Identification of *Cryptosporidium* species and genotypes in dairy cattle in Brazil

Identificação de espécies e genótipos de *Cryptosporidium* em bovinos leiteiros no Brasil

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Abstract

In this study, we identified *Cryptosporidium* species and genotypes present in dairy cattle in the central region of São Paulo state, Brazil. Fecal specimens were collected from 200 animals (100 calves and 100 cows) in ten dairy farms. Fecal samples were examined using microscopic examination (ME), enzyme immunoassay (EIA) and polymerase chain reaction (PCR). *Cryptosporidium* species and genotypes were determined by restriction fragment length polymorphism (RFLP) or DNA sequencing analysis of the SSU-rRNA and GP60 genes. The occurrence of *Cryptosporidium* spp. infection was 14% (28/200). The occurrence in calves (26%) was significantly higher than in cows (2%). Of the 27 *Cryptosporidium*-positive specimens submitted to genotyping, *C. andersoni* was identified in 23 (85.1%), *C. bovis* in three (11.1%), and the zoonotic *C. parvum* subtype IIaA15G2R1 in one (3.7%). The study demonstrates that *Cryptosporidium* spp. infection was common and widespread in dairy cattle in this region and that calves have a high prevalence of *C. andersoni*. Furthermore, the presence of *C. parvum* subtype IIaA15G2R1 indicates that dairy calves from this region should be considered a potential source of zoonotic *Cryptosporidium* oocysts.

Keywords: *Cryptosporidium* spp., cattle, PCR-RFLP, sequencing, genotyping.

Resumo

No presente estudo foram identificadas espécies e genótipos de *Cryptosporidium* originadas de bovinos leiteiros na região central do estado de São Paulo, Brasil. Amostras fecais foram coletadas de 200 animais (100 bezerros e 100 vacas) em 10 propriedades leiteiras. As amostras foram examinadas utilizando os métodos de microscopia óptica (MO), ensaio imunoenzimático (EI) e reação em cadeia da polimerase (PCR). As espécies e genótipos de *Cryptosporidium* foram determinados pelo método de polimorfismo no tamanho dos fragmentos de restrição (RFLP) ou sequenciamento dos genes SSU-rRNA e GP60. A infecção por *Cryptosporidium* spp. teve ocorrência de 14% (28/200). A ocorrência em bezerros (26%) foi significativamente maior do que em vacas (2%). Do total de 27 amostras positivas submetidas à caracterização genética, *C. andersoni* foi identificado em 23 (85.1%), *C. bovis* em três (11.1%) e *C. parvum* subtipo IIaA15G2R1 em uma (3.7%). O presente estudo demonstrou que a infecção por *Cyptosporidium* é comum e difundida em bovinos leiteiros nessa região e que bezerros possuem uma alta prevalência de *C. andersoni*. A presença de *C. parvum* subtipo IIaA15G2R1 indica que bezerros leiteiros dessa região devem ser considerados uma fonte de oocistos de *Cryptosporidium* com potencial zoonótico.

Palavras-chave: *Cryptosporidium* spp., bovinos, PCR-RFLP, sequenciamento, genotipagem.

Introduction

Cryptosporidium is an apicomplexan parasite that infects the microvillus border of the gastrointestinal epithelium of a wide range of vertebrate hosts, including humans and cattle. In farm animals, *Cryptosporidium* infection causes disorders of the digestive system and is a common cause of morbidity in neonatal calves (OLSON et al., 2004). *Cryptosporidium* spp. has been found in beef and dairy cattle worldwide (HAMNES et al., 2006; GEURDEN et al., 2007; THOMPSON et al., 2007). Longitudinal studies have found high prevalence rates in young calves in some herds, with infections levels frequently reaching 100% (SANTÍN et al., 2008). Cattle have been reported as the primary host for four species of

