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# Molecular and immunohistochemical detection of assemblage E, *Giardia duodenalis* in scouring North Dakota calves

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**Abstract**

Despite many reports on the shedding of *Giardia* parasites by scouring calves, the role of *Giardia* as a cause of calf diarrhea is still controversial. To elucidate the role of *Giardia duodenalis* in calf scours, diagnostic samples from 189 scouring calves were tested by different assays during a 1-year-study period. *Giardia* antigens were detected in 22/189 scouring calves by a fecal-based enzyme-linked immunosorbent assay and 10 of these were positive for assemblage E, *G. duodenalis* by polymerase chain reaction. *Giardia* trophozoites were demonstrated by immunohistochemistry in intestinal sections from five calves in which the parasites were spatially distributed in areas of microscopically detectable enteritis. Our data suggest that under certain circumstances, *Giardia* may cause intestinal lesions leading to calf scours. Gnotobiotic calf-based infectivity studies are needed if the pathogenicity of *Giardia* in calves is to be definitively determined.


*Keywords:* Calf scours; *Giardia duodenalis*; Immunohistochemistry

## 1. Introduction

*Giardia duodenalis* is an intestinal protozoan that commonly infects humans, and a wide range of domestic and wild mammals. Recent application of molecular methodologies has aided the detection of seven genotypes (assemblages) of *G. duodenalis*; assemblages A and B having the widest host ranges, including humans; assemblages C–G with limited host ranges including canids (C and D), hoofed-livestock (E), cats (F), and rats (G).

Reports from North America indicate a high prevalence of *G. duodenalis* in calves (Xiao et al., 1993; O'Handley et al., 2000; Wade et al., 2000a,b; Appelbee et al., 2003; Trout et al., 2004; McAllister et al., 2005). This is frequently accompanied by scouring (Xiao et al., 1993; O'Handley et al., 2000; Wade et al., 2000a,b; Appelbee et al., 2003; Trout et al., 2004; McAllister et al., 2005) thus underscoring the potential pathogenic nature of this parasite in calves. Research conducted in Ohio, Tennessee, New York, and Minnesota suggests that *Giardia* spp. may be an important pathogen in calves (Xiao et al., 1993; Wade et al., 2000a,b; Nydam et al., 2001; Smith et al., 2004). However, no attempts were made to demonstrate

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morphological changes in the small intestine of the calves studied. Interestingly, the Ohio study further reported that treatment of *Giardia*-infected scouring calves with metronidazole was associated with rapid cessation of the scours (Appelbee et al., 2003), which provides indirect evidence that *Giardia* spp. was the cause of the diarrhea in these animals. With the lack of empirical morphologic evidence however, the importance of this protozoal agent as an enteric pathogen in calves has remained speculative. Conversely, the role of this parasite in young scouring non-bovine domestic ruminants has been clarified. Data from experimentally infected lambs and goat kids clearly demonstrates that feed efficiency and weight gain are negatively impacted by *Giardia* infections (Olson et al., 1995).

Studies of tissues from humans and animals infected with *Giardia* spp. revealed a number of histologic changes including: villous atrophy and blunting, crypt cell hyperplasia, infiltration by inflammatory cells, and ultrastructural alterations in the microvillus border of enterocytes (Buret et al., 1990; Koudela and Vitovec, 1998). Decreases in the activity of certain intestinal brush border enzymes including alkaline phosphatase, lactase, maltase and sucrase, have also been reported (Smith et al., 2004; Buret, 2007). These findings suggest that intestinal malabsorption is a significant consequence of *Giardia* spp. infection. In fact, this protozoan may cause severe enteric disease without penetrating the intestinal epithelium (Buret, 2007). Recently it was demonstrated that *Giardia*-induced intestinal dysfunction involves apoptosis of enterocytes and down-regulation of the tight junction protein claudin-1 (Troeger et al., 2007). Although these mechanisms have not yet been described in calves, it is likely that similar to what happens in other young domestic ruminants, *Giardia* spp. has the potential to cause significant intestinal damage that can lead to clinically important calf scours. The objective of this study was to elucidate the role of *Giardia* spp. as a cause of intestinal damage in scouring calves.

