Article

Evaluation of the zoonotic potential of *Giardia duodenalis* in fecal samples from dogs and cats in Ontario

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Abstract — This study determined the distribution and zoonotic potential of *Giardia duodenalis* assemblage types among canine and feline fecal samples from Ontario. The effectiveness of *Giardia* assemblage typing methods by sequencing the genes of small subunit ribosomal RNA *(ssu-rRNA)*, β-giardin *(bg)*, glutamate dehydrogenase *(gdh)*, and triose phosphate isomerase *(tpi)* was evaluated simultaneously. From 2008 to 2010, 118 canine and 15 feline *Giardia* positive fecal samples were tested. The *ssu-rRNA* sequencing method typed 64% (75/118) and 87% (13/15) of the *Giardia*-positive canine and feline samples, respectively. Among the typeable samples, 68% (51/75) of canine samples contained *G. duodenalis* assemblage D and 31% (23/75) contained *G. duodenalis* assemblage C (both non-zoonotic assemblage types). Only 1% (1/75) of the typeable canine samples contained a potentially zoonotic assemblage B. In contrast, 100% (13/13) of the typeable feline samples contained potentially zoonotic assemblages A (n = 12) or B (n = 1).

Résumé — Évaluation du potentiel zoonotique de *Giardia duodenalis* dans les échantillons de fèces provenant de chiens et de chats de l'Ontario. Cette étude a déterminé la distribution et le potentiel zoonotique de types de regroupements de *Giardia duodenalis* parmi des échantillons de fèces canins et félins provenant de l'Ontario. L'efficacité des méthodes de typage des regroupements par le séquençage des gènes de petites sous-unités d'ARN ribosomales *(ssu-rRNA)*, de β-giardin *(bg)*, de glutamate déshydrogénase *(gdh)* et de triose-phosphate-isomérase *(tpi)* a été évaluée simultanément. De 2008 à 2010, 118 et 15 échantillons de fèces canins et félins positifs pour *Giardia* ont été testés. La méthode de séquençage *ssu-rRNA* a typé 64 % (75/118) et 87 % (13/15) des échantillons canins et félins positifs pour *Giardia* respectivement. Parmi les échantillons pouvant être typés, 68 % (51/75) des échantillons canins contenaient le regroupement D de *G. duodenalis* et 31 % (23/75) contenaient le regroupement C de *G. duodenalis* (tous deux des types de regroupement non zoonotiques). Seulement 1 % (1/75) des échantillons canins pouvant être typés contenaient un regroupement B potentiellement zoonotique. Par contraste, 100 % (13/13) des échantillons félins pouvant être typés contenaient des regroupements A (n = 12) ou B (n = 1) potentiellement zoonotiques.

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Introduction

Giardia duodenalis (also known as *G. intestinalis* and *G. lamblia*) is an intestinal protozoan parasite that infects a wide range of hosts, including humans, dogs, cats, and cattle. Transmission usually occurs through direct fecal-oral routes or through food or water contaminated with *G. duodenalis* cysts (1). A previous study reported that the prevalence of *G. duodenalis* infections in dogs in Ontario was 7.8% (2). This study also noted that in 78% of the cases where *Giardia* was identified, the dog showed no clinical signs.

Over the past few years the understanding of the epidemiology of *Giardia* has changed as 7 major assemblages of *G. duodenalis* (A to G) have been described (3). Assemblages A and B can infect a wide range of hosts, including humans and other mammals, and are therefore considered to be potentially zoonotic (4). In contrast, assemblages C to G are host-specific

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with minimal zoonotic potential. Assemblages C and D typically infect dogs, wolves, and coyotes but can also infect cats (4). Assemblage E infects mostly hoofed animals, such as cattle, sheep, and goats. Assemblage F is typically only found in cats, and assemblage G mainly infects rats (4).

In Canada, the assemblage types of *G. duodenalis* have been reported for bovine fecal samples in Ontario (1,5) and Alberta (6). Recently, a study reported the *G. duodenalis* assemblage types in canine fecal samples in Saskatchewan (7). However, there have been no reports on the zoonotic potential of *G. duodenalis* found in Ontario pets.

