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Original article

Giardia duodenalis assemblages in cats from Virginia, USA

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ABSTRACT

Giardia duodenalis is considered a species complex that is divided into 8 genetically distinct but morphologically identical assemblages (A-H). Assemblages C-H are generally host adapted, while A and B infect both people and animals and are considered potentially zoonotic. Furthermore, within assemblage A there are four subtypes (AI, AII, AIII, and AIV) of varying zoonotic potential; human isolates belong to AI and AII, while animal isolates belong to AI, AIII and AIV. Assemblages A, B, C, D, and F have all been reported from cats. The objective of this study was to determine the assemblage(s) of *G. duodenalis* present in cats from Virginia using multilocus genotyping and to assess if there were any differences among the assemblage(s) found in the populations of cats surveyed (free-roaming, shelter, owned) or their geographic location within Virginia. Samples that were positive for *G. duodenalis* cysts by microscopy using centrifugal flotation with $ZnSO_4$ solution and/or direct immunofluorescence assay were genotyped using PCR and sequencing targeting fragments of the SSU rRNA, gdh, bg, and tpi genes. In total, 54 cyst-positive samples were analyzed by PCR and sequencing: 43 produced amplicons, and 37 samples had interpretable sequence data at one or more loci. Assemblage F was detected in 21/37 samples, AI was detected in 12/37 samples, and in 4/37 samples both assemblages F and AI were detected. The potentially zoonotic assemblage AI was detected in cats from two widely separated animal shelters and from one free-roaming cat. These genotyping data demonstrate that potentially zoonotic *G. duodenalis* assemblages are present in cats in Virginia.

1. Introduction

The zoonotic potential of *Giardia duodenalis* from animals varies and is dependent on the parasite assemblage. Of the (at least) eight assemblages (A-H) that are genetically distinct but morphologically identical (Dado et al., 2012; Feng and Xiao, 2011; Sprong et al., 2009; Monis et al., 1999), assemblages A and B have the broadest host specificity and infect both humans and animals, as such they are considered potentially zoonotic (Ballweber et al., 2010; Bowman and Lucio-Forster, 2010;). Furthermore, within *G. duodenalis* assemblage A there are four subtypes (AI, AII, AIII, and AIV); human isolates belong to assemblages AI and AII, while animal isolates belong to AI, AIII and AIV (Sprong et al., 2009). Surveys of *G. duodenalis* prevalence in cats in the U.S. range from 0.58–13% (De Santis-Kerr et al., 2006; Vasilopulos et al., 2006; 2007). While most studies of cats that provide assemblage data show the predominant *G. duodenalis* assemblage to be cat specific Assemblage F, the potentially zoonotic assemblages A and B have been reported from cats in New York, Mississippi, Alabama and Ontario, Canada (McDowall et al., 2011; Vasilopulos et al., 2007; Lefebvre et al., 2006; van Keulen et al., 2002). With over 156 million cats in the United States, including about 50 million free-roaming or stray cats, (http://www.humanesociety.org/issues/feral_cats/) it has been suggested that cats may be a factor in human *G. duodenalis* infection, especially considering domestic cats close proximity to people (Paoletti et al., 2011).

Giardia duodenalis assemblages are determined via PCR and sequencing gene fragments at certain loci, specifically the small subunit ribosomal RNA (SSU rRNA), (Appelbee et al., 2003; Hopkins et al., 1997) β-giardin (bg), (Lalle et al., 2005) glutamate dehydrogenase (gdh), (Read et al., 2004) and triose phosphate isomerase (tpi) (Sulaiman et al., 2003) loci. Mixed infections with more than one

Abbreviations: IFA, imunofluorescence assay; MLG, multilocus genotyping; SSU, rRNA small subunit ribosomal RNA; bg, β-giardin; gdh, glutamate dehydrogenase; tpi, triose phosphate isomerase

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assemblage have been reported and cannot be differentiated with a single locus PCR (Ballweber et al., 2010). Currently it is recommended that researchers use multilocus genotyping (MLG) when attempting to determine isolate assemblages (Cacciò and Ryan, 2008; Covacin et al., 2011; Ryan and Cacciò, 2013) because of the variable ability of each locus to identify *G. duodenalis* assemblages (Gomez-Munoz et al., 2012).