## 2. Materials and methods

### 2.1. Study samples

The 189 study samples came from 1-day to 2-month-old scouring calves originating from both dairy and beef farms in North Dakota, and the bordering counties of Minnesota that are served by the North Dakota State University Veterinary Diagnostic Laboratory (NDSU-VDL). The samples included intestinal tissue and/or intestinal contents obtained from calves at necropsy and mailed-in samples submitted to the NDSU-VDL for routine scours screening.

### 2.2. SNAP Giardia-ELISA

The samples were tested for *Giardia* antigens using a SNAP *Giardia*-antigen test kit (IDEXX Laboratories, Inc., Westbrook, ME) which has a sensitivity and specificity of 95%. The testing was done according to the instructions provided with the test kit.

### 2.3. Pathogen culture and other screening tests

For bacteriologic culture, intestinal contents were inoculated on tryptose soy agar (TSA) II 5% sheep blood, and placed in an aerobic incubator at 35 °C for 12 h. Samples were also inoculated on Skirrow's media, and incubated in anaerobic conditions with a microaerophilic gas generating pack at 37 °C for 12 h. *Escherichia coli* isolates were further genotyped for six virulence factors (K99, F41, Intimin, Sta, Stx-1 and Stx-II) by a multiplex PCR assay based on the Qiagen(r) Multiplex PCR kit. All *Salmonella* isolates were sent to the National Veterinary Services Laboratories, Ames Iowa for serotyping. Presence of *Cryptosporidium* spp. was evaluated by the modified acid fast stain, and in calves aged 2 weeks old, fecal flotation was done to rule out coccidian and other parasites. Frozen intestinal sections were tested by direct immunofluorescent antibody (FA) assay to detect antigens from bovine virus diarrhea virus (BVD), rotavirus and coronavirus. A polyclonal porcine anti-BVD IgG antibody conjugated to fluorescein isothiocyanate (VMRD Cat. # 210-61-BVD) was used for the BVD FA test. Electron microscopy was done on intestinal contents for detection of rotavirus and coronavirus using standard protocols.

### 2.4. Tissue processing and histological evaluation

Representative tissue samples from different segments of the small and large intestine were fixed in 10% buffered formalin, processed routinely and paraffin embedded. Five-micron sections were deparaffinized, stained with hematoxylin and eosin (H&E), and evaluation was done via 40× and 60× objectives of a light microscope. For evaluation of the histological changes, intestinal sections from the scouring calves and a negative control slide from an age matched calf that died from lead toxicity, were evaluated by two board certified veterinary pathologists who separately described the microscopic changes in the samples, and later scored *Giardia* parasite loads in immunohisto-

chemically stained replicate sections. Results from these analyses and the parasite scores were recorded on a data sheet previously designed for this purpose. The scoring of the *Giardia* parasites was based on a scale ranging from 0 to 3 where: 0 = no parasites present, 1 = few parasites present, 2 = moderate numbers of parasites present and 3 = many parasites present.

