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Genotypic characterisation of *Giardia* from domestic dogs in the USA

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Abstract

The first large-scale urban survey of *Giardia* infections in dogs was undertaken in the USA. It involved several locations in the Western United States with *Giardia* isolates from microscopy-positive samples characterized by multi-locus PCR and sequencing. A high prevalence of *Giardia* was confirmed in asymptomatic domestic dogs, and for the first time, provides evidence that zoonotic assemblages/subgroups of *Giardia* occur frequently in domestic dogs living in urban environments, and more frequently than the dog specific assemblages.

Keywords: *Giardia duodenalis,* domestic dogs, USA, molecular epidemiology

Introduction

There is increasing evidence globally that infections with *Giardia* occur frequently in domestic dogs, and that it is often the most common enteric parasite of dogs in developed countries (Thompson et al. 2008; Scaramozzino et al. 2009; Ballweber et al. 2010). Prevalence rates vary and are influenced by the sampling strategies and diagnostic methods used (Epe et al. 2010). Although *Giardia* may be associated with gastrointestinal disorders in dogs (Barutzki et al. 2007; Epe et al. 2010), most concern has been directed to the public health significance of such infections since *Giardia* is potentially zoonotic (Thompson 2004; Caccio et al. 2005; Leonhard et al. 2007; Sprong et al. 2009; Ballweber et al. 2010).

Prevalence rates on their own are insufficient to gauge the public health risks of canine *Giardia* infections without data on the genotypes of *Giardia* that occur in dogs. *G. duodenalis* (*G. intestinalis*) is the most frequent form of *Giardia* found in mammals and represents a species complex comprising genotypes/assemblages that are host specific, and those that affect humans and a range of other mammalian species and are thus considered to have zoonotic potential (Monis et al. 2009). Dogs are susceptible to infection with assemblages C and D which are considered to be specific for dogs (Hopkins et al. 1997; Monis et al. 1998; Thompson 2004), while assemblage F seems to be specific for cats, E for livestock (Ey et al. 1997) and G for rats (Monis et al. 1999). In

47 contrast, assemblages A and B affect humans but are not human-specific and infect a
48 wider host range including dogs, cats, livestock and wildlife and are potentially zoonotic.
49 Assemblages A and B show genetic sub-structuring and some subgroups appear to have
50 more zoonotic potential than others (Monis et al. 2009; Sprong et al. 2009).

51

52 Molecular epidemiological studies have been undertaken in different parts of the world
53 and although these have demonstrated that dogs may be infected with zoonotic and/or
54 dog-specific assemblages of *Giardia*, it is not possible to extrapolate from one area to
55 another with respect to the public health risk of canine *Giardia* infections. The frequency
56 of infection with zoonotic assemblages is generally more common in household dogs
57 than in dogs from kennels (Leonhard et al. 2007; Claerebout et al. 2009; Scaramozzino et
58 al. 2009). However, surveys of household dogs have demonstrated differences between
59 geographical areas, with prevalences of infection with zoonotic genotypes varying
60 between 5.5% (Australia), 61% (Thailand), 80.5% (Belgium), 87% and 6.1% (Germany)
61 and 30.8% (Italy) (rev. in Leonhard et al. 2007; Ballweber et al. 2010). The reasons for
62 this variability are not clear but emphasise the need to undertake studies in different
63 geographical areas.

64

65 The public health significance of *Giardia* infections in dogs in the USA has been a
66 subject of concern and controversy for many years yet only one small survey has been
67 published, in which isolates of *Giardia* from 15 domestic dogs in Atlanta were all shown
68 to belong to assemblage D (Sulaiman et al. 2003). The results of this study are surprising
69 in light of the more recent study by Vasilopulos et al. (2007) which examined 250 cats

from Mississippi and Alabama, USA, and of 17 positive for *Giardia* found 6 infected with Assemblage A-I and 11 with Assemblage F (the cat specific genotype), emphasising the need for additional genotyping studies with larger sampling sizes to be undertaken on dogs in the USA.

