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First description of *Giardia duodenalis* in buffalo calves (*Bubalus bubalis*) in southwest region of São Paulo State, Brazil

Monally Conceição Costa de Aquino [a], Tatiani Vitor Harvey [b], Sandra Valéria Inácio [a], Walter Bertequini Nagata [a], Elis Domingos Ferrari [a], Bruno César Miranda Oliveira [a,c], George Rego Albuquerque [b], Giovanni Widmer [c], Marcelo Vasconcelos Meireles [a], Katia Denise Saraiva Bresciani [a,*]

[a] Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba, São Paulo, Brazil
[b] Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil
[c] Department of Infectious Disease & Global Health, Cummings School of Veterinary Medicine at Tufts University, North Grafton, Massachusetts, USA

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ABSTRACT

We performed molecular characterization of *Giardia duodenalis* in buffalo calves from the Southwest region of São Paulo State, Brazil. A total of 183 fecal samples of Murrah breed buffaloes up to six months of age were collected. We examined these samples by the polymerase chain reaction (PCR) targeting the small-subunit ribosomal RNA gene and positive samples were characterized using additional PCR assays targeting a portion of the beta-giardin, the glutamate dehydrogenase and the triose-phosphate isomerase genes. Based on the *SSU* rRNA nPCR, the presence of *G. duodenalis* was confirmed in 12 (6.56%) of fecal samples, of these, five, four and three samples were positive for the *tpi*, *bg* and *gdh* genes, respectively. Assemblage identification by sequencing was successful in 6 of 12 samples and sequence analysis showed 100% genetic similarity with *G. duodenalis* assemblage E. This observation represents the first detection of *G. duodenalis* assemblage E in buffaloes calves in Brazil.

1. Introduction

Giardia duodenalis is a unicellular flagellate parasite that infects the intestinal tract of a variety of mammals, including humans. The life cycle of this protozoan is direct, cysts are eliminated in the feces and are infective when ingested. Transmission occurs by the fecal-oral route or by ingestion of contaminated food and water (Ryan and Cacciò, 2013).

Giardia infection has a significant impact on both humans and animals (Thompson and Monis, 2012). Giardiasis may be asymptomatic or characterized by abdominal pain, watery diarrhea, steatorrhea, vomiting and nausea in human (Bartelt and Sartor, 2015). In ruminants, the infection may not show clinical signs or may cause chronic diarrhea, weight loss, lethargy, and drop in productivity (Ryan and Cacciò, 2013; Blanco et al., 2017). Giardiasis is recognized as one of the most prevalent waterborne diseases, with several documented outbreaks (Efstratiou et al., 2017).

* Corresponding author at: Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba. Rua Clóvis Pestana, 793, Jardim Dona Amélia, cep 16050-680, Araçatuba, São Paulo, Brasil.
E-mail address: katia.bresciani@unesp.br. (K.D.S. Bresciani).

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Eight genotypes (assemblages) of *G. duodenalis* have been described; assemblage A and B are found in humans and animals, while others assemblages are commonly associated with specific host species (Ryan and Cacciò, 2013). Some studies have reported the occurrence of animal assemblages in humans, such as assemblage F in Ethiopia (Gelanew et al., 2007), assemblage C in China and Slovakia (Štrkolcová et al., 2015) and assemblage E in Egypt (Foronda et al., 2008; Helmy et al., 2014; Abdel-Moein and Saeed, 2016), Brazil (Fantinatti et al., 2016) and Australia (Zahedi et al., 2017).

The occurrence and distribution of *G. duodenalis* assemblages have been extensively studied in cattle. These animals can be infected by assemblages A and B (Coklin et al., 2007; Fava et al., 2013), but assemblage E predominates (Wang et al., 2017; Jian et al., 2018). In buffaloes, giardiasis was documented in several countries, including Italy (Cacciò et al., 2007), Australia (Abeywardena et al., 2013), Sri Lanka (Abeywardena et al., 2014) and Egypt (Helmy et al., 2014). Although giardiasis is common in cattle in Brazil (Silva Jr et al., 2011; Volpato et al., 2018; Sevá et al., 2018), the genetic characterization of *Giardia* spp. has been documented (Toledo et al., 2017; Paz e Silva et al., 2012; Souza et al., 2007). To our knowledge, no studies on the molecular characterization of *G. duodenalis* infecting buffaloes in Brazil have been published.

Many genetic markers have been used for the characterization of *Giardia*, such as the small subunit of the ribosomal RNA (*SSU* rRNA), beta-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose-phosphate isomerase (*tpi*). The *SSU* rRNA gene sequence can be used to identify assemblages. Since this marker lacks resolution to characterize genetic variation within assemblages, *tpi*, *bg* and *gdh* sequences are used because sequence polymorphism enables the characterization of the genetic heterogeneity of this protozoan and enhances our understanding of its zoonotic potential (Ryan and Cacciò, 2013).