### 2.5. Pathogen-specific immunohistochemistry

For immunohistochemistry (IHC), a polyclonal goat anti-*Giardia* IgG antibody (Antibodies, Inc. # 51-280) was used to stain intestinal sections obtained from scouring and negative control calves. Briefly, deparaffinized 5 μm sections were dehydrated in a three ethanol gradient (70, 95 and 100%), followed by a 1 min wash in distilled water plus another wash in deionized water. Endogenous peroxidase activity was blocked by a 5 min incubation in 3% hydrogen peroxide after which slides were washed in Dako cytomation wash buffer [Tris buffer saline, with Tween 20, pH 7.6 (TBS)]. Following this step, the sections were permeabilized with proteinase K for 5 min, reacted with DAKO protein blocking solution (0.25% casein in phosphate buffered saline, pH 7.4) for 5 min and then incubated for 30 min with an anti-*Giardia* goat IgG polyclonal antibody diluted at 1:160 in DAKO cytomation antibody diluent (Tris–HCl with Tween 20). Following another wash step in TBS, the sections were incubated with a biotinylated anti-goat IgG (DAKO LSAB+ system) for 15 min, washed with TBS and the slides incubated for 15 min with streptavidin-labeled horseradish peroxidase. After another TBS wash, the sections were treated with two 5 min changes of Romulin red AEC chromogen, washed with deionized water, then with TBS after which they were counterstained with DAKO automation hematoxylin. The slides were further washed with deionized water, air dried and dehydrated in 10 dips of 95% ethanol, 10 dips in absolute alcohol plus a 1 min dip in xylene. The sections were secured with a coverslip and the slides examined by light microscopy. The IHC for the enteropathogenic viruses was based on a similar protocol that used as primary antibodies a coronavirus monoclonal antibody (mAb) (Z3A5/8F2; Kansas State University) diluted 1:400; and a rotavirus polyclonal goat antibody (AB 1129; Chemicon) diluted at 1:1600.

### 2.6. DNA extraction and polymerase chain reaction

Seventeen of the 22 *Giardia*-antigen-positive samples were sent to the Environmental Microbial Safety Laboratory, Agricultural Research Service, USDA, Beltsville, Maryland where a *Giardia*-specific polymerase chain reaction (PCR) test was performed using primers to the ssu-rRNA gene. Total DNA was extracted from each fecal sample using a DNeasyTissue Kit (Qiagen, Valencia, CA), with a slight modification of the kit protocol. Approximately 100 μl of fecal material was mixed with 180 μl of ATL buffer and thoroughly mixed. To this suspension, 20 μl of proteinase K (20 mg/ml) was added; after the suspension was incubated overnight at 55 °C, 200 μl of AL buffer was added. The remaining protocol followed manufacturer's instructions except that the nucleic acid was eluted in 100 μl of AE buffer to increase the quantity of recovered DNA.

A fragment of the ssu-rRNA gene (∼292 bp) was amplified by nested PCR as previously described (Hopkins et al., 1997). The primers used in the PCR assay were: forward primer (1–18) 5′CATCCGGTCGATCCTGCC3′ and reverse primer (268–292) 5′AGTCGAACCCTGATTCTCCGCCAGG3′. PCR products were analyzed on 1% agarose gel with ethidium bromide staining. PCR products were purified using EXO-SAP-ITtm (USB Corporation, Cleveland, OH) and sequenced with the same PCR primers used with the original nested amplification in 10 μl reactions, Big Dye(TM) chemistries. All of the PCR positive products were sequenced once, in both directions, on an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms from each strand were aligned using Lasergene software (DNASTAR, Inc., Madison, WI).

## 3. Results

During the 12-month study period that began in June 2006 and ended in May 2007, 22/189 (11.6%) samples tested positive for *Giardia* antigens by the fecal antigen ELISA method. These samples included intestinal contents obtained at necropsy of scouring calves, and intestinal tissue and fecal mail-in samples from scouring calves received at the NDSU-VDL. Except for one *Giardia*-positive case that was also positive for coronavirus and *E. coli* expressing the virulence factors F41 and Stx-I, no other pathogenic organisms were identified in all the other 21 *Giardia*-positive cases. The highest number of *Giardia*-positive cases was recorded during the warm months of June 2006 (four cases) and May 2007 (seven cases). Of all the *Giardia* positive cases, 95% were aged between 1 and 4 weeks, and no age was indicated for 5% of the calves. Of all the calf samples tested, virulent *E. coli* accounted for 16/189 (8.5%), *Salmonella* spp. for 11/189 (5.8%), *Cryptos-*

*poridium* spp. for 56/189 (29.6%), coccidia for 13/189 (6.9%), rotavirus for 47/189 (24.9%) and coronavirus for 24/189 (12.7%). A mixed infection of *Giardia* spp., *E. coli* and coronavirus was demonstrated in one calf.