Therefore the aim of the present study was to determine the frequency of *Giardia* assemblages in dogs presented at veterinary clinics for health screening in the Western United States where *Giardia* testing incidence is the highest recorded (ANTECH Diagnostics data on file).

Materials and Methods

Sampling strategy

Samples positive for *Giardia* by microscopy were obtained from 238 healthy dogs presenting for an annual check-up at veterinary clinics in the Western United States. The 238 samples for this study were chosen at random on various days in 2009 during which a total of 519,585 faecal samples were submitted and screened at ANTECH laboratories of which 35,172 (6.8%) were positive by microscopy for *Giardia* (ANTECH Diagnostics data on file and available from David.Aucoin@vcaantech.com). All dogs were considered asymptomatic for *Giardia* infection by the veterinarian on duty.

DNA extraction

DNA was extracted from faecal samples preserved in ethanol using the Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, USA) with the Maxwell® 16 SEV Instrument (Promega). In addition to the recommended protocol, 1 µl of the final elution was further diluted by adding 4 µl of Water-ultra pure grade (Fisher Biotech Perth, Australia). All PCR reactions were run prior to freezing the extracted DNA.

Amplification of 18S rRNA gene

PCR reactions used 2 µl of the diluted DNA template, 2.5 µl of 10 X Reaction Buffer, 2.5 µl of $MgCl_2$ (25 mM), 0.15 µl Taq-Ti hot start DNA polymerase (Fisher Biotech Perth, Australia), 1 µl of dNTPs (5 mM) (Promega), 1 µl of each primer (10 µM), 5% dimethyl sulfoxide (DMSO)(Sigma-Aldrich St. Louis, Missouri) and Water-ultra pure grade, to a final volume of 25 µl. The first-round PCR conditions were: 96°C for 5 min for 1 cycle, 96°C for 45 s, 50°C for 30 s and 72°C for 45 s for 35 cycles followed by 72°C for 7 min. The forward primer RH11, 5′- CATCCGGTCGATCCTGCC -3′ and reverse primer RH4, 5′- AGTCGAACCCTGATTCTCCGCCAGG -3′ were from Hopkins et al. (1997). Two micro liters from the first-round PCR reaction was used in the second-round PCR. Second-round PCR conditions were: 96°C for 5 min for 1 cycle, 96°C for 45 s, 55°C for 30 s and 72°C for 45 s for 35 cycles followed by 72°C for 7min with PCR primers forward GiarF, 5′- GACGCTCTCCCCAAGGAC -3′ and reverse primer GiarR, 5′- CTGCGTCACGCTGCTCG -3′ (Read et al. 2004). DMSO was used in the first round PCR only. Ultrapure Bovine Serum Albumin (BSA) Non-Aceylated was added to the second round PCR (1% [50mg/mL]).

Amplification of β-giardin gene

PCR reactions used 2 µl of the diluted DNA template, 2.5 µl of 10 X Reaction Buffer, 2.5 µl of $MgCl_2$ (25 mM), 0.15 µl T*th* Plus DNA polymerase (Fisher Biotech Perth, Australia), 1 µl of dNTPs (5 mM) (Promega), 1 µl of each primer (10 µM) and Water-ultra pure grade (Fisher Biotech Perth, Australia), to a final volume of 25 µl. The first-round PCR conditions were: 95°C for 5 min for 1 cycle, 95°C for 30 s, 50°C for 30 s and 72°C for 60 s for 40 cycles followed by 72°C for 7 min. The forward primer G7 5′-AAGCCCGACGACCTCACCCGCAGTGC -3′ and reverse primer G759 5′-GAGGCCGCCCTGGATCTTCGAGACGAC -3′ were from Cacciò et al. (2002). Two micro liters from the first-round PCR reaction was used in the second-round PCR. Second-round PCR conditions were: 96°C for 5 min for 1 cycle, 96°C for 45 s, 55°C for 30 s and 72°C for 45 s for 35 cycles followed by 72°C for 7min with PCR primers forward: 5′- GAACGAACGAGATCGAGGTCCG -3′ and reverse: 5′-CTCGACGAGCTTCGTGTT -3′, Lalle et al. (2005).