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Cryptosporidium with significant biological differences: *C. parvum*, *C. bovis*, *C. ryanae* (previously identified as the *Cryptosporidium* deer-like genotype) and *C. andersoni* (SANTÍN et al., 2004; FAYER et al., 2005, 2006, 2008). Recent studies have reported that susceptibility of cattle to *Cryptosporidium* spp. varies with host age (SANTÍN et al., 2004; MENDONÇA et al., 2007; BROGLIA et al., 2008). The species *C. parvum* infects the intestine of neonatal and pre-weaned calves (0-2 months old) and shows high infection rates in dairy herds (COKLIN et al., 2007; SANTÍN et al., 2008). Infections with *C. bovis* are common among post-weaned dairy calves (2-11 months-old) in many geographical regions (SANTÍN et al., 2004; FENG et al., 2007; FELTUS et al., 2008). *C. ryanae* has been found in pre-weaned and post weaned calves (FENG et al., 2007; FAYER et al., 2008; BROOK et al., 2009). Both *C. bovis* and *C. ryanae* are much more prevalent in older, post-weaned dairy cattle (FELTUS et al., 2008; AYINMODE et al., 2010), although they have a low but widespread prevalence in pre-weaned calves (THOMPSON et al., 2007; SANTÍN et al., 2008) and other age groups (FAYER et al., 2006; FENG et al., 2007). *C. andersoni* has large oocysts and infects the abomasum of juvenile (1-2-year-old heifers) and adult cattle (>24 months old) persisting for years with a long oocyst shedding duration (KVÁC; VÍTOVEC, 2003; ROBINSON et al., 2006). Despite the abundant prevalence information for *Cryptosporidium* around the world, there is little information on the *Cryptosporidium* species and genotypes present in infected cattle in Brazil. This study was undertaken to determine the occurrence and characterize the species of *Cryptosporidium* present in dairy cattle in south-central São Paulo state, Brazil.

Materials and Methods

1. Dairy farms and sample collection

Feces were collected from dairy cattle located on ten farms in three counties (Pardinho, Botucatu and Itatinga) of south-central São Paulo state, Brazil (Table 1). All farms were visited between February and August 2006. Dairy calves and cows were randomly selected for sampling. At each farm, 20 fecal specimens were collected from calves (2 weeks to 6 months of age) and cows (>24 months old). Two hundred cattle fecal samples were collected in total (100 from calves and 100 from cows). Feces were collected directly from the rectum of each animal into a plastic specimen cup that was immediately capped, labeled and placed on ice in an insulated container. Feces were transported to the laboratory of parasitic diseases, UNESP – Univ Estadual Paulista in Botucatu and processed on the same day of collection. Animals were generally grazed on pasture, housed in groups in large pens (either completely or partially covered by a roof) or housed in large free-stall barns.

2. Light microscopy examination

Cryptosporidium oocysts were identified microscopically in fecal smears after staining with a modified Ziehl–Neelsen stain (MARTINEZ; BELDA NETO, 2001).

3. Antigen-EIA

The CRYPTOSPORIDIUM TEST® antigen EIA kit (Techlab, USA) was used to demonstrate the presence of *Cryptosporidium* oocysts antigen in 10% formalin preserved samples. The diagnostic kit procedure was followed and the results were examined and interpreted according to the manufacturer's instructions. Results were read photometrically at 450 nm. A sample was considered positive when the O.D. was ≥0.150.

4. PCR analysis

Fecal specimens frequently contain PCR inhibitors and a large number of cells from the host (e.g., intestinal cells, blood cells). The adequacy of DNA isolation and purification was assessed for each isolate by first performing PCR amplification with specific primers sets targeting the mitochondrial DNA from bovine cells, as previously described (MARTELLINI et al., 2005). Mitochondrial

Table 1. City, farm designation, number of samples examined at each farm, number of positives, and prevalence of *Cryptosporidium* species/genotypes from 200 dairy cattle on 10 farms in the region of the counties of Pardinho, Botucatu and Itatinga, São Paulo state, Brazil.

City	Farm	N° samples	N° positives	Species/Genotype		
				C. andersoni	*C. bovis*	*C. parvum*
Pardinho	PAR 1	20	1 (5)	0	1 (100)	0
Pardinho	PAR 2	20	2 (10)	0	2 (100)	0
Botucatu	BTU 1	20	4* (20)	3 (100)	0	0
Botucatu	BTU 2	20	1 (5)	1 (100)	0	0
Botucatu	BTU 3	20	9 (45)	9 (100)	0	0
Botucatu	BTU 4	20	1 (5)	1 (100)	0	0
Botucatu	BTU 5	20	1 (5)	1 (100)	0	0
Itatinga	ITA 8	20	2 (5)	1 (50)	0	1 (50)
Itatinga	ITA 9	20	7 (35)	7 (100)	0	0
Itatinga	ITA 10	20	0 (0)	0	0	0
Total	10	200	28 (14)	23 (85.1)	3 (11.1)	1 (3.7)

The values in parentheses are percentages. *An isolate from one calf could not be typed by RFLP analysis or sequencing. The total species/genotype percentages are based on the total of 27 positive samples submitted to genetic characterization.