Of the ELISA-positive samples that were sent to a referral laboratory for PCR testing, 10/17 (58.8%) were positive for assemblage E *Giardia* DNA, 6/17 (35.3%) were negative and 1/17 (5.9%) could not be tested because of sample quality.

For the cases where fresh or formalin-fixed intestinal tissue samples were available, intestinal sections were examined by the principal investigator, and further evaluated by two board-certified veterinary pathologists who in a blinded fashion analyzed the H&E sections, and scored the parasite loads in the IHC sections. Low to moderate numbers of lymphoplasmacytic cells and rare eosinophils infiltrated into the submucosa and lamina propria and occasional crypts were distended by low numbers of inflammatory cells (Fig. 1). Results of the examination of correlate IHC stained sections included variable numbers of *Giardia* trophozoites that were admixed with sloughed enterocytes in the intestinal lumen of *Giardia* spp. positive calves (Fig. 1), or many parasites that were attached to the intact or disrupted villus enterocytes in the calf that was positive for *Giardia* spp., coronavirus and virulent *E. coli* (Fig. 2). In the latter, there were numerous *Giardia* trophozoites that invaded into the mucosa (Fig. 2). No comparable histologic changes or *Giardia* trophozoites were present in H&E and IHC sections of intestinal tissue obtained from the negative control (Fig. 3).

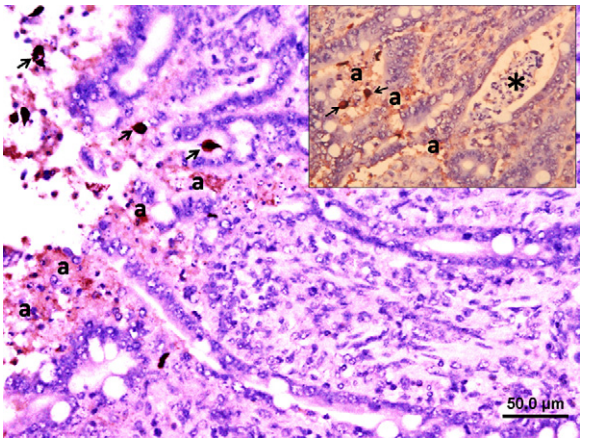


Fig. 2. Small intestine (jejunum); scouring calf: there are numerous *Giardia* trophozoites (black arrow heads) and *Giardia* antigens (a) in areas of mucosal necrosis and denudation. This calf tested positive for *Giardia* spp., coronavirus and virulent *Escherichia coli*. IHC, bar = 50 μm. *Inset*: a medium size crypt abscess is indicated with a black asterisk. IHC.

## 4. Discussion and conclusions

In this paper, we present data that demonstrates the morphologic distribution of *Giardia* parasites in intestinal sections from scouring calves testing negative to other enteric pathogens. In a single calf that was positive to coronavirus and virulent *E. coli* (Fig. 2), the *Giardia* parasites and/or antigen were spatially distributed in areas of microscopically demonstrable enteritis suggesting that under certain circumstances this protozoan may play a role in calf scours. This has

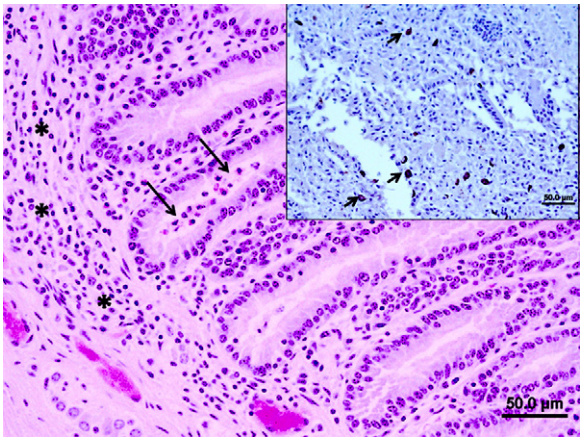


Fig. 1. Small intestine (jejunum); scouring calf: there are low to moderate numbers of lymphoplasmacytic cells along with rare eosinophils that infiltrate the submucosa and lamina propria (black asterisks). Occasional crypts are distended by low numbers of cellular debris (long black arrows). H&E, bar = 100 μm. *Inset*: many intact *Giardia* trophozoites (short black arrows) are present within the sloughed intraluminal cellular debris. IHC, bar = 50 μm.