Herein, we report the first molecular detection of *Giardia* in buffalo calves in Brazil. *G. duodenalis* was detected by PCR targeting the *SSU* rRNA gene. Positive samples were characterized using the *bg*, *gdh* and *tpi* genes, with subsequent sequencing to evaluate the importance of these animals as reservoirs of zoonotic assemblages.

2. Material and methods

2.1. Ethics committee

The study was approved by the Committee of Ethics in Animal Experimentation (CEEA) of São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba, São Paulo, Brazil.

2.2. Farms

The research was carried out in eight farms (Table 1) in Alambari (Fig. 1), a municipality located in the southwest region of São Paulo State. This locality has shown a dramatic increase in the number of buffalo farms. On seven farms (AN, CE, DA, JU, K, KB and ST), buffaloes were kept in an extensive system, feeding on silage and bran when enclosed in a corral. Water was available *ad libitum* from water sources located in the pasture and from fountains on the farm. In the remaining farm (R), calves drank water from an artesian well and this farm had semi-intensive husbandry system. The animals were kept in a corral designed for calves, and they were mostly fed with concentrate and released into a paddock contiguous to the corral for a few hours daily.

2.3. Collection and processing of fecal samples

In October 2016, fecal samples were collected from 183 Murrah breed buffaloes, which were categorized into four groups: 1) 5 to 60 days of age (75 samples); 2) 61 to 180 days of age (108 samples); 3) with diarrhea (53 samples); and 4) without diarrhea (130 samples). The only clinical sign presented by buffaloes was diarrhea. The fecal samples were obtained directly from the rectal ampulla of each calf with the aid of individualized plastic bags, which were immediately closed, identified and stored in a styrofoam box (thermal insulation) with ice and immediately transported to the laboratory. Approximately 200 mg of feces were frozen in microtubes at −20 °C and used for DNA extraction.

Table 1
Positividade para Giardia spp. em amostras fecais de bubalinos de cada fazenda do estudo com as respectivas coordenadas geográficas.

Farm	Coordinates	Samples	
		Total	Positives to *Giardia* spp.
NA	−23.613260; −47.903624	14	2
CE	−23.578333; −47.885609	11	2
DA	−23.5601812; −47.891139	26	2
JU	−23.521496; −47.901090	38	1
K	−23.537358; −47.912367	8	1
KB	−23.588194; −47.892419	31	2
ST	−23.596474; −47.881446	22	2
R[a]	−23.524700; −47.881068	33	0

[a] Dairy with semi-intensive breeding regime.