Previous studies have shown that the use of multiple polymerase chain reaction (PCR)-sequencing methods may detect mixed assemblage types attributed to mixed infections, where a single PCR sequencing method may not (8,9). The purpose of this study, therefore, was to employ several different PCR-sequencing methods to identify the prevalence of *G. duodenalis* assemblage types among canine and feline fecal samples in Ontario, by which to determine the zoonotic potential of these infections. Since multiple PCR-sequencing methods were used, the effectiveness of each method or combination of methods for typing *G. duodenalis* was also evaluated.

Materials and methods

Sample collection

Fecal samples used for this study were those submitted by veterinary clinics in Ontario to the Animal Health Laboratory (University of Guelph) from 2008 to 2010 for testing for *G. duodenalis* either by an antigen-enzyme-linked immunosorbent assay (ELISA) (ProSpecT; Remel, Lenexa, Kansas, USA), or by standard sucrose wet mount for cysts. All fecal samples (118 canine and 15 feline) that tested positive with either method were subjected to assemblage typing by PCR sequencing methods.

DNA extraction

Sample DNA was extracted from fecal samples using the Qiagen QIAmp DNA stool mini kit (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions with the following modifications: 200 mg of feces were mixed with 1.4 mL of lysis buffer (buffer ASL) until homogeneous, then incubated with shaking at 95°C for 5 min. The final elution volume was 100 μL.

Molecular typing

Previously described PCR methods specific for partial regions of 4 genes, including the genes of small subunit ribosomal RNA *(ssu-rRNA)*, β-giardin *(bg)*, glutamate dehydrogenase *(gdh)*, and triose phosphate isomerase *(tpi)*, were employed to amplify the target DNA extracted from fecal samples for sequence analysis. The PCR methods were modified to achieve optimal amplification.

The *ssu-rRNA* PCR was performed using a nested PCR targeting a 180 base pair (bp) region of the *ssu-rRNA* gene using the primers and amplification conditions described previously (10).

A 750 bp region of the *bg* gene was amplified using a previously published PCR method (11) with modified cycling conditions as follows: 1 cycle of 95°C for 5 min, 50 cycles of 94°C for 30 s, 66°C for 90 s, 72°C for 90 s, and 1 extension cycle of 72°C for 10 min.

A previously published PCR method was employed to amplify a 432 bp region of the *gdh* gene (11). The amplification conditions were modified as follows: 95°C for 5 min, 50 cycles of 94°C for 30 s, 54°C for 45 s, 72°C for 45 s, and a final extension of 72°C for 10 min.

A 450 bp region of the *tpi* gene was first amplified using a previously described PCR assay (12) with modified amplification conditions: 95°C for 5 min, 35 amplification cycles of 94°C for 45 s, 59°C for 45 s, 72°C for 60 s, and a final extension of 72°C for 10 min. If amplification was not successful, an alternative nested PCR targeting a 530 bp region of the *tpi* gene was used (13). For both the primary and secondary PCR, the amplification conditions were modified as follows: 95°C for 5 min, 35 amplification cycles of 94°C for 45 s, 59°C for 45 s, 72°C for 60 s, then 72°C for 10 min.

Both undiluted and a 1:10 dilution of the extracted DNA were tested with each of the 4 PCR assays to evaluate and reduce the influence of inhibitors.

All PCR products were analyzed by electrophoresis using 2% precast agarose gels (Invitrogen, Carlsbad, California, USA). If specific amplification occurred, the PCR products were purified with the EZNA Cycle Q spin purification kit (Omega Biotek, Norcross, Georgia, USA). Occasionally, nonspecific bands were seen along with the specific PCR products. In these cases, samples were electrophoresed again on 1.5% agarose (Invitrogen) gels and the amplicons of correct size were excised, and then purified using the Qiaquick gel extraction kit (Qiagen). Purified PCR products were submitted to a molecular biology service facility (Laboratory Services, University of Guelph, Guelph, Ontario) for sequencing in 2 directions using the PCR-specific forward and reverse primers.

Assemblage identification

The DNA sequences were assembled using Vector NTI software (Invitrogen), and then compared to other sequences published in GenBank using Blast software (National Centre for Biotechnology Information, NCBI). Identification of *G. duodenalis* assemblage was defined as $>$ 99% similarity of the sequence to the closest assemblage sequence in the GenBank database. If different assemblages were identified by different PCR sequence typing methods, or if the sequence was heterogeneous and contained overlapping nucleotides at assemblage-specific positions, the sample was considered to contain mixed assemblage types. To confirm the Blast search results, a database of published sequences of each assemblage type for each gene was constructed and each assemblage type was aligned with all sample sequences using the AlignX software (Vector NTI, Invitrogen).

