MOLECULAR CHARACTERIZATION OF *Giardia intestinalis* IN HEIFER CALVES REARED IN INDIVIDUAL SHELTERS WITHIN PADDOCKS AT THE MUNICIPALITY OF PIRAÍ IN THE STATE OF RIO DE JANEIRO, BRAZIL*

Thais Ferreira Fagundes[1+], Letícia Gabriela Poblete Vidal[2], Pedro Afonso Moreira Alves[3], Douglas McIntosh[4], Rita de Cássia de Alves Alcântara de Menezes[5], Adevair Henrique da Fonseca[5] e Maria Julia Salim Pereira[5]

ABSTRACT. Fagundes T.F., Vidal L.G.P., Alves P.A.M., McIntosh D., Menezes R. C.A.A., Fonseca A.H. & Pereira M.J.S. **Molecular characterization of *Giardia intestinalis* in heifer calves reared in individual shelters within paddocks at the Municipality of Piraí in the State of Rio de Janeiro, Brazil.** [Caracterização molecular de *Giardia intestinalis* em bezerras criadas em abrigos individuais dentro de piquetes no Município de Piraí, Estado do Rio de Janeiro, Brasil]. *Revista Brasileira de Medicina Veterinária, 35(Supl.2):17-21, 2013*. Programa de Pós-Graduação em Ciências Veterinárias, Anexo 1 do Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, BR 465, km 7, Seropédica, 23897-970, Brazil. E-mail: tha.fagundes@gmail.com

This study aimed to characterize the *G. intestinalis* genotypes in heifer calves from birth to 100 days old, reared in individual shelters within paddocks in the municipality of Piraí, Rio de Janeiro State. Molecular characterization of the protozoan genotypes was performed by analyzing the *β-Giardin* gene via PCR (nested-PCR). Samples from 30 heifer calves positive for Giardia cysts in parasitological examinations were selected for PCR analysis, and of these samples, 10 were digested with the enzyme *Hae*III. Following digestion the genotypes were confirmed by sequencing. The genotypes A and E were identified in 30% and 70% of the samples analyzed, respectively. As genotype A is infectious to humans and the affected calves can be a source of infection, this parasitosis is of great public health importance. This is the first study to characterize *G. intestinalis* genotypes present in heifer calves reared in individual shelters within paddocks in Rio de Janeiro State.

KEYWORDS. *Giardia intestinalis* assemblages, β-Giardin, dairy calves, individual shelters.

RESUMO. Este trabalho teve como objetivo caracterizar molecularmente os genótipos de *G. intestinalis* em bezerras criadas em abrigos individuais dentro de piquetes no município de Piraí, Estado do Rio de Janeiro. A caracterização molecular do protozoário foi realizada usando o gene β-giardin como alvo por PCR (nested-PCR). Amostras de 30 bezerras positivas para cistos de *Giardia* em exames pa-

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[1] Médica-veterinária, *DSc.* Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Anexo 1, Instituto de Veterinária (IV), Universidade Federal Rural do Rio de Janeiro (UFRRJ), *Campus* Seropédica, BR 465, Km 7, Seropédica, RJ 23897-970, Brasil. [+]Author for correspondence, E-mail: tha.fagundes@gmail.com - bolsista CAPES.

[2] Bióloga, *MSc.* PPGCV, Anexo 1, IV, UFRRJ, *Campus* Seropédica, BR 465, Km 7, Seropédica RJ 23897-970. E-mail: let_vidal@yahoo.com.br - bolsista CAPES.

