Article

Temporal changes in the prevalence and shedding patterns of *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts in a herd of dairy calves in Ontario

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Abstract — *Giardia duodenalis* and *Cryptosporidium* spp. infections, and the patterns of cyst and oocyst shedding, were observed in a herd of dairy calves in Ontario over a period of 3 mo. Cysts and oocysts were detected and enumerated in fecal samples using immunofluorescence microscopy; *Giardia* and *Cryptosporidium* DNA was detected using the polymerase chain reaction. The prevalence of *G. duodenalis* increased during the course of the study, reaching a peak of 93.1% when calves were 43 to 54 d old, and then decreased. Conversely, *Cryptosporidium* spp. prevalence was highest (75.9%) when calves were 11 to 22 d old, and subsequently decreased. The numbers of cysts and oocysts shed per gram of feces were positively correlated over time with the respective prevalence rates. Along with genotyping data, temporal changes in prevalence and shedding patterns should be considered when testing dairy calves for the presence and concentrations of cysts and oocysts, and when considering the potential for zoonotic transmission.

Résumé — Changements temporels dans la prévalence et les tendances d'excrétion des sporocystes de *Giardia duodenalis* et des oocystes de *Cryptosporidium* spp. dans un troupeau de veaux laitiers en Ontario. Les infections à *Giardia duodenalis* et à *Cryptosporidium* spp. et les tendances de l'excrétion des sporocystes et oocystes ont été observées dans un troupeau de veaux laitiers en Ontario pendant une période de plus de 3 mois. Les sporocystes et les oocystes ont été détectés et énumérés dans les échantillons fécaux à l'aide de l'immunofluomicroscopie; l'ADN de *Giardia* et de *Cryptosporidium* a été détectée en utilisant la réaction d'amplification en chaîne par la polymérase. La prévalence de *G. duodenalis* a augmenté pendant la durée de l'étude, atteignant un sommet de 93,1 % lorsque les veaux étaient âgés de 43 à 54 jours, et a diminué par la suite. Inversement, la prévalence de *Cryptosporidium* spp. était la plus élevée (75,9 %) lorsque les veaux étaient âgés de 11 à 22 jours, et a diminué par la suite. Au fil du temps, il y avait une corrélation positive entre le nombre de sporocystes et d'oocystes excrétés par gramme de fèces et les taux de prévalence respectifs. Les changements temporels de la prévalence et des tendances d'excrétion devraient être considérés, avec les données de génotypage, lors des tests auprès des veaux laitiers pour déceler la présence et les concentrations de sporocystes et d'oocystes et lors de la considération du potentiel de la transmission zoonotique.

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Introduction

Giardia duodenalis and *Cryptosporidium* spp. are common protozoan parasites that cause diarrhea in humans and other mammals, including livestock, cats, dogs, and wild animals worldwide (1–5). In recent years, these parasites have been the subject of numerous prevalence studies in livestock worldwide. The prevalence of both parasites has been reported to be high in cattle, particularly calves, in which 100% cumulative prevalences have been reported (6–10). In Canada, the prevalence in dairy calves ranges from 28% to 73% for *G. duodenalis,* and from 15% to 46% for *Cryptosporidium* spp. (11–14). In a study done in Quebec, 89% of the dairy farms in which calves were sampled were positive for *Cryptosporidium* spp. (15).

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Giardia duodenalis cysts have been reported in calves as young as 4 to 5 d old (6,16). The peak prevalences of giardiasis in dairy calves occur over a wide age range, from approximately 4 to 20 wk (7,17–21). A recent longitudinal study in dairy calves found that the highest prevalence of infection with *G. duodenalis* occurred in pre-weaned calves, with a peak prevalence at 4 and 5 wk of age (10). *Cryptosporidium* spp. infections occur in dairy calves as young as 2 to 6 d old (6,16,17,22,23). The majority of studies have reported *C. parvum* infections to be most prevalent in calves 2 to 4 wk old (7,9,11,22,23), although slightly earlier peaks have also been reported (17,18,24). Conversely, Hamnes et al (21) reported that *Cryptosporidium* infections peaked in older dairy calves that were ~8 to 12 wk old. Santín et al (25) reported 2 peaks in prevalence; one at 2 wk old and a second smaller peak at 6 mo. A longitudinal study of cryptosporidiosis in dairy cattle demonstrated that the highest prevalence of infection occurred in pre-weaned calves, with a significant decrease with the age of the animals (9). These authors reported peak prevalence at 2 wk old, and a second smaller peak at 18 wk.