Results

Table 1 shows the *G. duodenalis* assemblage types identified by PCR sequencing of each gene in canine and feline fecal samples collected in Ontario. The *ssu-rRNA* PCR sequencing method was able to genotype 64% (75/118) and 87% (13/15) of the *Giardia*-positive canine and feline samples, respectively.

Table 1. Assemblage types of *G. duodenalis* determined by PCR sequencing of 4 genes in canine and feline fecal samples collected between 2008 and 2010 in Ontario

Sample source	Gene sequenced	Samples tested	Samples typeable	*G. duodenalis* assemblage type						
				A	B	C	D	E	F	G
Dog	*ssu-rRNA*	118	75	—	1	23	51	—	—	—
	bg	118	37	—	—	3	33	—	1	—
	gdh	118	20	—	—	4	16	—	—	—
	tpi	118	24	—	—	4	20	—	—	—
Cat	*ssu-rRNA*	15	13	12	1	—	—	—	—	—
	bg	15	4	—	—	—	—	—	4	—
	gdh	15	2	—	—	—	—	—	2	—
	tpi	15	0	—	—	—	—	—	—	—

— = none were identified.

Among the typeable canine samples, assemblage D was the most prevalent, comprising 68% (51/75) of samples, followed by assemblage C, another canine-specific assemblage type (31%, 23/75). Only 1% (1/75) of the typeable canine samples contained assemblage B, a potentially zoonotic assemblage type. Assemblage A was not detected in any sample. Among feline samples, 100% (13/13) of the typeable samples, using the *ssu-rRNA* PCR sequencing method, were found to contain potentially zoonotic assemblage types of *G. duodenalis,* with 92% (12/13) and 8% (1/13) of the typeable samples being assemblage type A and B, respectively.

The *bg* PCR sequencing method was able to type 31% (37/118) of the canine samples; 89% (33/37) of the typeable samples were identified as assemblage D, whereas only 8% (3/37) were identified as assemblage C (Table 1). Interestingly, 1 canine sample (3%, 1/37) was identified as being the feline-specific assemblage F using this genotyping method. Among the feline samples, only 27% (4/15) were successfully typed with the *bg* PCR sequencing method, and all of these samples were found to contain the feline-specific assemblage F (Table 1).

Only 17% (20/118) of the *Giardia*-positive canine fecal samples were typeable by the *gdh* PCR sequencing method, and 80% (16/20) of these consisted of assemblage D (Table 1). Similarly, 13% (2/15) of the *Giardia*-positive feline fecal samples were typeable by this method, and both typeable samples contained assemblage F (Table 1).

The *tpi* PCR sequencing method was able to type 20% (24/118) of the *Giardia*-positive canine samples, of which 83% (20/24) and 17% (4/24) contained the canine-specific assemblages D or C, respectively (Table 1). This assemblage typing method was not able to type any of the *Giardia*-positive feline fecal samples.

Among the 118 canine fecal samples with the *ss-rRNA, bg, gdh,* and *tpi* PCR sequencing methods, only 12 samples were positive with all 4 PCR sequencing methods; 15 and 11 samples were positive with 3 and 2 PCR sequencing methods, respectively. Forty-two samples were positive with only 1 PCR sequencing method, whereas 38 of these samples were negative with all 4 methods.

Among the 15 feline fecal samples tested with the *ss-rRNA, bg, gdh,* and *tpi* PCR sequencing methods, no samples were positive with all 4 PCR sequencing methods, and only 2 samples were positive with 3 of the methods. One sample was positive with 2 of the 4 PCR sequencing methods, and 2 samples were negative with all 4 sequencing methods. Most (10/15) of these samples were positive with only 1 PCR sequencing method.

