Genetic characterisation of *Giardia duodenalis* in dairy cattle in Brazil

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**Abstract:** The intestinal protozoan parasite *Giardia duodenalis* (Lambl, 1859) Kofoid & Christiansen, 1915 [syn. *Giardia intestinalis* and *Giardia lamblia*] has emerged as a widespread enteric pathogen in humans and domestic animals. In recent years, *G. duodenalis* has been found in cattle worldwide and longitudinal studies have reported cumulative prevalence of 100% in some herds. In the present study, we determined the prevalence and genetic characterisation of *G. duodenalis* in 200 dairy cattle from 10 dairy farms in São Paulo state, Brazil. All faecal specimens were screened for the presence of *G. duodenalis* using microscopy examination, enzyme immunoassay (EIA) and polymerase chain reaction (PCR). DNA was extracted from faecal samples and *G. duodenalis* were identified by amplification of the small subunit ribosomal (SSU-rDNA) and glutamate dehydrogenase (GDH) genes followed by restriction fragment length polymorphism (RFLP) or sequencing analysis. *Giardia* was identified in eight farm locations (80% prevalence). Overall, 15/200 (7.5%) animals were positive for infection, only one of which was a cow. *Giardia duodenalis* genotype E was present in 14 of the animals tested. Zoonotic genotype AI was present in one positive sample. Genotype E and genotype A represented 93% and 7% of *G. duodenalis* infections, respectively. This study demonstrates that *G. duodenalis* infection was prevalent in dairy calves in São Paulo state and that the non-zoonotic genotype E predominates in cattle in this region. Nevertheless, calves naturally infected in Brazil can shed *Giardia* cysts that can potentially infect humans, and thus, they may represent a public health risk.

**Keywords:** *Giardia duodenalis*, cattle, PCR-RFLP, sequencing, genotyping

*Giardia duodenalis* (Lambl, 1859) Kofoid & Christiansen, 1915 [syn. *Giardia intestinalis* and *Giardia lamblia*] is an intestinal parasite commonly identified in mammals, including humans. The protozoan is prevalent in many parts of the world and is commonly isolated in the faeces of calves (Becher et al. 2004, Trout et al. 2005). This microorganism has been found in beef and dairy cattle worldwide (Appelbee et al. 2003, Barwick et al. 2003). Prevalence rates can vary, but this variation reflects differences in management, climate and study design (Olson et al. 1997, 2004). Studies reported that these parasites were highly prevalent in calves with infection rates as high as 100% in some herds (O’Handley et al. 1999, Ralston et al. 2003). An association was demonstrated between parasite infection, resultant diarrhoea and significant production losses (Olson et al. 1995, O’Handley et al. 2000, Huetink et al. 2001).

Taxonomy of the genus is mainly based on morphology and genetic profiles (Thompson et al. 2000). According to these criteria, six *Giardia* species have been recognized: *G. agilis* in amphibians, *G. muris* and *G. microti* in rodents, *G. psittaci* and *G. ardeae* in birds and *G. duodenalis* in mammals (Thompson 2004). *Giardia duodenalis* is the only species found in both humans and other mammals, including domestic and farm animals such as dogs, cats, cattle, pigs, sheep and horses (Thompson 2000, Hunter and Thompson 2005).

Molecular genetic studies have demonstrated that *G. duodenalis* is a complex species comprising at least seven major genotypes (Monis et al. 2003). Genotypes A and B appear to have the widest host ranges, including humans, cattle, other domesticated animals and wild animal species; genotypes C and D infect dogs, genotype E infects hoofed livestock, genotype F infects cats, and genotype G infects rats (Thompson 2000, Monis and Thompson 2003). Genotype A consists of isolates that can be grouped into two distinct clusters; AI consists of a mixture of closely related animal and human isolates which are geographically widespread, and most attention regarding the zoonotic potential of *Giardia* has focused on the AI subgroup (Olson et al. 2004, Thompson 2004). In contrast, the second subgroup, AII consists entirely of
human isolates. Genotype B comprises two subgroups, III and IV, and the latter appears to be human-specific (Monis et al. 2003, Read et al. 2004).

Genotype E is the predominant genotype found in beef and dairy cattle in Australia, Canada, the United States and the Netherlands (O’Handley et al. 2000, Huetink et al. 2001, Appelbee et al. 2003, Trout et al. 2006) but a small percentage of genotype A has been found in these same studies. Despite the abundance of *G. duodenalis* around the world, there is little information on the genotypes in infected cattle in Brazil. This study was undertaken to determine the occurrence and genetic variants of *G. duodenalis* in dairy cattle in south-central São Paulo state, Brazil.