The SSU rRNA gene is a highly conserved multicopy gene that is considered the most sensitive locus for genotyping (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012; McDowall et al., 2011). The bg, gdh, and tpi are other loci commonly used in MLG. They are much less conserved compared to the SSU rRNA but are adequately discriminatory to distinguish subtypes within assemblages (Cacciò and Ryan, 2008; Covacin et al., 2011; Scorza et al., 2012; Sprong et al., 2009). However, several investigators have observed that the variability found in these other loci (bg, gdh, tpi) may produce mismatches in the binding region, which then result in reduced sensitivity of PCR (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012). Several researchers who utilized MLG have found the SSU rRNA locus to be the most sensitive (Traub et al., 2004; McDowall et al., 2011). There is also the possibility that some primers result in preferential amplification of certain assemblages (Cacciò and Ryan, 2008; Scorza et al., 2012). Read et al. (2004) found that isolates genotyped to species-specific assemblages using the SSU rRNA locus, but when genotyped using the gdh they were determined to be potentially zoonotic isolates of assemblages A and B. The authors suggested that this was due to the less conserved nature of the gdh locus, and also due to the different target fragment sizes of the two loci, with the gdh locus targeting a larger fragment size (Read et al., 2004).

At this time there are five published studies describing the assemblage(s) of *G. duodenalis* found in cats in the United States (Fayer et al., 2006; McGlade et al., 2003; Scorza et al., 2012; van Keulen et al., 2002; Vasilopulos et al., 2007). Each of these studies were based on fewer than 20 cat samples that were sequenced and analyzed, and in four of these studies only one locus was analyzed. Three used the SSU rRNA locus, (Fayer et al., 2006; McGlade et al., 2003; van Keulen et al., 2002), one used the gdh locus, (Vasilopulos et al., 2007) and one used the gdh, bg, and tpi loci (Scorza et al., 2012). The objective of the present study was to determine the assemblage(s) of *G. duodenalis* present in cats from Virginia using MLG and to assess if there were any differences among the assemblage(s) found in the population of cats surveyed (free-roaming, shelter, owned) or their geographic location within Virginia at the time of sample collection.

2. Materials and methods

Fecal samples were utilized that had been collected as part of other parasite prevalence surveys (Monti et al., 2017; Taetzsch et al., 2018). In total, 54 cyst-positive samples were analyzed by PCR and sequencing: 43 produced amplicons, and 37 samples had interpretable sequence data at one or more loci. The samples originated from animal shelters in northeastern Virginia (Prince William, Fauquier, and Stafford counties), from both a shelter and trap-neuter-release program in eastern Virginia (Richmond), and from an animal shelter and veterinary hospital in western Virginia (Blacksburg).

2.1. Morphologic testing and cyst isolation

Giardia duodenalis cyst-positive samples were identified by centrifugal flotation using $ZnSO_4$ fecal flotation solution (Zajac and Conboy, 2011) and/or direct immunofluorescent assay (IFA) following the manufacturer's instructions (MERIFLUOR® *Cryptosporidium/Giardia* IFA, Meridian Bioscience Inc.)

Cysts were concentrated from positive samples using sugar gradient density separation to isolate cysts (Scorza et al., 2012). Briefly, 2 g of feces were mixed with phosphate buffered saline solution containing ethylenediaminetetraacetic acid (PBS-EDTA), filtered through cheesecloth and then pipetted onto sugar solution (s.g. = 1.26) in a 15 mL conical tube and centrifuged at 800 ×*g* for 10 min. The top layer of the filtrate and the interface were then placed in a new tube and centrifuged for 10 min at 1200 ×*g*. The supernatant was discarded and the pellet was washed twice more with PBS-EDTA. After the final wash the supernatant was discarded and the resulting cyst pellet was suspended in 1 mL of PBS-EDTA and stored at 4 °C until DNA extraction was performed.

2.2. DNA extraction and PCR

DNA was extracted from the concentrated fecal samples using a commercial kit (Qiagen, DNeasy Tissue Kit, Venlo, Netherlands). Reagents from the kit were used, but with modification as outlined below (Santin et al., 2006). Fifty µL of the cleaned fecal sample were added to 180 µL of ATL buffer, vortexed, and then 20 µL of proteinase K (20 mg/mL) added, and the sample mixed again. Samples were incubated overnight at 55 °C, and mixed with 200 µL of AL buffer. DNA was eluted in 100 µL of AE buffer and stored at −20 °C until PCR was performed.