Several studies have also reported temporal changes in the fecal counts of cysts and oocysts in dairy calves. The numbers of *Giardia* cysts shed by calves peaked at 2 wk of age, and high numbers continued to be shed until 7 wk (6). Similarly, in British Columbia, the highest cyst counts were found in dairy calves that were 2 to 4 wk old (11). Conversely, in a study done in Alberta, *Giardia* cyst counts did not reach a maximum until dairy calves were 89 d old (7). *Cryptosporidium* oocyst counts generally peak in calves at an earlier age than do *Giardia* cyst counts. Xiao and Herd (6) reported that oocyst counts peaked at 1 wk of age and then declined to low numbers by 3 wk. Similarly, Huetink et al (17) reported the highest oocyst counts in calves between 9 and 29 d old. In British Columbia, the highest counts were found in dairy calves from 0 to 2 wk old for *Cryptosporidium* (11), whereas in Alberta *Cryptosporidium* oocyst counts peaked in dairy calves between 10 and 19 d old, and subsequently declined and remained low (7).

The purpose of the present study was to determine the prevalence and shedding patterns, over time, of *Giardia duodenalis* and *Cryptosporidium* spp., in dairy calves housed at an agricultural college in Ontario, Canada, and their implications on animal-to-animal and zoonotic transmission.

Materials and methods

Source and collection of specimens

Male Holstein dairy calves used in this study originated from several farms near Kemptville, Ontario. At the source farms, calves were fed colostrum from their dams or pooled colostrum from the herd, and were removed from their dams within 12 h of birth. They were purchased in the summer of 2005, transported to Kemptville College by 3 d of age, and entered into this study (sampling day 0) at ages ranging from 11 to 22 d. All calves were housed in individual hutches and provided feed and water ad libitum.

From July to October, 2005, 29 calves were sampled 6 times each. Using an individual disposable latex glove, feces were collected directly from the rectum of each animal and transferred into a plastic cup. Cups were capped, labelled with the animal's ear tag number, and immediately placed into an insulated container packed with ice or cold packs. Specimens were subsequently refrigerated and processed within 1 to 3 d of collection. All procedures involving the calves were approved by the University of Guelph Animal Care Committee.

Cyst and oocyst concentration from feces

A sucrose flotation technique (7) was used with the following modifications. To 5 g of feces, 10 mL of phosphate-buffered saline (PBS), pH 7.4, was added and thoroughly mixed with an applicator. The suspension was passed through 4 layers of gauze (A.R. Medicom, Montreal, Quebec) and layered over 5 mL of 1 M sucrose (Sigma-Aldrich Canada, Oakville, Ontario) solution (specific gravity 1.13) into a clean polypropylene conical Falcon tube (Becton Dickinson, Oakville, Ontario). The samples were centrifuged at 800 × *g* for 5 min; the interface and the upper layer of liquid were transferred with a disposable pipette to a clean tube and centrifuged at 800 × *g* for 5 min. The supernatant was decanted, leaving a final pellet volume of 1 mL.

Immunofluorescence microscopy for *Giardia duodenalis* and *Cryptosporidium* spp.

After cyst and oocyst isolation and concentration, a 20-μL sample of the concentrate was spotted onto a microscope slide (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solutions (20 μL each of Giardi-a-Glo and Crypt-a-Glo, Waterborne, New Orleans, Louisiana, USA) were applied to the slide, which was then incubated in a humid air chamber at room temperature in the dark for 45 min. After incubation, the slide was briefly irrigated with PBS and covered with a 22 × 22 mm glass cover slip. Examinations were performed with a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics. *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts were identified and enumerated at 200× magnification. The number of cysts and oocysts per gram of feces was calculated using the following modification of the formula of O'Handley et al (7).

$$N = (s \times pv)/(vol \times wt) \quad \textit{Equation 1}$$

where: N = number of oocysts per gram feces, s = number of oocysts counted on the slide, pv = pellet volume (approximately 0.5 mL), vol = volume of sample examined (0.02 mL), and wt = weight of fecal sample (g).

DNA extraction

Total DNA was extracted from each sucrose flotation concentrated sample using the DNeasy Tissue Kit (Qiagen, Mississauga, Ontario). Concentrated sample (200 μL) was transferred to 1.5 mL microcentrifuge tubes and lysed overnight at 56°C using 180 μL of lysis buffer and 20 μL of Proteinase K (20 mg/mL) supplied with the DNeasy Tissue Kit. The remaining protocol followed manufacturer's instructions except that the nucleic acid was eluted in 100 μL of elution buffer to increase the quantity of recovered DNA.

