes a group of emerging opportunistic pathogens that has a wide range of vertebrates and invertebrate as hosts. Most of the species infect invertebrates and fish, but 14 species in 8 genera infect humans (Didier and Weiss, 2006). In humans, two species are the most commonly identified, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. Of those, *E. bieneusi* is the most frequently reported worldwide mainly associated with chronic diarrhea and wasting syndrome (Didier and Weiss, 2011). It is considered as an opportunistic pathogen in HIV patients and other immunosupressed individuals (Nissapatorn and Sawangjaroen, 2011). *E. bieneusi* 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 genotyping of *E. bieneusi* using sequencing analysis of the ribosomal internal transcriber spacer (ITS) has shown more than 100 genotypes, some *E. bieneusi* genotypes are host-adapted while others have no host specificity and are considered zoonotic (Santín and Fayer, 2009).

Since the first report of *E. bieneusi* in cattle in 8 calves in Germany (Rinder et al., 2000), *E. bieneusi* has been identified as a common parasite of dairy and beef cattle and it has been identified in cattle from countries in Africa, Asia, Europe, and North America. In cattle, host-specific as well as zoonotic *E. bieneusi* genotypes have been reported (Table 1). It is still not clear the role that cattle play in the

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Table 1
E. bieneusi genotypes reported in humans, cattle and other hosts including findings from this study.

E. bieneusi genotype (GenBank accession number)	Synonyms (GenBank accession number)	Host	Location
Type IV (AF242478)	K (AF267141), Peru2 (AY371277), PtEBIII (DQ885579), BEB5 (AY331009), BEB5-var (AY331010)[a], CMITS1	Humans, cattle, cats, rhesus monkey, and dogs	Cameroon, China, Colombia, Gabon, England, France, Germany, Iran, Japan, Korea, Malawi, Netherlands, Niger, Nigeria, Peru, Portugal, Uganda, and USA
Peru6 (AY371281)	PtEb I (DQ425107), PtEb VII (DQ885583)	Humans, cattle, birds, and dogs	Peru, Portugal, and USA
D (AF101200)	WL8 (AY237216), PigEBITS9 (AF348477), Peru9 (AY371284), PtEb VI (DQ885582), CEbC (EF139197)	Humans, cattle, pigs, beaver, fox, muskrat, raccoon, falcon, horse, dog, mice, rhesus monkey, and baboon	Abu Dhabi, Argentina, Brazil, China, Colombia, Congo, Czech Republic, Gabon, Cameroon, England, India, Iran, Japan, Kenya, Korea, Malawi, Netherlands, Niger, Nigeria, Peru, Portugal, Russia, South Africa, Spain, Thailand, Tunisia, USA, Vietnam
BEB4 (AY331008)	CHN1 (HM992509)	Humans, cattle, and pigs	Argentina, China, Czech Republic, South Africa, and USA
EbpA (AF076040)	F (AF132833)	Humans, cattle, pigs, horses, and mice	Czech Republic, Germany, Japan, Nigeria, Switzerland, and USA
I (AF135836)	BEB2 (AY331006), CEbE (AF139199)	Humans and cattle	Argentina, China, Czech Republic, Germany, Korea, South Africa, and USA
J (AF135837)	BEB1 (AY331005), PtEb X (DQ885586), CEbB (EF139196)	Humans, cattle, and birds	Argentina, China, Germany, Korea, Portugal, and USA
CHN3 (HM992511)		Humans and cattle	China
CHN4 (HM992512)		Humans and cattle	China
BEB3 (AY331007)		Cattle	USA
BEB3-like (JQ923448)		Cattle	South Africa
PtEb XI (DQ885587)		Cattle	Portugal
BEB6 (EU153584)		Cattle and goats	Peru and USA
BEB7 (EU153585)		Cattle	USA
4948 FL-2 2004 (DQ154136)		Cattle	USA
CEbA (EF139195)		Cattle	Korea
CEbD (EF139198)		Cattle	Korea
BEB8 (JQ044398)		Cattle	USA
BEB9 (JQ044399)		Cattle	USA
CEbF (EF139194)		Cattle	Korea
M (AF267143)		Cattle	Germany
N (AF267144)		Cattle	Germany
CAF1 (DQ683746)	PEbE	Humans and pigs	Gabon, Niger, and Korea
EbpC (AF076042)	E (AF135832), WL13 (AY237221), Peru4 (AY371279), and WL17 (AY237225)	Humans, cattle, pigs, beavers, otters, muskrats, raccoons, and foxes	Argentina, Germany, Japan, Peru, Switzerland, Thailand, USA, and Vietnam
Peru 16 (EF014427)		Humans and guinea pigs	Peru
Peru10 (AY371285)		Humans and cats	Colombia and Peru
WL11 (AY237219)	Peru5 (AY371280)	Humans, cats, dogs, and foxes	Colombia, Peru, and USA
WL15 (AY237224)	WL16 (AY237224) and Peru14 (EF014430)[a]	Humans, beavers, foxes, raccoons, and muskrats	Peru and USA
O (AF267145)		Humans and pigs	Germany and Thailand
S6 (FJ439682)		Humans and mice	Czech Republic, Germany, and Malawi
Peru8 (AY371283)		Humans, chickens, and mice	Czech Republic, Germany, Malawi, and Peru
CZ3 (GU198951)		Humans and mice	Czech Republic and Germany
C (AF101199)	II (AF242476)	Humans and mice	Czech Republic, France, Germany, Netherlands, and Switzerland
PigITS5 (AF348173)	PEbA	Humans, pigs, and mice	Czech Republic, Germany, Japan, Korea, and USA
WL12 (AY237220)		Humans, beavers, and otters	Brazil and USA
BEB10 (KF675191)		Cattle	Argentina

