Executive summary

The overall objective of this study was to determine the distribution of *Giardia duodenalis* assemblage types among canine and feline fecal samples from Ontario and therefore their zoonotic potential. The specific objectives were:

1. To determine the prevalence of *G. duodenalis* infection in Ontario cats.
2. To determine the assemblage types and assemblage A subtypes of *G. duodenalis* identified from Ontario cat samples so as to determine the zoonotic potential.
3. Optimizing and evaluate assemblage A subtyping methods.

In this project, we evaluated 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*). 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, i.e. 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).

Project final report

Please see appendixes 1 at the end of this report.

Personnel: N/A

Test development and validation

MOL-224 *Giardia duodenalis* genotyping

Equipment purchases: N/A

Surveillance activities: From 2008 to 2010, 118 canine and 15 feline *Giardia* positive fecal samples were tested. 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).

**Emergency preparedness activities:** NA

**Final budget reconciliation** – actual $19,800 vs. proposed $19,800.

**Communications** - Publications and/or presentations that resulted from this project.

**Peer review paper published:**


**Results were presented at scientific conferences:**

1. Animal Health Forum, November 24, 2010, Guelph, Ontario (poster presentation)
2. CAHLN Annual Meeting, June 5-8, 2011, Guelph, Ontario (oral and poster presentation)
3. CPHAZ Symposium, Jun 9, 2011, Guelph (invited speaker and poster)

**Appendix 1**

**Evaluation of the Zoonotic Potential of *Giardia duodenalis* in Fecal Samples from Dogs and Cats in Ontario**

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Abstract:
The purpose of this study was to determine the distribution of *Giardia duodenalis* assemblage types among canine and feline fecal samples from Ontario and therefore their zoonotic potential. Simultaneously, we evaluated 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). 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, i.e. 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).

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

Over the last few years the understanding of the epidemiology of *Giardia* has changed as seven 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 many other mammals, and are therefore considered to be potentially zoonotic (4). In contrast, assemblages C to G are host-specific 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 (12, 13). Therefore, the purpose of this study 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, and therefore 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 to the Animal Health Laboratory, University of Guelph (Guelph, ON, Canada) from veterinary clinics in Ontario between 2008 to 2010 for testing for *G. duodenalis* either by an antigen-ELISA (ProSpecT, Remel, KS), 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:**

DNA was extracted from fecal samples using the Qiagen QIAmp DNA stool mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions with the following modifications: 200 mg of feces were mixed with 1.4 mL of lyse 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 four different 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 *ssu-rRNA* gene using the primers and amplification conditions described previously (8).

A 750 bp region of the *bg* gene was amplified by a previously published PCR method (9) with modified cycling conditions as follows: 1 cycle of 95°C for 5 min, 50 cycles of 94°C for 30 sec, 66°C for 90 sec, 72°C for 90 sec, and one 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 (9). The amplification conditions were modified as follows: 95°C for 5 min, 50 cycles of 94°C for 30 sec, 54°C for 45 sec, 72°C for 45 sec, and a final extension of 72°C for 10 min.

A 450 bp region of the *tpi* gene was firstly amplified by a previously described PCR assay (10) with modified amplification conditions: 95°C for 5 min, 35 amplification cycles of 94°C for 45 sec, 59°C for 45 sec, 72°C for 60 sec, 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 (11). 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 sec, 59°C for 45 sec, 72°C for 60 sec, then 72°C for 10 min.

Both undiluted and a 1:10 dilution of the extracted DNA were tested with each of the four PCR to evaluate and reduce the influence of inhibitors.
All PCR products were analyzed by electrophoresis using 2% precast agarose gels (Invitrogen, Carlsbad CA). If specific amplification occurred, the PCR products were then purified with the EZNA Cycle Q spin purification kit (Omega Biotek, Norcross GA). Occasionally, nonspecific bands were seen along with the specific PCR products. In this case, samples were electrophoresed again on 1.5% agarose (Invitrogen) gels and the amplicons of correct size were excised, 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, ON, Canada) for sequencing in two directions using the PCR-specific forward and reverse primers.