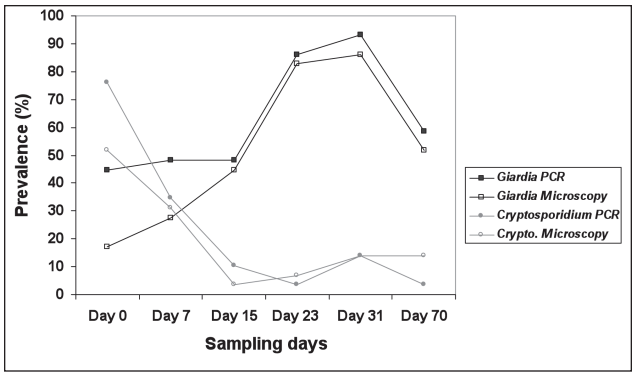

Figure 1. The prevalence of calves shedding *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts in relation to sampling day.

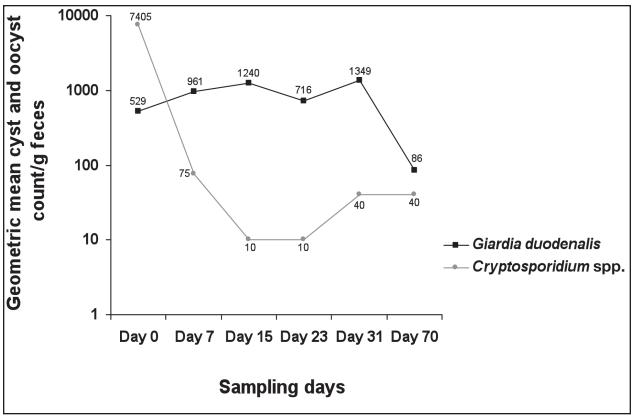

Figure 2. The geometric mean numbers of *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts shed by dairy calves over 6 sampling days at Kemptville College, Ontario.

Amplification of *Giardia* 16S rRNA gene fragments by PCR

Fragments of the 16S rRNA gene were amplified using modifications of previously described polymerase chain reaction (PCR) protocols. A nested-PCR protocol was used with first round primers Gia2029 (5′-AAGTGTGGTGCAGACGGACTC-3′) and Gia2150c (5′-CTGCTGCCGTCCTTGGATGT-3′) amplifying a 497-bp product (26); and secondary primers RH11 (5′-CATCCGGTCGATCCTGCC-3′) and RH4 (5′-AGTCGAACCCTGATTCTCCGCCAGG-3′) generating a 292-bp fragment (27). Polymerase chain reactions consisted of 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 5% (v/v) dimethyl sulfoxide, 2 U *Taq* polymerase, and 0.5 μM of each forward and reverse primer in a reaction volume of 50 μL. Following an initial hot start at 96°C for 2 min, 35 cycles, each consisting of 96°C for 45 s, 58°C for 30 s, and 72°C for 45 s were performed. A final extension step at 72°C for 4 min was included. For the second round of PCR, the PCR mixtures, the number of cycles, temperatures and times were identical except that the annealing temperature was decreased to 55°C for 30 s. Products from PCR were analyzed on 1% (w/v) agarose gels and visualized by ethidium bromide (Bio-Rad Laboratories, Mississauga, Ontario) staining. Sequencing of DNA was done to confirm PCR positives and to determine the genotypes of *Giardia duodenalis* present. The methods and results were previously reported (14).

Amplification of *Cryptosporidium* 18S rRNA gene fragments by PCR

To amplify the 18S rRNA gene fragments a nested PCR protocol was followed, using the following primers previously described by Xiao et al (28): 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCTAATCCTTCGAAACAGGA-3′ for primary PCR, and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ for nested PCR, to generate an 830 bp fragment. Amplification of PCR was performed in 50 μL volumes as per Santín et al (25) with a slight modification. In brief, 5 μL DNA was added to 1× PCR buffer, 4.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U *Taq,* 2.5 μL fetal BSA (0.1g/10 mL), and 1 μM of each forward and reverse primer. Samples were subjected to 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min, with an initial hot start at 94°C for 3 min, and a final extension step at 72°C for 7 min. The nested PCR mixture was identical, except that 1 μL DNA and a final concentration of 3 mM $MgCl_2$ were used. A total of 40 cycles, each consisting of 94°C for 30 s, 58°C for 90 s, and 72°C for 2 min, were performed with an initial hot start at 94°C for 3 min, and a final extension step at 72°C for 7 min. Products from PCR were analyzed on 1% (w/v) agarose gels and visualized by ethidium bromide [Bio-Rad Laboratories (Canada), Mississauga, Ontario] staining. Sequencing of DNA was done to confirm PCR positives and to determine the species of *Cryptosporidium.* The methods and results were previously reported (14).