Adapted from Santín et al. (2012).
[a] Unpublished.

Table 2
E. bieneusi in pre-weaned calves on dairy farms in Buenos Aires, Argentina.

Municipalities	No. of samples	No. of positive samples	*E. bieneusi* genotype identified
Tandil	18	1	BEB4
Tres Arroyos	3	2	I, J
Bolivar	2	0	
Alvarado	1	0	
Gral Lamadrid	12	1	BEB10
Gral Pueyrredón	1	1	EbpC
Juarez	2	0	
Azul	15	0	
Lobería	2	1	I
Rauch	9	3	D, J
Olavarría	5	1	J

epidemiology of *E. bieneusi*, and in due to the fact that there is no prevalence data and/or molecular characterization available for this parasite in Argentina, this study was carried out to investigate the presence of *E. bieneusi* in calves from dairy farms in Buenos Aires, Argentina.

2. Materials and methods

2.1. Study area

This study was carried out from 2008 to 2010 in 23 dairy farms located in different municipalities at the south of Buenos Aires province, Argentina (Table 2). Farms were selected because they are located on an area with an important dairy industry. Farms were chosen randomly and all animals included in the study were less than 37 days old. A total of 70 fecal samples were collected directly from the rectum of each calf using sterile latex gloves into individual plastic containers that were labeled with the calf's identification number. Fecal specimens were transported immediately to the laboratory and stored at 4 °C.

2.2. Specimen preparation for PCR and gene sequencing

Feces were sieved and subjected to sucrose flotation to remove debris and concentrate spores (Smith, 2008). Total DNA was extracted using a DNAeasy Tissue Kit (Qiagen, Valencia, California). The protocol, as described below, utilized the manufacturer's reagents with slight modifications (Fayer et al., 2010). To 50 μl of fecal suspension 180 μl of ATL buffer was added and mixed by vortexing. Twenty microliters of proteinase K (20 mg/ml) was added, the sample was mixed and incubated overnight at 55 °C before 200 μl of AL buffer was added. The protocol then followed manufacturer's instructions with the exception that to increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer.