[3] Médico-veterinário, *MSc.* Estação Experimental de Seropédica, PESAGRO-RIO, Rodovia BR 465, Km 7, Seropédica, RJ 23897-000. E-mail: gir.leiteiro@ig.com.br

[4] Microbiologista, PhD. Departamento de Parasitologia Animal (DPA), Anexo 1, IV, UFRRJ, *Campus* Seropédica, BR 465, Km 7, Seropédica, RJ 23897-970. E-mails: pus972@yahoo.co.uk

[5] Médico-veterinário, *DSc.* DPA, Anexo I, IV, UFRRJ, Campus Seropédica, BR 465, Km 7, Seropédica, RJ 23897-970. E-mails: cássia@ufrrj.br; fonseca@ufrrj.br; m.salim@ufrrj.br

rasitológicos foram selecionadas para realização da PCR e dez dessas foram submetidas à digestão com a enzima *HaeIII*. Após a digestão, os genótipos foram confirmados por sequenciamento. Os genótipos A e E foram identificados em 30% e 70% das amostras analisadas, respectivamente. Como o genótipo A é infectante para o homem e bezerras infectadas podem servir de fonte de infecção, esta parasitose reveste-se de importância em saúde publica. Este é o primeiro estudo que caracteriza os genótipos de *G. intestinalis* presentes em bezerras criadas em abrigos individuais em piquetes no Estado do Rio de Janeiro.

PALAVRAS-CHAVE. Genótipos de *Giardia intestinalis*, β-Giardin, bezerras leiteiras, abrigos individuais.

INTRODUCTION

Giardia intestinalis (syn. *G. lamblia, G. duodenalis*) is a protozoan that may be the cause of diarrhea symptoms observed in various hosts and is found in dairy calves in herds from various countries (Guimarães et al. 2001, Mark-Carew et al. 2012, Geurden et al. 2012, Muhid et al. 2012). This protozoan has at least 8 genotypes (classified as A through H), which are morphologically indistinguishable (Thompson et al. 2000, Feng & Xiao 2011), although genetic and biological differences exist between the genotypes. Therefore, it is necessary to classify the genotypes by the varying degrees of biological potential to infect humans and other animal species (Xiao et al. 1993, Monis et al. 1999, Feng & Xiao 2011). Genotypes A and B infect humans, bovines, and other feral and domesticated species; genotypes C and D infect dogs; genotype E infects ungulates; genotype F infects cats; genotype G infects rats; and genotype H infects seals (Monis & Thompson 2003, Feng & Xiao 2011).

In Brazil, studies on molecular characterization of *G. intestinalis* in cattle herds are limited to the State of São Paulo (Souza et al. 2007, Paz e Silva et al. 2012), and therefore, knowledge regarding the genotypes circulating the Brazilian cattle herds may still be considered incipient.

Association between the type of rearing and management system and *Giardia* occurrence in calves has been observed (Silva Junior et al. 2011, Muhid et al. 2012), because this infectious agent can identify favorable developmental environments depending on where the animals are housed. Among these housing systems are the individual shelters within paddocks that are denominated "casinhas" in Brazil (Oliveira et al. 2005)

Therefore, the present study aimed to characterize *G. intestinalis* genotypes that infect heifer calves reared in individual shelters within paddocks in the municipality of Piraí, Rio de Janeiro State, Brazil.

MATERIALS AND METHODS

The samples used in this work were obtained from a longitudinal study based on weekly samples derived from the same individuals conducted by Fagundes (2013), which briefly consisted in following up a cohort of 118 newborns. The animals were crossbred heifer calves, characterized by varying degrees of black and white Holstein breed and reared from birth to 100-days-old in individual shelters within paddocks at a dairy farm in the municipality of Piraí, Rio de Janeiro State, Brazil.

The heifer calves were separated from their dams immediately after birth and received six liters of colostrum by bottle, which was divided into two fractions. Colostrum derived uniquely from cows was tested using a colostrometer, stored in a freezer and subsequently thawed before administering to calves. Each calf house had two buckets: one for supplying feed (offered since birth) and one for milk (offered until weaning at 90 days-old) and water derived from an artesian well. The buckets were cleaned in the morning using only water. These heifer calves were monitored from September 2009 to April 2011, with a total of 1,457 stool samples being analyzed. *Giardia* infection was diagnosed via the identification of fecal cysts using the formol-ether centrifugal sedimentation technique and *Giardia* cysts were observed in 80% (94/118) of the heifer calves using a light microscope at 1000X magnification with immersion oil.