Table 2. Samples with non-congruent results by different typing methods

Sample source	Sample ID	*G. duodenalis* assemblage types identified by different gene sequences			
		ssu-rRNA	*bg*	*gdh*	*tpi*
Dog	2008–01	C & D[a]	D	—	C
	2009–01	C	C & D[a]	C & D[a]	D
	2009–02	C	C & D[a]	C	D
	2009–03	C	C & D[a]	C	C & D[a]
	2009–04	C & D[a]	F	—	D
	2010–01	C & D[a]	D	—	D
	2010–02	C & D[a]	C & D[a]	—	C & D[a]
Cat	2010–03	A	F	—	—
	2009–05	A & F[a]	F	—	—
	2009–06	A & F[a]	F	F	—
	2009–07	A & F[a]	F	—	—

— = none detected.
[a] Identified by the presence of heterogeneous sequences.

Among all 88 typeable canine and feline samples, 12.5% (11/88) had non-congruent assemblage types among the different PCR sequence typing methods and were therefore considered to contain mixed assemblage types (Table 2). In addition, some samples contained heterogeneous sequences with overlapping nucleotides at assemblage-specific positions. These were also considered to contain mixed assemblage types (Table 2). Most of the canine samples (6/7) that were identified as having mixed assemblage types were a C and D mixture. One canine sample contained a mixture of assemblages C, D and F (Table 2). All mixed assemblage types in feline samples ($n = 4$) were an A and F mixture (Table 2). The *ssu-rRNA* and the *bg* PCR sequencing methods together identified all mixed assemblage types in all of the samples (Table 2). The *gdh* and *tpi* PCR sequencing methods were able to type fewer samples and did not identify any new assemblage types that had not been identified by the *ssu-rRNA* and the *bg* PCR sequencing methods. Therefore, *gdh* and *tpi* PCR sequencing methods were of little value in this study.

Discussion

The distribution of *G. duodenalis* assemblages in different animal species and humans has been studied extensively (1,6–10,12–16).

To date, there has been no report describing the assemblage types of *G. duodenalis* from canine and feline fecal samples in Ontario. Results from this study indicate that the 2 most prevalent assemblage types among canine samples in Ontario are the canine-specific assemblages C and D, which are non-zoonotic genotypes. Only 1 of the 75 typeable canine fecal samples contained a potentially zoonotic *G. duodenalis* assemblage (type B). This finding is similar to those in studies from Europe, which concluded that assemblages C and D were the most prevalent among canine samples that were tested (9,12,14). Among 28 canine samples that were genotyped in Sweden, 96% were typed as either assemblage C or D, or a C and D mixture (12). Among 600 *G. duodenalis* isolates from canine samples collected from public and veterinary health institutions across Europe, 68% were either assemblage C or D, 23% were assemblage A and 9% were assemblage B (9).

To date, few studies on the assemblage type of *G. duodenalis* in canine samples have been conducted in the Americas. One study, which tested 15 dogs in Georgia, USA found that 100% carried *G. duodenalis* assemblage D (13). A study from Brazil found that *G. duodenalis* assemblages C or D were present in 100% of the canine samples tested (16). In contrast to these reports, some studies have found that the most prevalent assemblage type in canine samples was assemblage A. However, in general, this appears to be associated with areas of low socioeconomic status. Himsworth et al (7) showed that, in a remote indigenous area of Saskatchewan, Canada, over half of the canine fecal samples tested contained *G. duodenalis,* all of which (*n* = 13) were assemblage A. The authors found that the dogs had free-range access throughout the community and therefore might have frequented a landfill where they could have come in contact with human or animal feces (7); it has been suggested that dogs carrying *G. duodenalis* zoonotic assemblage types A or B typically become infected in environments that are contaminated with feces of humans or other animals (17). Claerebout et al (15) studied the assemblage type of *G. duodenalis* isolated from various populations of dogs in Belgium. They found that 80% of healthy *Giardia*-positive household dogs carried assemblage A, whereas 94% of *Giardia*-positive dogs reared in kennels and 80% of *Giardia*-positive dogs with clinical signs indicative of giardiosis were infected with assemblages C or D. The authors suggested that canine assemblages C and D may be more commonly isolated from dogs reared in kennels because large numbers of dogs are in such close contact, and the canine-specific assemblages may outcompete other assemblage types.

In contrast to canine fecal samples, feline fecal samples more frequently contain the potentially zoonotic assemblage types of *G. duodenalis*. The study described here showed that all of the typeable feline fecal samples (*n* = 13) contained *G. duodenalis* assemblage types A, B, or mixed types A and F. A previous study conducted in Italy found that 100% of the *G. duodenalis* isolated from feline fecal samples (*n* = 10) were assemblage A (18). Other studies from Europe, Brazil, and Mississippi found that 27% to 42% of the positive feline fecal samples contained *G. duodenalis* assemblage A; 57% to 67% contained assemblage F (9,16,19). However, these studies show that a much lower percentage (2%) of *G. duodenalis* isolated from feline fecal samples were typed assemblage B (16).