Data analysis

Statistical analyses of the data were performed using the statistical software package Splus6.2 (Insightful Corporation, Seattle, Washington, USA) and MS Excel Spread Sheet for Windows (Office 2000 version, for *t*-test). For data analysis of infection over time, the method of estimation for the cumulative probability function applying Weibull distribution was used. For persistence of infection, a chi-squared test for equality of proportions with 4 degrees of freedom was used.

Results

All 29 calves (100%) shed *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts during the study period. The youngest calf on sampling day 0 was 11 d old, and was negative for both *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts. However, 4 other calves (13.8%), including a 12-day-old animal, shed both *G. duodenalis* and *Cryptosporidium* spp. on sampling day 0.

On sampling day 0, 13 of the 29 calves (44.8%) were positive for *G. duodenalis* based on PCR of a fragment of the 16S rRNA gene, and 5 (17.2%) were positive by microscopy (Figure 1). The percentage of calves shedding *G. duodenalis* rose steadily with time and reached a peak of 93.1%, by PCR, on sampling day 31 when calves were 43 to 54 d old (Figure 1). By sampling day 31, all 29 calves had shed *G. duodenalis* cysts on at least 1 occasion. Thereafter, the prevalence of *G. duodenalis* cysts in

the calves began to decrease, but they were still shedding cysts at the end of the study (sampling day 70).

The number of calves shedding *Cryptosporidium* spp. on sampling day 0 was 22 out of 29 (75.8%) based on PCR of a fragment of the 18S rRNA gene, whereas 15 out of 29 (51.7%) calf samples were positive by microscopy (Figure 1). The prevalence of *Cryptosporidium* spp. was highest on sampling day 0, when the calves were 11 to 22 d of age, and then declined during sampling days 15 to 23. After a small increase on day 31, a drop in the number of positive calves occurred again on sampling day 70, when calves were 81 to 92 d old (Figure 1). Statistical analysis gave a chi-squared value of 22.3 on 5 degrees of freedom, with a *P*-value of 0.0005, demonstrating a significant variation in the prevalence of cysts and oocysts with respect to sampling days.

The results of the cyst and oocyst enumerations indicated that, individually, animals shed from 0 to 19,920 *G. duodenalis* cysts, and from 0 to 75 000 *Cryptosporidium* spp. oocysts, per gram of feces during the course of this study (results not shown). The geometric mean cyst and oocyst counts over time are shown in Figure 2. On sampling day 0, only 2 (6.9%) calves shed $>$ 1000 *G. duodenalis* cysts/g of feces, whereas 14 calves (48.3%) shed $>$ 1000 *Cryptosporidium* spp. oocysts/g, with 3 shedding $>$ 50 000 oocysts/g. During sampling days 7 to 31, the geometric mean number of *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts shed/g of feces increased and decreased, respectively (Figure 2), positively correlating with the respective prevalence rates. Statistical analysis based on log counts using ANOVA gave an F-value of 4.65 on 5 and 56 degrees of freedom, with a *P*-value of 0.0013, demonstrating a significant variation in the number of cysts and oocysts/g of feces with respect to sampling days.

With respect to repeated measurements of the presence and concentration of *G. duodenalis* and *Cryptosporidium* spp. in the fecal samples, statistical methods describing the onset of infection (Weibull distribution) and persistence of infection (Pearson chi-squared test) were used. The Weibull distribution estimated that, on or before 40 d of age, an average of 90% of calves will start shedding *G. duodenalis* cysts. As sampling times were at fixed intervals, no data on the presence of *G. duodenalis* cysts or *Cryptosporidium* spp. oocysts in calves were available between collection days, and the estimation procedure took this into account.

Discussion

The present study represents the first temporal study on the prevalence and concentrations of *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts in dairy calves in eastern Canada, and showed clear trends over time which may be useful in describing the infection dynamics of these pathogens, as well as having implications regarding zoonotic transmission. Both *G. duodenalis* and *Cryptosporidium* spp. were detected in each of the 29 calves at some time during the study. This finding is in agreement with earlier studies reporting a 100% cumulative prevalence of these parasites in calves (6–10).