The polymerase chain reaction (PCR) amplification was performed using a set of nested primers specific for *E. bieneusi* 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 μl) contained 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl (pH = 9), 0.2 mM dNTPs, 50 pmol of each primer, 2.5 U of Taq (Qbiogene Inc., Carlsbad, California), and 2.5 μl 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. 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 μl 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 KF675191–KF675196.

3. Results

Feces from 70 calves from dairy farms located at Buenos Aires province were examined for *E. bieneusi* by PCR (Table 2). Overall, 10 calves (14.3%) were found infected. Of 23 dairy farms in 11 municipalities, calves excreting spores of *E. bieneusi* were found on 8 farms in 7 municipalities. All PCR positive were sequenced to determine the genotype(s). Nucleotide sequences were compared with those in the GenBank database by BLAST analysis. Sequence analysis revealed the presence of six different genotypes; 4 previously reported in cattle, D, I, J, and BEB4; one reported in cattle for the first time, EbpC; and a novel genotype (BEB10) reported in this study for the first time (Table 2). Genotype BEB10 nucleotide sequence differs only in one nucleotide

in the ITS region with genotype CHN5 reported in dogs in China (HM992513; Zhang et al., 2011).

The most prevalent genotype was J (4 specimens), followed by I (2 specimens). The rest of the genotypes were identified each in one specimen. Mixed infections were not detected.

4. Discussion

The present findings demonstrate for the first time the presence of Microsporidia in cattle in South America. It also constitutes the first molecular characterization of *E. bieneusi* in Argentina.

E. bieneusi was found in 10 of 70 calves examined (14.3%) and on 8 of the 23 farms. The positive farms were located on 7 of 11 municipalities included in the study that covered a wide area of Buenos Aires province. These results confirm that *E. bieneusi* is a common parasite of pre-weaned calves in Buenos Aires and suggest that calves may represent a potential source of infection for humans and other animals. The prevalence obtained in this study was higher than the one reported in pre-weaned calves (3%) in the United States (Fayer et al., 2003). However, prevalence was similar to the one obtained in a study that covered the same farms in post-weaned calves (13%) (Santín et al., 2004).

Sequence analysis of the ITS identified six different genotypes, D, I, J, BEB4, EbpC, and a novel genotype named BEB10. Five of the 6 genotypes identified in this study have been previously found in humans (D, I, J, EbpC, BEB4). The two most common genotypes identified in this study, genotype J and I, were previously thought to be cattle-specific. However, genotype J has also been reported in chickens and humans (Reetz et al., 2009; Zhang et al., 2011) and genotype I genotype in humans (Zhang et al., 2011).

Genotypes I, J and BEB4 were previously found in cattle in several countries (Table 1), but this is the first report of these genotypes in South America. Genotype D was previously reported in cattle in Korea and South Africa (Lee, 2007, 2008; Abu Samra et al., 2012). In South America, genotype D has never been reported in cattle until now, and it has only been found in earlier studies in South America in horses and humans from Colombia and Peru, respectively (Sulaiman et al., 2003; Bern et al., 2005; Cama et al., 2007; Santín et al., 2010). Genotype EbpC was previously found in humans, monkeys, pigs, and other wildlife animals (Table 1), but this constitutes the first report of this genotype in cattle.

A novel genotype, named BEB10, was identified in one calf. In conclusion, this study demonstrates for the first time the presence of *E. bieneusi* in cattle in South America, and constitutes the first molecular characterization of this parasite in Argentina. Five of the 6 genotypes identified in the study are zoonotic, and its presence in calves suggest that they may represent a public health risk for humans through contamination of ground and surface water with zoonotic *E. bieneusi* genotypes. More studies are needed in order to assess the role of calves as a source of zoonotic genotypes of *E. bieneusi* to humans in Argentina.

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