Previous studies showed that assemblage A contained considerable variation in *bg* gene sequences. As a result, assemblage A has been divided into 4 subtypes with most assemblage AII and some AI being found in humans, and some AII, most AI, and all AIII and AIV typically being found in animals (4,17). In order to define the true zoonotic potential of the feline isolates typed in this study as assemblage A, more research is needed to determine the subtypes of *G. duodenalis* assemblage A present in the samples. Previous studies have also suggested that the *ssu-rRNA* loci may not be able to discriminate among the assemblage A subtypes (17). Therefore, it may be necessary to use another typing method to analyze the subtype if *G. duodenalis* assemblage A is identified by the *ssu-rRNA* PCR sequencing method.

Other researchers have concluded that genotyping *G. duodenalis* is problematic (10,12). In the work described here, only 67% of the canine *Giardia*-positive samples, identified by antigen-ELISA or microscopic examination of cysts, were typeable. In work carried out in Germany, Leonhard et al (10) found that 92% of *G. duodenalis* isolates (*n* = 55) were typeable, while a study from Sweden demonstrated that 87% of isolates were typeable (12). Different DNA extraction methods have been investigated for their ability to remove PCR inhibitors and to lyse *Giardia* cysts more effectively; however, these methods did not significantly improve the sensitivity of PCR methods (20,21). More work is still required in this area so that robust, highly sensitive, PCR methods become available for use in commercial diagnostic laboratories.

This study showed that the *ssu-rRNA* PCR sequencing method in conjunction with the *bg* PCR sequencing method was able to type the highest number of samples, and to identify all mixed infections, while the *gdh* and *tpi* PCR sequencing methods had little value. Similarly, Leonhard et al (10) concluded that 92% of positive samples were typeable with the *ssu-rRNA* PCR sequencing method, but only 43% were typeable using the *gdh* PCR sequencing method. Likewise, Lebbad et al (12) concluded that while 78% of isolates tested were typeable with a combination of *bg, gdh,* and *tpi* PCR sequencing methods, only 12% were typeable by sequencing 1 or 2 of these genes. The *ssu-rRNA* is a highly conserved multi-copy gene, which may explain why *ssu-rRNA* PCR sequencing is more successful than other sequencing methods (4). The *bg, gdh,* and *tpi* are single-copy genes, and their sequences may be more variable, causing mismatches at primer binding sites, and resulting in unsuccessful amplification (4).

In the work described here, a total of 11 samples were found to contain different assemblage types by different PCR sequencing methods. Non-congruent results generated by analysis of different genes have been described previously by Read et al (8). In their study, 25% of the canine and feline samples tested were typed as different assemblages when the results from the *ssu-rRNA* and *gdh* loci were compared (8). Similarly, a study on 1440 combined cat, dog, cattle, goat, sheep, pig, and wildlife samples found that 13% of the *Giardia* isolates had non-congruent assemblage assignments when the same 4 genes as used in this study were examined (9). It has previously been

suggested that differences in assemblage identification could be attributed to mixed assemblage types, and that genotyping methods based on different genes may have a propensity to detect 1 assemblage type over another in mixed infections (8,9). Mixed assemblage types based on heterogeneous sequences with overlapping nucleotides at assemblage-specific positions has also been described (12,22). Sprong et al (9) explain that allelic sequence heterozygosity may also be responsible for the presence of overlapping nucleotides. Although *G. duodenalis* displays a very low level of allelic sequence heterozygosity (9), more analysis is needed to exclude the possibility that allelic sequence heterozygosity existed in samples with mixed assemblage types identified in this study.

Results from this and previous studies show that potentially zoonotic assemblages may be assigned with one PCR sequencing method while a "species-specific" assemblage type may be assigned by another PCR sequencing method (9). A multi-gene assemblage typing approach such as the one described in this study can be used to determine if a sample contains mixed *G. duodenalis* assemblage types, including the potentially zoonotic assemblages. However, assemblage A should be further subtyped in order to identify the true zoonotic potential. CVJ

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