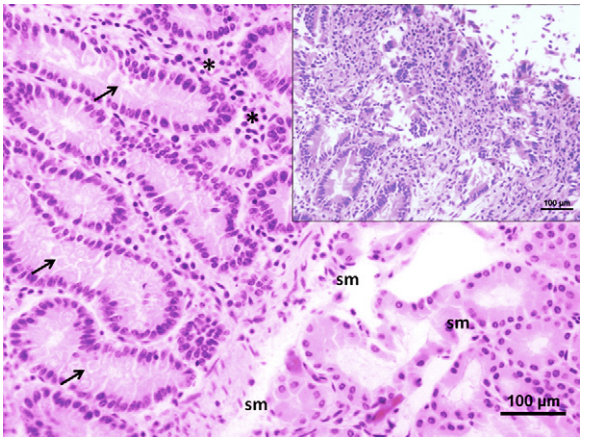


Fig. 3. Small intestine (jejunum); negative control calf: very low numbers of lymphoplasmacytic cells infiltrate the lamina propria (black asterisks). Unlike the *Giardia*-positive-scouring calf in Fig. 1, the submucosa (sm) and intestinal crypts (black arrows) are remarkably devoid of inflammatory cell infiltrates. H&E bar = 100 μm. *Inset*: there is notable absence of *Giardia* trophozoites. IHC.

been previously implied but unproven by data collected in a number of studies (Xiao et al., 1993; O'Handley et al., 2000; Wade et al., 2000a,b; Appelbee et al., 2003; Trout et al., 2004; McAllister et al., 2005). Our results are corroborated by the detection of *Giardia* DNA (Fig. 4) in some of the samples previously found positive for *Giardia* antigens by a fecal-based ELISA. Whereas the histological changes in intestinal sections from the four of the five *Giardia*-positive calves were not definitive, the intestinal lesions in the only calf with a *Giardia*, coronavirus and *E. coli* mixed infection (Fig. 2) included occasional crypt abscesses; increased mitotic activity in the crypt enterocytes consistent with crypt cell hyperplasia; villous attenuation; infiltration of the lamina propria by low to moderate numbers of lymphocytes, plasma cells, and lesser numbers of eosinophils and macrophages; along with moderate to marked sloughing of the enterocytes in some cases. Whereas these changes cannot be definitively attributed to the *Giardia* infection, some of them are similar to those reported in other young *Giardia*-infected scouring non-bovine animals (Quigley et al., 1994; Smith et al., 2004); and a contributory role of the intralesional *Giardia* trophozoites to the development of severe enteritis in the calf cannot be ruled out. A review article on the mechanisms of epithelial dysfunction in human intestinal giardiasis (Olson et al., 1995) reports that this parasite can cause severe enteric disease without penetrating the intestinal epithelium. Based on the conclusions by these workers, it is probable that the numerous *Giardia* trophozoites seen in the pure *Giardia* infection may have played a partial role in the development of scours in those calves; this assertion will be investigated in future infectivity studies. The presence of *Giardia* parasites as seen in the various intestinal sections of *Giardia*-positive calves may have compromised the integrity of the intestinal mucosa in a way that most probably led to maldigestion and malabsorption leading to diarrhea. Many of the *Giardia* trophozoites seen in the present study were either found attached to the intact villi, or denuded mucosal areas and/or admixed with sloughed enterocytes within the lumen. Troeger et al. (2007) reported that *Giardia*-induced intestinal dysfunction in humans involves down-regulation of the tight junction protein claudin-1, and enterocyte apoptosis. Although we did not demonstrate these mechanisms in the present study, most of the *Giardia*-positive calves had a high level of enterocyte sloughing. This suggests that enterocyte sloughing as reported in humans (Troeger et al., 2007) may be an important pathophysiological mechanism in intestinal giardiasis of calves. However, this needs to be experimentally verified in infectivity studies based on gnotobiotic calves as postmortem autolysis may have confounded the intestinal changes in the present study. Future studies are needed to demonstrate these and other potential enteropathogenic mechanisms of giardiasis in calves if appropriate therapeutic interventions are to be adopted. Moreover, calves could potentially be used as a model for human giardiasis if these mechanisms were established to be similar in both species.