**MATERIALS AND METHODS**

**Dairy farms and sample collection**

Faeces were collected from dairy cattle located on 10 dairy 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 in accordance with the availability of animals on the properties. At each farm, 20 faecal specimens were collected from calves (2 weeks to 6 months of age) and cows (>24 months). Two hundred cattle faecal samples were collected in total (100 from calves and 100 from cows). Faeces were collected directly from the rectum of each animal with a gloved hand and transferred into a plastic cup. 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.

**Light microscopy examination**

Zinc sulfate flotation (Faust et al. 1938) was employed to detect the presence of *Giardia* cysts in the faeces. Approximately 1–2 gram of each fresh faeces was mixed with distilled water, vortexed thoroughly and filtered into a disposable plastic tube (15 ml in volume). The filtrate was centrifuged at 1,500×g for 1 min. After this wash step, the supernatant was discarded and a ZnSO4 solution was added to the sediment, mixed thoroughly and centrifuged at 1,500×g for 1 min. Zinc sulfate solution was added up to the rim of the tube and a cover glass (size: 18 mm × 18 mm) was put on top of the tube. After 3 min, the cover glass was removed from the tube, placed on a microscope slide and stained with iodine. The preparations were examined under ×100 magnification for the presence of cysts. The identity of parasitic stages was confirmed by examining them under ×400 magnification. Animals were considered positive if a *G. duodenalis* cyst with the correct morphology (12–14 μm in length with an axostyle and two or four nuclei) was detected in the sample.

**Antigen-EIA**

The GIARDIA II® antigen EIA (Techlab, USA) was used to identify the presence of cysts in 10% formalin preserved samples. The diagnostic kit procedure was followed. The results were examined and interpreted according to the manufacturer’s instructions. One hundred microlitres of a 10% formalin-preserved stool sample were used for the assay. Bound antigen was detected by a horseradish peroxidase-conjugated secondary monoclonal antibody and tetramethylbenzidine substrate. Results were read photometrically at 450 nm. A sample was considered positive when O.D. was >0.150 at 450 nm.

**Isolation of DNA**

DNA was extracted directly from 200 mg of each faecal sample using the QI Amp DNA StoolMini Kit (Qiagen, Germany) following the manufacturer’s recommended protocol with slight modifications. Briefly, the samples were boiled for 5 min, exposed to four cycles of freezing in liquid nitrogen for 5 min and then boiled for an additional 5 min. Cycling between freezing and boiling temperature served to disrupt the cell walls of the cysts and to release the DNA. Elution of the DNA was performed with 100 μl of elution solution to increase the quantity of recovered DNA. DNA was stored at −20°C until it was used in the PCR assays.

**PCR analysis**

Faecal specimens frequently contain PCR inhibitors and a large number of cells from the host (e.g., intestinal and blood cells). The adequacy of DNA isolation and purification was assessed for each isolate by first performing PCR amplification with specific primer sets targeting the mitochondrial DNA from bovine cells, as previously described (Martelinni et al. 2005). Mitochondrial DNA gene targets were chosen for this assay because individual host cells contain numerous copies of mitochondrial DNA. The successful amplification of this target demonstrates the absence of inhibitors in a DNA sample originating from faeces.

**Polymerase chain reaction**

A 292-bp region of the small subunit ribosomal gene (SSU-rDNA) was amplified by PCR as previously described (Hopkins et al. 1997, Leonhardt et al. 2007). Each specimen was analyzed at least twice by PCR.

**GDH semi-nested PCR**

A fragment of the GDH gene for *Giardia* (432 bp) was amplified by a semi-nested PCR using primers GDH E, GDH H and GDH F as previously described (Read et al. 2004) with some modifications. PCRs reactions were performed in a total volume of 25 μl, with the primary PCR reaction mixture containing 12.5 μl 2× GoTaq (Promega, USA) Green Master Mix buffer (pH 8.5, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP,
A 100-bp ladder (Fermentas, USA) and positive and negative controls were included in each gel. Gel images were visualized under UV light and were captured with a gel documentation system (Alphalmlager, AlphaEasy FC, Alphalnotech Corporation®). PCR products were purified using a Wizard® SV Gel and PCR Clean-up System (Promega, USA). Sequencing reactions were performed on the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). All specimens gave the expected length of the bovine mitochondrial PCR product and these data indicate that PCR inhibitors were not responsible for negatives results. The protozoan cysts were detected in six out of all faecal specimens collected (3%). Enzyme immunoassay (EIA) identified the same six samples that were positive with the conventional morphological technique. Among the 200 cattle samples tested, 15 (7.5%) produced positive PCR signals (GDH and SSU-rDNA genes). No samples that were negative in the PCR assay were positive using microscopy and ELISA. Agreement between diagnostic tests was moderate (Kappa = 0.552). All specimens gave the expected length of the bovine mitochondrial PCR product and these data indicate that PCR inhibitors were not responsible for negatives results. The protozoan was detected on 80% (8/10) of the farms visited. The prevalence of G. duodenalis infection varied across farms, with the lowest (5%) on PAR-1, BTU-5, ITA-9 and ITA-10 and the highest prevalence (20%) on PAR-2. Positive G. duodenalis samples for each county and farm are shown in Table 1.