DNA gene targets were chosen for this assay because individual host cells contain numerous copies of mitochondrial DNA. The successful of amplification of this target demonstrates the absence of inhibitors in a DNA sample originating from feces.

5. Isolation of DNA

The QIAamp® DNA Stool Mini Test Kit (Qiagen, Germany) was used according to the manufacturer's instructions with a slightly modified protocol. The modifications include four cycles of freezing in liquid nitrogen for 5 minutes and boiling for 5 minutes to disrupt the oocyst cell walls to release DNA. The DNA was eluted from the silica column by applying 100 μL AE-buffer and centrifugation at 16,000 rcf for 1 minute (to increase the quantity of recovered DNA). DNA was stored at –20 °C until it was used for PCR assays

6. SSU-rRNA gene PCR

Cryptosporidium species and genotypes were identified using nested PCR-based protocol. The secondary PCR product from the small subunit (SSU) rRNA gene (with a size ranging from 826 to 864 bp depending on the isolate) was amplified by a nested PCR as previously described (XIAO et al., 2001).

7. GP60 gene PCR

A fragment of the 60 kDa glycoprotein (GP60) gene was amplified by nested-PCR (PENG et al., 2003; GLABERMAN et al., 2002) using primers 5'-ATAGTCTCCGCTGTATTC-3' and 5'-GCAGAGGAACCAGCATC-3' (primary PCR) and 5'-TCCGCTGTATTCTCAGCC-3' and 5'-GAGATATATCTTGGTGCG-3' (secondary PCR). These primers were designed based on sequences conserved among all known *C. parvum* GP60 alleles.

8. SSU-rRNA PCR-RFLP analysis

PCR-RFLP analysis of the SSU-rRNA gene secondary PCR products was performed using *Ssp*I, *Vsp*I, *Dde*I and *Mbo*II in order to differentiate *Cryptosporidium* species and genotypes (XIAO et al., 2001; FENG et al., 2007; PLUTZER; KARANIS, 2007). The master mix used for restriction digestion contained 2 μL of reaction buffer, 5 units of each enzyme, endonuclease and nuclease-free water according to the manufacturer´s instructions (Fermentas, USA). Restriction digestion was carried out at 37 °C, overnight. Species/genotypes assignments were made by comparing the RFLP profiles to known profiles reported in the literature.

9. SSU-rRNA gene and GP60 DNA sequencing analysis

Selected PCR products for the SSU-rRNA and GP60 genes were sequenced in both directions with the forward and reverse primers used in the secondary PCRs. Sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3500 Genetic Analyzer® (Applied Biosystems, USA). The quality of the generated electropherograms was analyzed using ABI Sequencing Analysis® v5.3 software. The accuracy of the data was confirmed by bidirectional sequencing with the forward and reverse primers used in the secondary PCR. Sequence electropherograms from each strand were aligned and inspected using MEGA v.5 software (TAMURA et al., 2007). The nucleotide sequences of the SSU-rRNA and GP60 genes reported in this paper were aligned using Clustal W. The sequences obtained were compared against a database (GenBank at www.ncbi.nlm.nih.gov/BLAST) using BLAST software to determine the species and genotype of *Cryptosporidium*. Phylogenetic and molecular evolutionary analyses were performed with MEGA v.5 and phylogenetic inference was done using the neighbor-joining (NJ) method. Genetic distance was calculated with the Kimura 2-parameter model.

10. Cryptosporidium parvum subtype identification using the GP60 gene

Cryptosporidium parvum subtypes were determined by sequence analysis of the GP60 gene and named using the protocol previously described (SULAIMAN et al., 2005). This nomenclature is based on the number of TCA (designated by the letter A), TCG (designated by the letter G) and ACATCA (designated by the letter R) repeats in the microsatellite region. For example, a sequence with 15 TCA, 2 TCG and 2 ACATCA repeats was assigned A15G2R2 (SULAIMAN et al., 2005).

11. Nucleotide sequence accession numbers

The SSU-rRNA gene nucleotide sequences were deposited in GenBank under accession numbers JF937701 to JF937708 for *C. andersoni* isolates, JF957617 and JF957618 for *C. bovis* isolates. The *C. parvum* SSU-rRNA and GP60 gene nucleotide sequences were deposited in GenBank under accession numbers JN120853 and JF937700, respectively.