To determine the assemblage(s) of *G. duodenalis* in each sample we utilized multilocus genotyping. Loci of four genes were utilized for PCR and sequencing using previously described primers and cycling conditions with the following changes: 24 µL/reaction of a commercial master mix (Promega GoTaq® Green Master Mix) and 24 µL of water were used in place of the master mix described in the original publications (Appelbee et al., 2003; Hopkins et al., 1997; Lalle et al., 2005; Read et al., 2004; Sulaiman et al., 2003); 2 µL of DNA was used as the template as outlined by Scorza et al. (2012). The genes targeted were the small subunit ribosomal RNA (SSU rRNA) (Appelbee et al., 2003; Hopkins et al., 1997), b-giardin (bg) (Lalle et al., 2005), glutamate dehydrogenase (gdh), (Read et al., 2004) and triose phosphate isomerase (tpi) (Sulaiman et al., 2003). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Negative and positive controls were included for all PCR assays and electrophoresis analysis.

2.3. Purification of amplicons and sequencing

Positive amplicons underwent gel extraction using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, Venlo, Netherlands) following the manufacturer's instructions. The resulting product was sequenced at the Virginia Bioinformatics Institute, Virginia Tech utilizing the same PCR primers. The sample was sequenced in both directions and analyzed using a sequence alignment editor. Isolate sequences were compared with sequences in the GenBank database by BLAST analysis (http://www.ncbi.nlm.nih.gov/) and aligned with reference sequences from the literature (Table 1) for identification.

3. Results

In total 54 cyst-positive samples from individual cats were analyzed using the SSU rRNA, gdh, bg, and tpi PCR assays. Of the 54 samples analyzed, 43 produced amplicons of which 37 had interpretable sequence data at one or more loci. The samples with interpretable sequence data originated from animal shelters (n = 10) in northeastern

Table 1
GenBank accession numbers for sequences used for genotyping at each locus.

Assemblage	GenBank accession numbers for reference sequences			
	SSU rRNA	gdh	bg	tpi
F	AF199444	AF069057	AY647264	EU781003
A[a]/AI	DQ414242	DQ414242	AY655702	L02120

[a] For the SSU rRNA locus assemblage subtype cannot be determined.

Table 2
Multilocus genotyping results of isolates.

No. of cats	SSU rRNA	gdh	bg	tpi	Geographic location
3	F	F	F	F	Northern Virginia Shelter (Prince William
3	F	–	–	–	County, VA)
1	F	F	–	F	
1	F	F	–	–	
1	F	–	–	F	
1	–	–	F	–	
2	F	F	F	–	Eastern Virginia Trap-neuter-release program
2	F	–	–	–	(Richmond, VA)
1	F	–	F	–	
1	F	F	–	F	
1	A	AI	AI	AI	
4	–	AI	–	–	Eastern Virginia Shelter (Richmond, VA)
1	–	AI	F	–	
1	F	F	–	–	Western Virginia (VA-MD College of
1	F	–	–	F	Veterinary Medicine, Blacksburg, VA)
3	–	AI	F	–	Western Virginia Shelter (Blacksburg, VA)
2	–	AI	–	–	
4	A	AI	AI	AI	
1	–	AI	–	–	
1	F	–	F	–	
1	F	–	–	–	
1	F	–	–	F	

–: no sequence data for that locus.

Virginia (Prince William, Fauquier, and Stafford counties), from both a shelter ($n = 5$) and trap-neuter-release program (free-roaming cats; $n = 7$) in eastern Virginia (Richmond), and from an animal shelter ($n = 13$) and animal hospital ($n = 2$) in western Virginia (Blacksburg). Combining information from all four loci there were 21 samples that were genotyped as assemblage F, 12 as assemblage A, and 4 that genotyped to both assemblages F and A (Table 2). Twenty-five samples had sequence data at the SSU rRNA locus, 25 at gdh, 17 at bg, and 13 at the tpi locus. At the SSU rRNA, bg, and tpi loci, assemblage F was most commonly identified, but assemblage AI was detected more often than assemblage F by the gdh locus. Most isolates were the same assemblage across all four loci, but four samples had both assemblages A and F present. All isolates had 98–100% identity to their respective GenBank accession numbers listed in Table 2.

Twenty-one samples were genotyped as only assemblage F and of those samples 13 (62%) were from cats in animal shelters, 6 (29%) were from free-roaming cats, and 2 (9%) were from client owned animals. There were 11 samples that were genotyped as only assemblage AI; one sample was from a free-roaming cat and the others were from two animal shelters several hundred miles apart, one in eastern Virginia and one in western Virginia, (Table 1). Four additional samples from the same two shelters genotyped as both assemblages F and AI. There were no potentially zoonotic assemblages of *G. duodenalis* recovered from other locations.