**Prevalence related to age**

In this study, infection with G. duodenalis was more prevalent in younger animals than in older ones (P = 0.0013). Prevalence observed in calves (14%) was higher than in cows (1%). The prevalence related to the age of calves was approximately 30% for calves at 61–90 days of age and ~17% for calves at 0–30 days of age (Fig. 1). However, the point prevalence of G. duodenalis appears not to vary within calves age groups (p = 0.0634).

**Presence of diarrhoea in calves**

Diarrhoea was recorded in 17% (17/100) of the calves at the time of the sampling. G. duodenalis was detected in 17.6% (3/17) of diarrhoeic calves and in 13.2% (11/83) of non-diarrhoeic calves. The percentage did not differ significantly; thus, it was not found to be statistically associated with infection (P = 0.927).
Genetic characterisation

All *G. duodenalis* positive isolates could be typed by RFLP and/or sequencing analysis. Two different restriction patterns from the *NcoI* digestion of semi-nested PCR products were obtained. Two genotypes were identified: genotype E (14 samples) and genotype A (one sample). Across all farms, 93% (14/15) of the *G. duodenalis* positive animals were infected with genotype E, whereas 7% (1/15) were infected with genotype A. Genotype E was found on all *Giardia* positive farms sampled. Genotype A was found on a farm in Pardinho in a 4 month-old calf.

Selected isolates in the study were submitted to sequencing analysis, which confirmed our RFLP-genotyping results. The *BRAcalf*14 (accession no. JF957619) isolate SSUr-DNA sequence exhibited 100% match with the genotype A isolate from a dairy calf in the USA (GenBank accession no. AY655700; Trout et al. 2004). The other isolate sequenced, *BRAcalf*20 (accession no. JF957620) exhibited the A–G transition at nucleotide position 92, indicative of the livestock genotype. Its sequence was identical to the previously established genotype E (GenBank accession no. AY655701; Trout et al. 2004).

The GDH PCR positive faecal sample of genotype AI (*BRAcalf*14, GenBank accession no. JF957621) from the current study had 100% similarity with a positive *G. duodenalis* genotype AI isolate (GenBank accession no. EF507642) from cattle in São Paulo from another study (Souza et al. 2007).

A double peak T/C (two overlapping nucleotides) occurred in the electropherograms of three nucleotides sequences at the GDH locus: isolates *BRAcalf*12 (GenBank accession number JN160733), *BRAcalf*53 (GenBank accession number JN160735) and *BRAcalf*84 (GenBank accession number JN160738). This feature is similar to that detected in the isolate ad-133 (GenBank accession number AY178740) at the same position.

DISCUSSION

The occurrence of *Giardia duodenalis* infections in cattle was described in several geographic regions. Studies in Canada (Olson et al. 1997, Appelbee et al. 2003, Ralston et al. 2003, Coklin et al. 2007), Australia (O’Handley et al. 2000, Becher et al. 2004), Belgium (Geurden et al. 2004), New Zealand (Hunt et al. 2000), the United States (Trout et al. 2004, 2005, 2006, 2007), Italy (Berrilli et al., 2004), Denmark (Langkjaer et al. 2007), the Netherlands (Huetink et al. 2001) and Portugal (Mendonça et al. 2007) have identified animals infected with this parasite.

The prevalence of *G. duodenalis* infection in cattle varies markedly. While many point prevalence studies (only one sample collected from each animal) of cattle report a significant percentage of *Giardia*-infected animals, cumulative prevalence studies (more than one sample collected of each animal) often observe close to 100% infection rates (Xiao and Herd 1994, O’Handley et al. 1999, Ralston et al. 2003, Santin et al. 2009).

In previous studies, the average prevalence of *G. duodenalis* in dairy cattle after faecal examination ranged from 22 to 60% (O’Handley et al. 2000, Appelbee et al. 2003, Trout et al. 2004, 2005, 2006, 2007, Coklin et al. 2007, Geurden et al. 2008). This finding is higher than the average prevalence (7.5%) reported in the current study, but point prevalence data are expected to underestimate the actual prevalence because they are influenced by a range of factors including method and study design, geographical differences, age composition of sampled calves, number of samples from each farm, total number of samples, herd size and sampling season (Hammes et al. 2006).

As observed by Ralston et al. (2003) and others (Appelbee et al. 2003, Geurden et al. 2004, Trout et al. 2007, Santin et al. 2009), cyst excretion can be intermittent. It is likely that the point prevalence data presented herein underestimates the actual numbers of infected animals on a given farm because only a single faecal sample from each animal was collected.