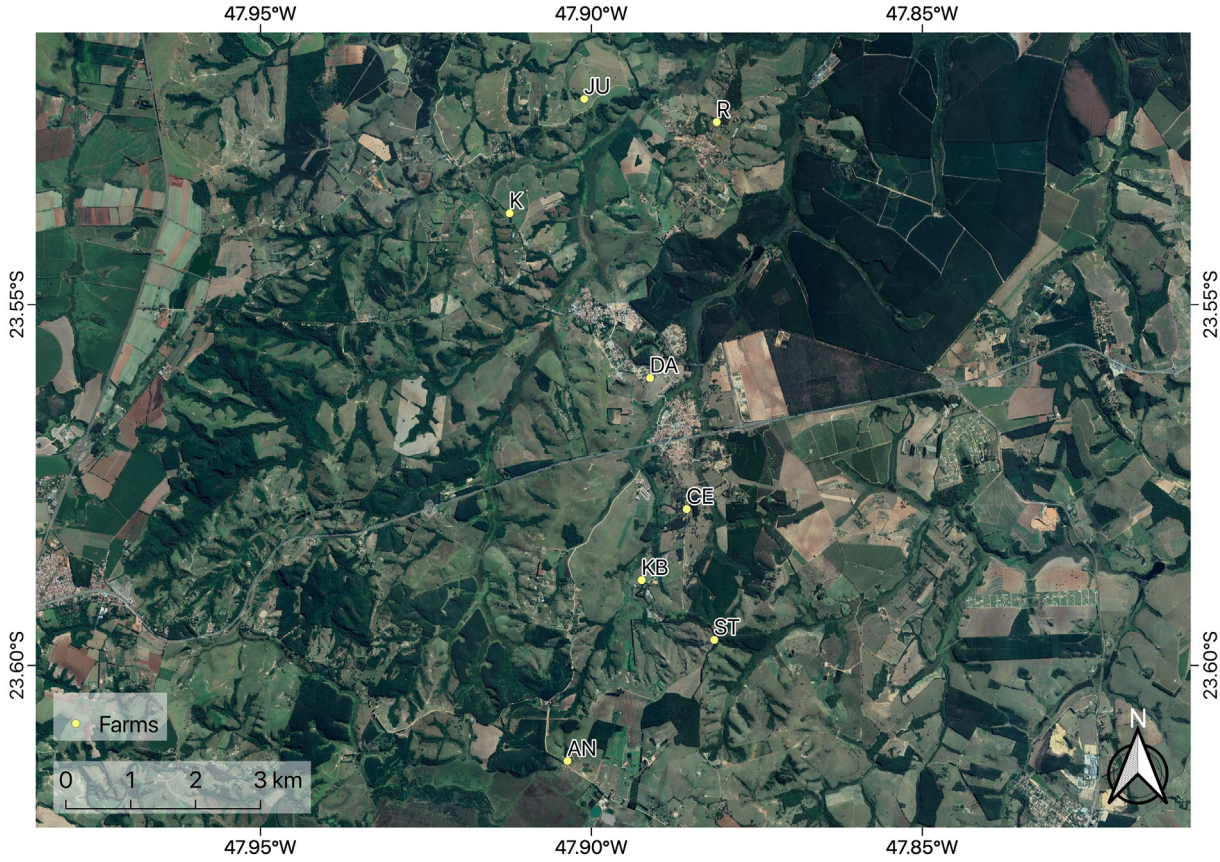

Fig. 1. Sampled farms distribution in Alambari city, São Paulo state, Brazil.

2.4. DNA extraction and PCR

Fecal DNA was extracted from 200 mg of feces using the QIAamp DNA Stool Mini Kit® (Qiagen GmbH, Hilden, Germany) following the protocol described by the manufacturer. Each fecal sample was initially diluted in 1.4 ml of ASL buffer, and 5 cycles of freezing in liquid nitrogen for 1 min and thawing in a thermomixer for 3 min at 99 °C was applied. Fecal DNA was eluted in 50 μL of AE buffer and stored at −20 °C.

Genetic characterization of *Giardia* was initially performed by nested PCR (nPCR) targeting the *SSU* rRNA gene. PCR positive samples were subsequently analyzed with the *bg*, *gdh* and *tpi*-specific nPCRs. The nPCR protocols for amplification of these markers were previously described (Supplementary Table S1).

2.5. Amplicon purification and sequencing

The amplified fragments were visualized by GelRed® stained gel electrophoresis (Biotium, Fremont, USA), purified using the QIAquick® Gel Extraction kit (Qiagen GmbH, Hilden, Germany) and sequenced by capillary electrophoresis on ABI3730 apparatus (Applied Biosystems, Foster City, USA) using POP7 polymer and BigDye v3.1® (Applied Biosystems, Foster City, USA). Amplicons were sequenced in both directions with the oligonucleotides primers of the secondary reaction. For determination of the

Table 2
Giardia nPCR positive samples according to molecular marker.

Molecular markers	Samples											
	1	2	3	4	5	6	7	8	9	10	11	12
ssu rRNA	+	+[a]	+[a]	+[a]	+[a]	+[a]	+	+	+	+[a]	+	+
tpi	−	+[a]	−	+[a]	−	+	+	−	−	+	−	−
Bg	+[a]	+[a]	−	−	−	+[a]	−	−	−	−	−	+
Gdh	−	+[a]	+[a]	−	+	−	−	−	−	−	−	−

[a] Samples with successful sequencing.

consensus sequence, we used the CodonCode Aligner software version 4.0.1 (CodonCode Corporation Dedham®, MA, USA). Amplicon consensus sequences were aligned with Clustal W (Thompson et al., 1997) in BioEdit (Hall, 1999), using homologous sequences downloaded from GenBank as references. Sequences were deposited in Genbank under accession numbers MG958615 (*SSU* rRNA), MG958616 (*gdh*), MG958617 (*bg*), MG958618 (*tpi*). Only one sequence of each gene was deposited in GenBank, since they had 100% similarity to each other.

2.6. Statistical analysis

Data analysis consisted of descriptive and inferential statistics (Fisher's exact test) to verify the association between the occurrence of *Giardia* with each of the variables studied (fecal consistency and age group). The 95% confidence interval was calculated using the Wilson (Score) interval (Sergeant, 2017) and the statistical analysis was considered significant when $P < 0.05$.

3. Results

Based on the *SSU* rRNA nPCR, *Giardia* spp. DNA was detected in 6.56% (12/183) of fecal samples of the calves analyzed in our study. Of these samples, five, four and three samples were positive for the *tpi*, *bg* and *gdh* genes, respectively (Table 2). All sequences were identical with *G. duodenalis* assemblage E sequences previously described.