12. Data analysis

The occurrence rate of *Cryptosporidium* spp. throughout the study was calculated as the number of infected individuals divided by the number of individual's sampled × 100. Agreements between diagnostic methods and the presence or absence of diarrhea in the populations infected or not infected with *Cryptosporidium* spp. were compared using the Kappa test. The farm occurrence was calculated as the number of farms with at least one positive sample compared with the total number of farms in the study.

Results

The occurrence of *Cryptosporidium* spp. infection in our study was 14% (28/200). Data relating to the number of calves examined at each farm location, the number of positive specimens at each

location and the species or genotype of *Cryptosporidium* identified by PCR-RFLP and/or sequencing at the SSU-rRNA and GP60 genes are shown in Table 1.

1. Detection of Cryptosporidium by microscopy, ELISA and PCR

All 200 fecal specimens were tested using three diagnostic methods: microscopy, ELISA and PCR. We identified *Cryptosporidium* oocysts microscopically in 17 fecal samples after staining with a modified Ziehl–Neelsen stain. The ELISA method was significantly less sensitive, detecting parasites in only two samples. The PCR method proved to be the most sensitive (kappa=0.024) and was able to detect *Cryptosporidium* DNA in 28 samples. All *Cryptosporidium*-positive specimens generated the expected SSU-rRNA gene products by nested PCR (~830-860 bp). None of the samples that were positive by microscopy/ELISA were negative by PCR. All specimens produced the expected bovine mitochondrial PCR product, indicating that PCR inhibitors were not responsible for the negatives results.

2. Farms and handling

Cryptosporidium spp. was detected in animals from 90% of the farms sampled in this study (9/10). The occurrence of *Cryptosporidium* spp. in samples of each farm varied from 5% -45%. No clear association was found between the type of housing and prevalence of infection.

3. Relationship between Cryptosporidium infection and age

The level of occurrence in calves was 26% (26/100), which was higher than the occurrence of 2% (2/100) in cows. In specimens collected from 0 to 2 month-old calves, the occurrence was 23.8% (5 of the 21 calves in this category). Of the specimens collected from 2 to 6 month-old calves, 26.5% (21 of the 79 calves in this category) were positive for *Cryptosporidium* spp. As a percentage value, the lowest level of infection was found in 0 to 30 day-old calves, with *Cryptosporidium* spp. found in 16.6% (1/6) of samples in this category. Eight samples (36.3%) collected from calves at approximately 90 to 120 days (8/22) were positive for *Cryptosporidium* spp. In 150 to 180 day-old calves (average 6 months old) the occurrence was 60% (3/5).

4. Presence of diarrhea in calves

Diarrhea was observed in 17% (17/100) of calves at the time of sampling. *Cryptosporidium* spp. was detected in 23.5% (4/17) of diarrheic samples and in 26.5% (22/83) of non-diarrheic samples; therefore diarrhea was not found to be statistically associated with infection (kappa=0.025) and had no specific correlation with any *Cryptosporidium* species.

5. Species and genotypes by PCR-RFLP

Restriction analysis produced a banding pattern diagnostic of *C. andersoni* for 23 (85.1%) PCR products. Of the remaining samples, *C. bovis* was identified in three (11.1%) and *C. parvum* in one (3.7%). The isolate from one calf could not be typed by RFLP analysis or sequencing.

6. Species and genotypes on farms

The non-zoonotic *C. andersoni* was present in samples from seven of the 10 farms examined and was particularly prevalent on farms BTU-3 and ITA-9, with high numbers of calves infected. The infection rates with *C. andersoni* on each farm varied from 0 to 45%. This species was responsible for 100% of the infected animals from six farms. *Cryptosporidium bovis* was the only *Cryptosporidium* species identified in samples from two farms in Pardinho. *Cryptosporidium parvum* was identified in specimens from only one farm.

7. Relationship between Cryptosporidium species/genotypes and age

Cryptosporidium andersoni was found in 100% of positive specimens from calves at 1, 5 and 6 months of age and in 100% of positive specimens from adult cattle. The species *C. parvum* was found in only one calf, which was 3 months of age. In calves 4 months of age or older, only *C. bovis* and *C. andersoni* were found. For 0 to 6 month-old calves, *C. andersoni* was the most prevalent species. *C. parvum* was not identified in specimens from 0 to 2 month-old calves. *Cryptosporidium bovis* was identified in 4.7% (1/21) of specimens from 0 to 2 month-old calves and in 2.5% (2/79) of specimens from 0 to 6 month-old calves. The percentage of each identified species and genotype of *Cryptosporidium* relative to the age of the animal is shown in Figure 1.