The percentage of the calves shedding *G. duodenalis* cysts increased over the course of the study with the highest prevalence occurring among calves 43 to 54 d old. Weibull distribution estimated that most calves would be shedding *G. duodenalis* cysts on or before day 40 of the study. Thereafter, the prevalence of *G. duodenalis* infections decreased, but approximately half of the calves were still shedding cysts when the animals were 81 to 92 d old. These findings are similar to previously published studies reporting the highest prevalence of *G. duodenalis* infections in dairy calves approximately 4 to 12 wk old (7,10,18,19,21). Other studies have reported the highest prevalence of *G. duodenalis* infection in older dairy calves approximately 12 to 20 wk old (17,20). However, in a study in British Columbia, giardiasis was not found to be age dependent as approximately 80% of dairy calves from 2 to 24 wk old were infected (11). These temporal differences in peak prevalence of giardiasis among different studies may be associated with a number of variables including the breed of calves, husbandry practices, and/or the *G. duodenalis* genotypes involved.

The prevalence patterns over time seen in the present study for *Cryptosporidium* spp. infections in calves differed from that for *G. duodenalis.* The highest *Cryptosporidium* spp. prevalence occurred when the age of the animals ranged from 11 to 22 d. However, since the youngest calf in the study was 11 d old it is possible that the peak shedding period for *Cryptosporidium* spp. may have been missed in a number of calves. This finding is in agreement with earlier studies (9,11,17,22,23). Subsequently, the prevalence of *Cryptosporidium* spp. declined and remained low for the duration of the study. A similar decline in prevalence with age has been described in previous studies (18,21,22,24). These results support the conclusion by O'Handley et al (7) that giardiasis may be chronic in dairy calves, whereas cryptosporidiosis may be self-limiting.

Polymerase chain reaction analysis demonstrated a higher prevalence of infection with both *G. duodenalis* and *Cryptosporidium* spp. than with immunofluorescence microscopy, but these differences were not statistically significant on any of the sampling days (*t*-test, $P > 0.05$). Similarly, PCR was reported to be more sensitive than immunofluorescence microscopy in the detection of *Giardia* and *Cryptosporidium* in dairy calves by Santín et al (9,10).

The geometric mean cyst and oocyst counts in the present study were similar to those reported in other Canadian studies (7,11,12). Furthermore, the concentration of cysts and oocysts in the feces over time was positively correlated with the prevalence patterns for both parasites. Other studies have also reported a parallel between the prevalence of infection and the intensity of cyst and oocyst excretion in dairy calves (6,22,23). O'Handley et al (7) reported that the mean number of *Giardia* cysts in calf feces remained high throughout their study, whereas the number of *Cryptosporidium* oocysts decreased to low numbers 2 wk after infection.

Becher et al (18) concluded that calf-to-calf contact was the most likely source of transmission of *Giardia* and *Cryptosporidium* among dairy calves. In the present study, however, these pathogens were highly prevalent although the calves were housed in individual hutches, and did not have direct contact with other animals. Huetink et al (17) concluded that indirect calf-to-calf transmission by means of vectors is an important route for

acquiring infection, at least with *Cryptosporidium.* Mechanical vectors may include workers or students who frequently handle one calf after another. Other vectors involved in the transmission of *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts can include wild mice (29), or, in the summer, houseflies (30), which may contaminate the calves' feed or water. A further possibility is that cysts and oocysts could have been spread from one hutch to another during run-off following heavy rainfalls in the summer. Hutches may have also been contaminated with cysts and oocysts from previous occupants. The hutch area of Kemptville College has been used for many years, and is often populated with different groups of calves during the spring and summer. In the present study, up to approximately 20 000 *G. duodenalis* cysts and up to 75 000 *Cryptosporidium* spp. oocysts/g feces were shed by the calves. Both *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts are resistant to adverse environmental conditions, such as temperature extremes, and are capable of remaining infectious for relatively long periods after being passed in feces (1,31). Another important source of infection in calves is cow-to-calf transmission which can occur at birth (17). This source of infection, however, cannot account for the delayed shedding of *Cryptosporidium* oocysts seen in some calves, nor the subsequent increase in *Giardia* prevalence.

In addition to the high prevalence of both *G. duodenalis* and *Cryptosporidium* spp. in dairy calves in the present study, DNA sequence analyses demonstrated the presence of the zoonotic genotype, *G. duodenalis* Assemblage B, as well as a predominance of the zoonotic species, *C. parvum.* We reported these genotyping results in a previous paper (14). A predominance of *C. parvum* has also been reported in dairy calves in Ontario by Trotz-Williams et al (13,32).

Further studies are warranted to evaluate the public health significance of these findings. Results of this study indicate that infection patterns differ between *G. duodenalis* and *Cryptosporidium* spp. and that prevalence rates and intensity of shedding for both parasites depend on the age of the animals. Along with genotyping data, temporal changes in prevalence and shedding patterns should be taken into account when testing dairy calves for the presence and concentrations of cysts and oocysts, and when considering the potential for zoonotic transmission.

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