In the present study, *G. duodenalis* was detected on 80% (8/10) of the farms sampled, with prevalence ranging from 0 to 20% in the farms. In four widespread geographic studies in 28 states in the United States, *G. duodenalis* was present in 100% of farms with the prevalence in faecal samples analyzed by PCR ranging from 3% to 93% in each herd (Trout et al. 2004, 2005, 2006, 2007).

Presence of diarrhoea

Infection rates were very similar in the faecal samples of non-diarrhoeic (13.2%) and diarrhoeic (17.6%) calves.
As in others studies (Hunt et al. 2000), no relationship was observed between the presence of *Giardia* cysts and the consistency of the individual faecal specimen.

**Age-related *Giardia* infection**

As described by Trout et al. (2004), O’Handley et al. (2000), Appelbee et al. (2003) and Becher et al. (2004), *G. duodenalis* infections have been frequently reported in pre-weaned calves (under 2 months of age). However, post-weaned calves (3 to 11 month-old), heifers (12 to 24 months) and adult cows (>24 months) have also been repeatedly observed as hosts for this parasite (Trout et al. 2005, 2006, 2007, Hamnes et al. 2006, Castro-Hermida et al. 2007).

In our study, the prevalence reached 17% in 0 to 30 day-old calves, approached 14% in 30 to 60 day-old calves, and was around 29% in 61 to 90 day-old calves. The total number of 0 to 2 month-old calves was 21 and prevalence of *Giardia* in this subgroup was 14% (3/21). In 3 to 6 month-old calves, a total of 14% (11/79) specimens were positive. No significant difference was observed. In a North American study, *G. duodenalis* cysts were rare in cows and the highest occurrence was found in animals at 4 to 5 weeks old (Santín et al. 2009). On the other hand, Huetink et al. (2001) in the Netherlands reported that calves 4 to 5 months of age were the most frequently infected age group. Similar to previous studies (O’Handley et al. 1999, Trout et al. 2005, Hamnes et al. 2006), we observed that 30% of sampled calves at 3 month-old were infected. In pre-weaned, post-weaned, and adult dairy cows on 14 dairy farms in seven eastern states in the United States, *Giardia* was detected in 40%, 52%, and 27% of the animals, respectively (Trout et al. 2004, 2005, 2007).

Our results conform to those of previous studies that concluded that giardiasis in calves was generally established during the first months of life and increased when animals were exposed to cysts in the environment (Huetink et al. 2001). Prevalence significantly decreased in adult cows. Infection with *G. duodenalis* in adult animals was only 1% (1/100). A lower prevalence was found in adult dairy cattle compared with young calves, which was also consistent with previous reports (O’Handley et al. 1999, 2000, Appelbee et al. 2003, Mendonça et al. 2007). A high prevalence of *G. duodenalis* was observed by Castro-Hermida et al. (2007) and Trout et al. (2007) in adult dairy cattle.

**Genotypes**

Cattle are susceptible to infection by three major genotypes of *G. duodenalis*: the zoonotic genotypes (A and B) and the host-related, non-zoonotic genotype E (Thompson et al. 2000, Monis and Thompson 2003). Similar to our study, genotype E is most prevalent in dairy cattle from Australia, the United States, Canada and New Zealand and has a wide distribution among cattle farms (Appelbee et al. 2003, Becher et al. 2004, Trout et al. 2004, 2005, 2006, 2007). Others studies revealed various prevalence rates of genotype A in dairy cattle (O’Handley et al. 2000, Uehlinger et al. 2006, Langkjaer et al. 2007).

In a North American molecular epidemiologic study in pre-weaned and post-weaned calves and adult dairy cattle, *G. duodenalis* genotype A was detected at varying levels on 14 farms studied (Trout et al. 2004, 2005, 2006, 2007). This potentially zoonotic genotype represented 15% to 25% of the total *Giardia* detected in these farms. In the present study, zoonotic genotype A1 was detected on only one farm and in approximately 6.6% of the positive animals (1/15). The current study revealed that dairy cattle on the visited farms had a high occurrence of *G. duodenalis* genotype E, but the zoonotic genotype A was also reported.

**Zoonotic significance**

Cattle have been proposed as a potential source of human *Giardia* infections through direct contact or contamination of surface water supplies (O’Handley et al. 2000, Thompson et al. 2000, Thompson 2000, Hunter and Thompson 2005). Recent studies have shown high levels of the potentially zoonotic *G. duodenalis* genotype A in dairy cattle (Uehlinger et al. 2006, Geurden et al. 2008). In those studies, the majority of the dairy calves were infected with *G. duodenalis* genotype A. The results of this study confirm that dairy cattle in Brazil can shed *Giardia* cysts potentially infectious to humans. Further studies in other endemic regions in Brazil are required to fully evaluate the prevalence of zoonotic genotypes and the public health impact of infections with *Giardia* in dairy cattle.

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