In the present study, a DNA sample obtained from fecal material and another extracted from a sample of cell culture, provided by the Oswaldo Cruz Institute and the Laboratory of Cellular Ultrastructure Hertha Meyer - Institute of Biophysics Carlos Chagas Filho, UFRJ, respectively, were used as positive controls for *Giardia.*

Samples from 30 heifer calves positive for *Giardia* cysts via light microscopy were concentrated following the methodology applied by Almeida (2004).

The pellets formed after concentration of cysts were resuspended in 300 µl of ice cold phosphate buffered saline (pH 7.2) in screw-capped 1.5 mL microcentrifuge tubes containing 50 mg of glass beads (Sigma-Aldrich; product # G8772). The tubes were placed in a mini-beadbeater-16 apparatus (Biospec; Bartlesville, OK, USA), and the cells were disrupted using a single cycle of agitation (60 seconds). Cell lysis was completed by adding 300 µl cell disruption solution (20 mM Tris-HCl, 20 mM EDTA, and 1% sodium dodecyl sulfate) with 20 µl proteinase K (20 mg/ml; Sigma Aldrich) followed by incubation for 3 hours at 56^{0}C. DNA was extracted by performing single rounds of phenol and phenol chloroform treatment followed by precipitation with an equal volume of isopropanol for 20 minutes at room temperature. Precipitated (at 16,000 x g for 15 min) DNA pellets were desalted twice with 70% ethanol and resuspended overnight at 4^{0}C in 50 µl molecular biology grade water (Sigma-Aldrich) and subsequently stored at -20^{0}C.

The primers, reaction components, and cycling conditions described by Cacciò et al. (2002) were used to amplify a 753-base pair (bp) fragment of the β-*giardin* gene. To confirm the

identity of the 753-bp amplicon, a nested PCR reaction designed to generate a product of 511 bp was performed using 1 µl of product derived from the first reaction employing the primers reported by Ramos (2010) (BG1A1F(F2) 5'-GAACGAACGAGATCGAGGTCCG-3' and BG1A1R (R2) 5'-CTCGACGAGCTTCGTGTT- 3'). The reaction mixture and cycling conditions employed for the primary PCR were maintained for the nested assay. An aliquot (10 µl) of each PCR reaction was examined via 1.2% agarose gel electrophoresis to confirm the presence of the appropriate amplicons.

After confirming the species with the two amplification reactions, the 10 samples with the highest DNA concentrations were selected for digestion with the restriction endonuclease *Hae*III to determine the genotype. Aliquots (5 µl) of the 753-bp PCR products were digested with 10 U of *Hae*III (Invitrogen, Carlsbad, CA, USA) for 2 hours at 37°C. The restriction products were separated via electrophoresis on 12% polyacrylamide gels followed by staining with ethidium bromide. Following digestion the genotypes were confirmed by sequencing using the same primers employed in the PCR, performed at the Sector for DNA Sequencing, Centre for Human Genome Studies, Biosciences Institute, University of São Paulo, Brazil. Sequence alignments were performed using the software Sequencher™ (Version 5.0, Genecodes Corporation, CA, USA). All sequences were entered into the BLAST search algorithm and the NCBI nucleotide database to determine gene identity.

RESULTS AND DISCUSSION

DNA quantification of 30 positive samples for *G. intestinalis* cysts revealed an average DNA concentration of 45 ng/µl (varying from 7.1 to 86.5 ng/µl).

Based upon digestion with *Hae*III the genotypes A and E were identified in 30% (3/10) and 70% (7/10) of the samples, respectively (Figure 1). Subsequent nucleotide sequence analysis of the five samples shown in Figure 1, confirmed the genotypes determined from the digestion analysis.