8. Sequencing analysis of DNA sequences

DNA sequencing of selected PCR products confirmed the results of the RFLP analysis. Partial sequences of the SSU-rRNA and GP60 genes were compared with *Cryptosporidium* sequence data obtained from GenBank. The SSU-rRNA sequences of *C. bovis*, *C. parvum*, and *C. andersoni* were identical to those previously reported for these species (FAYER et al., 2005; THOMAZ et al., 2007; SEVÁ et al., 2010). The sample that tested positive for the *C. parvum* genotype by SSU-rRNA PCR was subgenotyped by sequence analysis of the GP60 gene. The GP60 nucleotide sequences of the *C. parvum* isolate (BRAcalf72) revealed 100% identity with *C. parvum* subtype IIaA15G2R1, GenBank accession number EU549719 (QUILEZ et al., 2008).

Discussion

In the current cross-sectional study, *Cryptosporidium* spp. was identified in 14% (28/200) of animal tested by analysis of
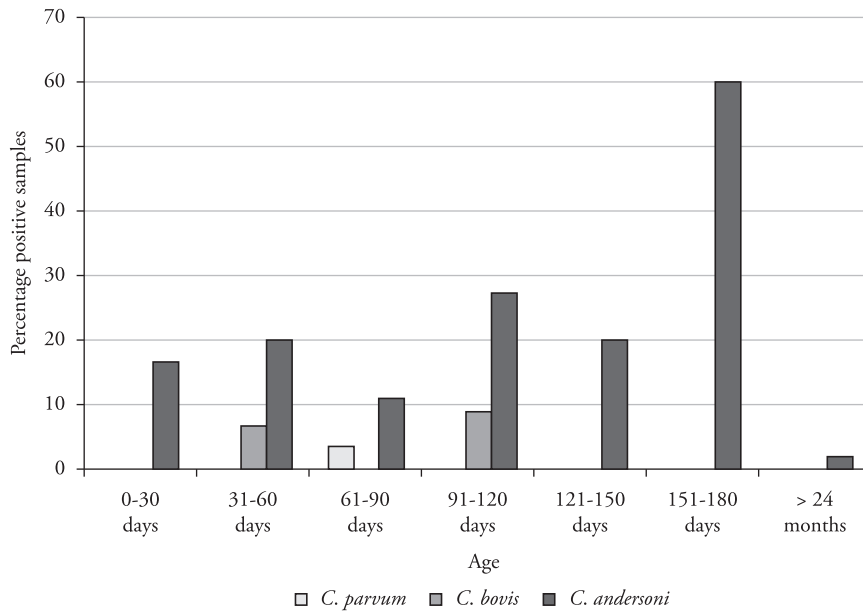
Figure 1. Relationship between age and occurrence of *Cryptosporidium* spp. in dairy cattle as determined by microscopy, ELISA and PCR. Data are based on specimens from 10 farms in south-central São Paulo state, Brazil.

a single fecal sample using microscopy, ELISA and PCR. Our results, as well as those of previous studies (MORGAN et al., 1998), indicate that the detection of *Cryptosporidium* oocysts in fecal smears by microscopy is less sensitive than the detection of DNA by PCR. In longitudinal studies, *Cryptosporidium* spp. has been found to be highly prevalent in dairy cattle (SANTÍN et al., 2008). In the present study, the occurrence of *Cryptosporidium* spp. in calves (26%) was significantly higher than in cows (2%). In epidemiological surveys in USA, Hungary, Germany, Ireland, Iran and Spain, the average prevalence of *Cryptosporidium* spp. in young calves ranged from 18.8% to 57.8% (SANTÍN et al., 2004; THOMPSON et al., 2007; BROGLIA et al., 2008; QUILEZ et al., 2008; KESHAVARZ et al., 2009) and the prevalence in adult cattle in Spain, Portugal and the USA ranged from 4.5% to 8.4% (FAYER et al., 2006; CASTRO-HERMIDA et al., 2007; MENDONÇA et al., 2007).