Assemblage Identification:

The DNA sequences were assembled using Vector NTI software (Invitrogen), 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 genes 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. Among the typeable canine samples, assemblage D was the most prevalent assemblage type, 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, one 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 four PCR sequencing methods, 15 and 11 samples were positive with three and two PCR sequencing methods respectively. Forty-two samples were positive with only one PCR sequencing method, whereas 38 of these samples were negative with all four 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 four PCR sequencing methods, and only two samples were positive with three of the methods. One sample was positive with two of the four PCR sequencing methods, and two samples were negative with all four sequencing methods. The majority (10/15) of these samples were positive with only one PCR sequencing method.

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 mix. One canine sample contained a mix of assemblages C, D and F (Table 2). All mixed assemblage types in feline samples (n=4) were an A and F mix (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 much less 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, 6-8, 10-16). 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 two most prevalent assemblage types among canine samples in Ontario are the canine-specific assemblages C and D, i.e. non-zoonotic genotypes and 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 (10, 13, 14). Among 28 canine samples that were genotyped in Sweden, 96% were typed as either assemblage C or D, or a C and D mix (10). 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 (13). To date, few studies describing the assemblage type of G. duodenalis in canine samples have been conducted in America. One study, which tested 15 dogs in Georgia, United States, found that 100% carried G. duodenalis assemblage D (11). Another study from Brazil found that G. duodenalis assemblages C or D were present in 100% of the canine samples tested (16). In contrast to the above 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 low socio-economic status areas. Himsworth et al. (7) showed that, in a remote indigenous area of Saskatchewan, Canada, over one-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 different populations of dogs in Belgium. They found that 80% of healthy Giardia-positive household dogs carried assemblage A, however 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 out compete other assemblage types.

In contrast to canine fecal samples, it appears that 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 27-42% of the positive feline fecal samples contained G. duodenalis assemblage A; 57-67% contained assemblage F (13, 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 four subtypes with most assemblage AII and some A1 being found in humans, whereas some AII, most A1 and all AIII and AIV typically found (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 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 (8, 10). 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._ (8) found that 92% of _G. duodenalis_ isolates (n=55) were typeable, while a study from Sweden demonstrated that 87% of isolates were typeable (10). 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 are 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 were able to type the most number of samples, and able to identify all mixed infections, while the gdh and tpi PCR sequencing methods had little value. Similarly, Leonhard _et al._ (8) 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._ (10) 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 one or two 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 has been described previously by Read _et al._ (12). 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 (12). Similarly, a study on 1440 combined cat, dog, cattle, goat, sheep, pig and other wildlife samples found that 13% of the _Giardia_ isolates had non-congruent assemblage assignments when the same four genes as used in this study were examined (13). 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 one assemblage type over another in mixed infections (12, 13). Mixed assemblage types based on heterogeneous sequences with overlapping nucleotides at assemblage-specific positions has also been described (10, 22). Sprong _et al._ (13) explains 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 (13), 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 (13). 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.

References:


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

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Gene sequenced</th>
<th>Sample tested</th>
<th>Sample typeable</th>
<th>G. duodenalis assemblage type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>ssu-rRNA</td>
<td>118</td>
<td>75</td>
<td></td>
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</tr>
<tr>
<td>Dog</td>
<td>bg</td>
<td>118</td>
<td>37</td>
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</tr>
<tr>
<td></td>
<td>gdh</td>
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<td>20</td>
<td>-</td>
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<tr>
<td></td>
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<td>-</td>
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<td>13</td>
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<td>1</td>
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<tr>
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<td></td>
<td>tpi</td>
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</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Sample ID</th>
<th>G. duodenalis assemblage types identified by different gene sequences</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>ssu-rRNA</td>
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<td>Dog</td>
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<td>C &amp; D&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td>2009-03</td>
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<tr>
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<td>2009-04</td>
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<td></td>
<td>2009-07</td>
<td>A &amp; F&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Identified by the presence of heterogeneous sequences.