Although *G. intestinalis* genotype E is most frequently identified in cattle (Souza et al. 2007, Barigye et al. 2008, Muhid et al. 2012, Paz e Silva et al. 2012), a high prevalence of genotype A has been reported in this species in various parts of the world (Trout et al. 2004, Santín et al. 2009, Geurden et al. 2012). Meanwhile, genotype B, which has also been reported in these animals (Lalle et al. 2005, Cocklin et al. 2007, Winkworth et al. 2008), seems to be less common. In the present study, we only identified genotypes A and E, which correlates with the results reported by Souza et al. (2007) and Paz e Silva et al. (2012) both performed in Brazil.

The results of several cross-sectional studies (Trout et al. 2004, 2005, 2006, 2007) that report genotype A as the most prevalent in suckling calves and genotype E as the most prevalent in weaned calves, heifers, and cows indicate a possible relationship between the genotypes and animal age. However, in a longitudinal study in which 30 cattle were monitored from birth until 2 years of age, Santín et al. (2009) have reported a higher prevalence of genotype E in suckling calves, while genotype A was not found at this age. These authors state that genotypes A and E occur simultaneously or consecutively and that protocols used in several studies only detect mixed infections when genotypes are present in approximately equal amounts; otherwise, only the predominant genotype is detected. Thus, it is suggested that genotype A is underreported because genotype E is present at higher levels. More recently, in a longitudinal study among 34 farms, Mark-Carew et al. (2012) detected genotypes A and E with all samples identified as belonging to genotype A in calves less than 84-days-old. In the current study, 10 samples all from suckling calves, were molecularly characterized and exhibited genotypes A or E, and mixed infections were not detected.

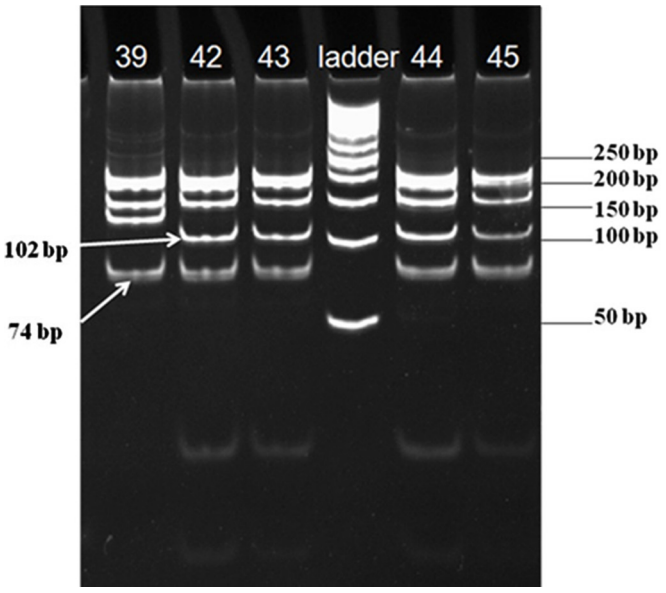

Figure 1. Representative banding patterns visualized following electrophoretic separation of *Hae*III digested PCR products (735 bp) from samples 39 (genotype A) and samples 42, 43, 44 and 45 (all genotype E). The ladder used was the 50 base-pair GeneRuler™ (Fermentas, Ontario, Canada).

It should be considered that disparities between the present study and others can be attributed to several factors, including region studied, study type, sample type, sample size, and molecular characterization methods used. As *G. intestinalis* genotype A is infective to humans and its transmission can spread either by direct contact or via contaminated water and food, this parasitosis is an important public health concern because affected calves can represent a

source of infection for both individuals involved in work with livestock and the population in general. Therefore, there is a need for owners to be vigilant in treating these animals' waste, thereby avoiding the contamination of water and food sources. This is the first study to characterize *G. intestinalis* genotypes present in heifer calves reared in individual shelters within paddocks in Rio de Janeiro State. However, additional studies should be conducted that involve several farms in different regions with more accurate data regarding the genotypes present in calf herds reared in this type of shelter.

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