1. Prevalence of species and genotypes

Previous studies aiming to genetically characterize *Cryptosporidium* spp. in cattle in Brazil have revealed the presence of *C. parvum* (HUBER et al., 2007; THOMAZ et al., 2007; MEIRELES et al., 2011), *C. bovis* (THOMAZ et al., 2007; MEIRELES et al., 2011), *C. ryanae* (MEIRELES et al., 2011) and *C. andersoni* (SEVÁ et al., 2010; MEIRELES et al., 2011) in isolates originating from animals in São Paulo and Rio de Janeiro state. In the majority of studies worldwide, *C. parvum* has been found to be most prevalent and widely distributed species in neonatal and pre-weaned dairy cattle (PENG et al., 2003; SANTÍN et al., 2004; HAMNES et al., 2006; TROTZ-WILLIAMS et al., 2006; COKLIN et al., 2007; GEURDEN et al., 2007; PLUTZER; KARANIS, 2007; THOMPSON et al., 2007). In the present study we found a very low prevalence of *C. parvum* in young calves from

farms in the south-central region of São Paulo state. This low level of infection may be a consequence of the small number of fecal specimens collected from calves in each age category. *C. bovis* was identified in a small number of fecal samples from calves in the present study. This species has been found to be present at low levels in cattle of all age groups in the USA, Europe, Asia and Africa (FAYER et al., 2005; SANTÍN et al., 2004; FENG et al., 2007; THOMPSON et al., 2007; AYINMODE et al., 2010; WANG et al., 2011) and it is now clear that this species is also present at low levels in dairy cattle in Brazil, as reported in previous Brazilian surveys (THOMAZ et al., 2007; MEIRELES et al., 2011). *C. andersoni* has previously been identified in fecal samples from dairy cattle in Brazil (THOMAZ et al., 2007; MEIRELES et al., 2011) and worldwide (KVÁC; VÍTOVEC, 2003; FAYER et al., 2005; ROBINSON et al., 2006). This animal-specific *Cryptosporidium* species predominates in older calves, heifers and adult cattle (SANTÍN et al., 2004; FAYER et al., 2006; ROBINSON et al., 2006). However, in a study of dairy herds in São Paulo State (SEVÁ et al., 2010) a high frequency of *C. andersoni* in 0-6 month-old calves was found; a result that is markedly different from frequencies reported in others locations where *C. parvum* predominates. Our data support these findings and indicate that calves of all ages are susceptible to infection with *C. andersoni*; thus, this species is not specifically associated with adult cattle.

2. Cryptosporidium parvum subtypes

The *C. parvum* subtype IIaA15G2R1 was detected in the present study. This subtype is commonly found in both calves and human worldwide (FELTUS et al., 2006; THOMPSON et al., 2007; WIELINGA et al., 2008) and has been shown to be the most prevalent *C. parvum* subtype in calves in some

herds in North America, Europe and Asia (ALVES et al., 2006; TROTZ-WILLIAMS et al., 2006; FENG et al., 2007; BROGLIA et al., 2008; QUILEZ et al., 2008; SANTÍN et al., 2008; BROOK et al., 2009). In Brazil, this subtype is widely distributed in calves in northwestern São Paulo state (MEIRELES et al., 2011).

3. Zoonosis

Our findings demonstrate that pre-weaned calves are not an important source of zoonotic cryptosporidiosis for humans in this region. However, additional studies in other regions of Brazil will be required to fully evaluate the prevalence of zoonotic species of *Cryptosporidium* in dairy calves and to determine the public health impact of these infections. Although *C. andersoni* appears to be the most prevalent species in cattle in this region, the presence of *C. parvum* (a zoonotic species common in humans) is of potential public health significance. Nevertheless, as observed in previous studies (FAYER et al., 2006; FENG et al., 2007), adult dairy cattle are a relatively low-risk source of infection for humans, because they usually harbor host-specific *Cryptosporidium* species.

Conclusions

The present study demonstrated that *Cryptosporidium* spp. is prevalent and widespread in dairy cattle farms in south-central São Paulo state, Brazil. However, our results may reflect regional variation; clearly, additional data are needed on *Cryptosporidium* species and genotypes in cattle from others dairy farms in Brazil. Cattle in the studied region are primarily parasitized by *C. andersoni*, a species restricted to ruminants host and not found in humans. In conclusion, our results provide useful information about the distribution of *Cryptosporidium* species and genotypes in dairy cattle populations and contribute to our knowledge of the epidemiology of cryptosporidiosis in Brazil and throughout the world.

Acknowledgments

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