It is not surprising that all the PCR positive calves exclusively carried the ruminant specific assemblage E, *G. duodenalis* (Fig. 4). Various workers have reported a majority of *Giardia* isolates from calves belong to this assemblage (Xiao et al., 1993; Wade et al., 2000a; Berrilli et al., 2004) with only a minority of the cases testing positive for assemblages A or B (O'Handley et al., 1999; Appelbee et al., 2003; Lalle et al., 2005; Trout et al., 2005; Coklin et al., 2007). The data obtained in the current study suggest that the zoonotic risk of *Giardia* infection in calves may be small.

In the present study, IHC of intestinal sections allowed in situ visualization of *Giardia* trophozoites in intestinal sections from scouring calves. From a diagnostic viewpoint, this technique appears to have potential for confirming a *Giardia* diagnosis in dead scouring calves testing positive for *Giardia* antigens by the fecal ELISA. Only a few studies have attempted to

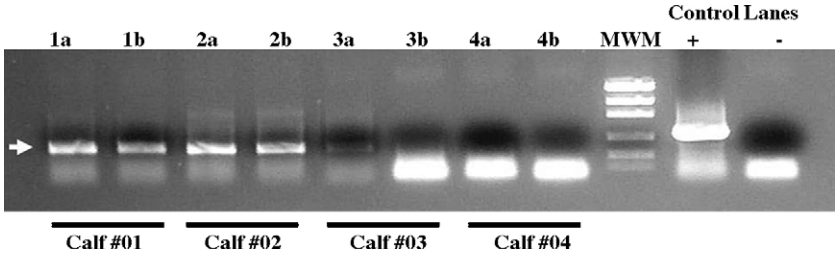


Fig. 4. Electrophoresis of PCR products in a 1% agarose gel that was stained with ethidium chloride. Duplicate samples from each calf were loaded onto the gel. Lanes 1a, 1b, 2a and 2b are positive for assemblage E *Giardia* DNA (white arrow) while lanes 3a, 3b, 4a and 4b are negative.

investigate coinfections of *Giardia* spp. with other enteric pathogens of calves (Quigley et al., 1994; Wade et al., 2000b). In view of this information gap, the likelihood of synergism between *Giardia* and other enteropathogenic agents in calves needs to be thoroughly investigated. Enhanced mucosal invasion by *Giardia* trophozoites was seen in intestinal sections from the only calf that tested positive for coronavirus and virulent *E. coli*. While this observation has no statistical significance and needs to be further substantiated, it seems reasonable to suspect that coinfections of *Giardia* with viruses, bacteria and parasites might synergistically act to produce more severe intestinal lesions in calves. Plans are underway to conduct controlled infectivity studies to clarify this supposition.

In summary, we are reporting the molecular and IHC detection of *Giardia* as the sole infectious agent in a number of scouring calves routinely screened for various enteric pathogens. All the infections in the study were associated with assemblage E, *G. duodenalis* which is ruminant specific and does not infect humans. We recommend that diagnosticians should screen for *Giardia* in dead scouring calves and assign a giardiasis diagnosis especially to those calves testing negative for other enteric pathogens. Our data strongly suggest that IHC may be a useful diagnostic tool for confirming a *Giardia* diagnosis in dead calves with history of clinical diarrhea. The attributes of treating *Giardia*-positive scouring calves with anti-protozoan agents needs to be revisited and thoroughly evaluated.

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