SSU rRNA sequences (MG958618) were identical to sequences obtained from bovines in Brazil (JF957620), while the *gdh* sequences (MG958616) were identical to the sequences already described in buffaloes (KJ124966) and humans (KJ124968) from Egypt. In addition, *bg* (MG958617) and *tpi* (MG958615) sequences were identical to sequences found in buffaloes from Egypt (KJ125024) and Australia (KF019197), respectively.

Giardia spp. positivity was 5.38% (7/130) in buffalo calves with normal fecal consistency and 9.43% (5/53) in those with diarrhea. According to age group, *Giardia* spp. occurrence was 6.67% (5/75) in 5 to 60 days old calves and 6.48% (7/108) in 61 to 180 days old calves.

There was no significant association between *Giardi*a infection and age and *Giardi*a infection and fecal consistency. Of the eight farms surveyed, *G. duodenalis* was detected in seven farms (AN, CE, DA, JU, K, KB and ST), all practicing an extensive management system.

4. Discussion

Our study represents the first molecular detection of *G. duodenalis* in buffalo calves in Brazil. The molecular characterization of the *SSU* rRNA, *tpi*, *bg* and *gdh* genes revealed the exclusive presence of assemblage E. Sample 2 (Table 2) from KB farm was the only one that amplified with the four PCR markers. The SSU rRNA gene is suitable for screening due to its high number of copies, its sensitivity is higher than assays targeting single-copy sequences (Ryan and Cacciò, 2013).

In our study, a single fecal sample per animal was collected, and the SSU nPCR found 6.56% *Giardia*-positive samples. In a previous PCR based study with one fecal sample, conducted in Australia and Sri Lanka, the occurrence of *G. duodenalis* was 13% (Abeywardena et al., 2013) and 0.7% (Abeywardena et al., 2014), respectively. Therefore, the occurrence may be underestimated, since the cyst shedding has been reported to be intermittent (Appelbee et al., 2003). The occurrence of *Giardia* can vary due to multiple factors, such as the age of the animals, management practices, climatic and geographical conditions, experimental design and according to the diagnostic test (Feng and Xiao, 2011).

The presence of *G. duodenalis* was not associated with diarrhea in our study, as already observed in buffalo calves (Helmy et al., 2014). The presence of *Giardia* cysts in diarrheal stools does not provide sufficient evidence to conclude that giardiasis is the cause of diarrhea in ruminants, which is often multifactorial, with more than one pathogen involved (O'handley and Olson, 2006).

Although the occurrence of *G. duodenalis* in our survey was not significantly associated with age, we observed *G. duodenalis* in buffalo calves up to six months of age, and one positive animal was only five days old. Giardiasis typically occurs in cattle aged 5–10 weeks, but the elimination of *Giardia* cysts has been observed in animals as young as four days (Xiao and Herd, 1994).

Based on phylogenetic analysis, all sequences obtained in the present study were classified as assemblage E, considered the dominant assemblage in the bovine species (Appelbee et al., 2003; Paz E Silva et al., 2012; Wang et al., 2017; Jian et al., 2018). Research with buffalo calves also found a predominance of assemblage E in other countries, such as Sri Lanka (Abeywardena et al., 2014), Egypt (Helmy et al., 2014) and Italy (Cacciò et al., 2007). In contrast, in Australia assemblage A was reported to be dominant (Abeywardena et al., 2014). It is important to note that recently assemblage E was also found in humans in Brazil (Fantinatti et al., 2016), Egypt (Foronda et al., 2008, Helmy, et al., 2015; Abdel-Moein and Saeed, 2016) and Australia (Zahedi et al., 2017). These buffalo calves can eliminate oocysts of *G. duodenalis* assemblage E in the environment and contaminate water of rivers and infect people by indirect contact. Feces of infected calves can potentially infect people by direct contact or indirectly (Abdel-Moein and Saeed, 2016).

In our study *G. duodenalis* assemblage E was detected only in dairies using an extensive husbandry system (AN, CE, DA, JU, K, KB and ST). In these farms, the buffalos had access to natural sources of water. On one of the farms (R), where we did not find this parasite, the animals were fed with concentrate and had access to water from artesian wells. Although we did not compare the same number of dairies that adopted similar management systems, we cannot rule out the possibility that the prevalence of giardiasis was influenced by the type of farm management.

5. Conclusion

We detected for the first time, *Giardia duodenalis* assemblage E in dairy buffalo calves in Brazil. Considering the detection of assemblage E in buffaloes and the zoonotic potential of this genotype, preventive management measures should be adopted to control the transmission of this disease in the herd.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2019.e00062.

Declaration of Competing Interest

The authors declare no conflict of interest.

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