4. Discussion

This report demonstrates that some cats in both heavily populated eastern Virginia and more rural western Virginia are infected with the potentially zoonotic *G. duodenalis* assemblage AI. This finding is consistent with reports from other parts of the United States (Table 3). Because of limitations of sample size, differences in assemblage(s) among the populations of cats sampled (owned, shelter, or free-roaming) could not be meaningfully evaluated. Similarly, any differences in prevalence of assemblages that were seen cannot be definitively established from these data because cats were possibly infected with *G. duodenalis* before entering shelters or acquired infections at that facility. The data collected from the free-roaming cats are probably more accurate reflection of assemblage prevalence as the cats were not housed in a common facility.

Although we detected both the species-specific *G. duodenalis* assemblage F and the potentially zoonotic sub-assemblage AI, we did not find the diversity of assemblages in cats that others have reported around the world (Table 3). Cats infected with *G. duodenalis* assemblages F and AI have been reported from genotyping studies using the gdh locus in Mississippi and Alabama (Vasilopulos et al., 2007) and assemblages A and B have been reported in a study utilizing the SSU rRNA locus in cats from New York (van Keulen et al., 2002). Outside the United States, G. *duodenalis* assemblages A, B, C, D, and E have been found in cats in addition to the species-specific assemblage F.

This study provides further support for the importance of utilizing a MLG approach for *G. duodenalis* which is advocated by others (Ballweber et al., 2010; Ryan and Cacciò, 2013; Thompson and Monis, 2012). If genotyping had been undertaken using only one locus, sub-assemblage AI would have been underreported in these samples.

The SSU rRNA, bg, and tpi loci all identified the same five cats as infected with sub-assemblage AI, but the addition of the gdh locus allowed for eleven additional samples to be identified as sub-assemblage AI. The SSU rRNA locus is generally considered best for the detection of *G. duodenalis* because of its multicopy and highly conserved nature (Gomez-Munoz et al., 2012). However, in contrast to other studies where the gdh gene provided limited genotyping information (Covacin et al., 2011; McDowall et al., 2011), in our study, as in Scorza et al. (2012) the gdh locus proved to be a valuable and informative target, as it identified the most sub-assemblage AI infections in addition to the five samples that all of the loci identified. Another study found the SSU rRNA locus to be the most sensitive for genotyping ($n = 107$), followed by the gdh gene ($n = 83$), then the bg and tpi genes which were equal ($n = 77$) (Gomez-Munoz et al., 2012). This variability in genotyping information obtained from each locus could be due to differences among laboratories. Interestingly Covacin et al. (2011) also found that the bg gene seemed to preferentially amplify assemblages A and B as opposed to the species-specific assemblages, but that was not the case in the present study. In the present study the bg locus identified more samples as assemblage F ($n = 7$), five samples with assemblage A, and none with assemblage B, even though cats can be infected with assemblage B as van Keulen et al., (2002) reported.

5. Conclusion

This is the first report of a MLG study of four loci to determine the assemblage(s) of *G. duodenalis* present in cats in the United States. By using the recommended MLG we have identified that cats in Virginia have potentially zoonotic *G. duodenalis assemblages* in addition to the species-specific assemblage F, and these results can provide the basis for further epidemiologic studies on the importance of cats in transmission of human *G. duodenalis* infection.

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Declaration of conflicting interests

The authors declared that there is no competing interest.

Table 3
Giardia duodenalis assemblages identified in cats throughout the world.

Location	No. of samples genotyped	Assemblage(s)	Loci tested	Reference
Canada and USA-New York	9	A, B	SSU rRNA	van Keulen et al., 2002
Italy-Lazio region	1	A	SSU rRNA	Berrilli et al., 2004
Australia-Western Australia	12	A, B, C, D, E	SSU rRNA, gdh	Read et al., 2004
Japan-Aomori	3	F	gdh	Itagaki et al., 2005
USA-Maryland	18	F	SSU rRNA	Fayer et al., 2006
USA-Alabama and Mississippi	17	A, F	gdh	Vasilopulos et al., 2007
Brazil-Sao Paulo	19	F	gdh	Souza et al., 2007
Canada-Ontario	15	A, B, F	SSU rRNA, gdh, bg, tpi	McDowall et al., 2011
Poalnd-Warsaw	4	A, B, D	gdh	Jaros et al., 2011
USA-Colorado	13	A, C, D, F	gdh, bg, tpi	Scorza et al., 2012
Japan-Aomori	41	A, C, D, F	SSU rRNA, gdh, bg, tpi	Ito et al., 2017
Spain-Álava	3	F	gdh, bg	de Lucio et al., 2017
Germany	20	A, D, F	SSU rRNA, gdh, tpi	Sommer et al., 2018

Ethical statement

The work described in this study did not use any animals. All fecal samples were collected as a part of routine cleaning and husbandry from the cages, runs, etc. where animals were housed.

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