Amplification of Glutamate Dehydrogenase gene (GDH)

PCR reactions used 2 µl of the diluted DNA template, 2.5 µl of 10 X Reaction Buffer, 2.5 µl of $MgCl_2$ (25 mM), 0.2 µl T*th* Plus DNA polymerase (Fisher Biotech Perth, Australia), 1 µl of dNTPs (5 mM) (Promega), 1 µl of each primer (10 µM) and Water-ultra pure grade (Fisher Biotech Perth, Australia), to a final volume of 25 µl. The first-round PCR conditions were: 94°C for 5 min for 1 cycle, 94°C for 30 s, 50°C for 30 s and 72°C for 60 s for 40 cycles followed by 72°C for 7 min. Two micro liters from the first-round PCR reaction was used in the second-round PCR. Cycling conditions for second-round PCR were: 94°C for 5 min for 1 cycle, 94°C for 30 s, 60°C for 30 s and 72°C for

60 s for 40 cycles followed by 72°C for 7 min. The primers for the first round PCR, GDHeF, 5'- TCAACGTYAAYCGYGGYTTCCGT -3'and GDHiR 5'- GTTRTCCTTGCACATCTCC -3' as well as the primers for the second PCR reaction GDHiF 5'- CAGTACAACTCYGCTCTCGG -3' and GDHiR were from Read et al. (2004).

Sequencing

PCR products were purified using a Wizard SV gel and PCR Clean-up system (Promega, Madison, USA) as per the manufacturer's instructions except for the final elution which was reduced from the recommended 50 µl to 20 µl-30 µl. Sequence reactions were performed using the Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. PCR products were sequenced with the second round primers (1µl [10 µM]). The cycling conditions for nucleotide sequencing: 1 cycle of 96°C for 2 min and 25 cycles at 96°C for 10 s, 60°C for 5 s and 60°C for 4 min. All PCR and sequencing reactions were run on a TaKaRa Thermal Cyclier Dice™ Version III. Reactions were electrophoresed on an ABI 3730 48 capillary machine.

Sequences were analysed and contigs of nucleotide sequences were made using Sequencher 4.8 (Gene Codes, Ann Arbor, MI, USA). Sequences were compared with sequences in GenBank by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul et al. 1997). In addition to the BLAST search, sequences were aligned in sequencher with sequences from GenBank with known sub-assemblage information to

confirm the sub-assemblage genotype and to determine the presence of multiple genotypes (Table 1).

Results

Of the 238 samples that were found microscopy positive in ANTECH laboratories prior to shipment to Australia, 148 were positive by PCR, of which 128 samples provided interpretable sequence data. Of these 128 samples, it was possible to identify a total of 296 *Giardia* infections of which 15% were assemblage C, 16% assemblage D, 28% assemblage A, and 41% assemblage B (Figure 1, Table 2).

Multiple genotypes were amplified from 83% of the 128 samples. Results for 57% of the multiple genotypes were from two or more loci, 25% of these amplifications from different loci provided the same genotype result. Sub-genotypes identified with the β-giardin gene were from assemblage A, sub-genotypes A2, A3, A5, A8, and for assemblage B, B1, B2, B3 and B4 (Table 2).

18S preferentially amplified assemblages B, D and C whereas β-giardin preferentially amplified assemblages A, B (Table 2). β-giardin appeared to be more sensitive amplifying single and multiple infections (Figure 1). The glutamate dehydrogenase gene (GDH) provided limited results with genotype information for only 17 samples (Table 2).

Discussion

The present study is the first large-scale urban survey of *Giardia* infections in dogs undertaken in the USA. It involved several locations in the Western United States and has not only confirmed the high prevalence of *Giardia* in asymptomatic domestic dogs, but has also shown, for the first time, that zoonotic assemblages/subgroups occur frequently in domestic dogs living in urban environments, and more frequently than the dog specific assemblages.

Multiple infections with zoonotic assemblages were most common, followed by mixed infections with zoonotic and dog assemblages, followed by single infections with B, C or D (Table 2). The results from this study emphasize that it is not possible to extrapolate from one geographical region to another in terms of the prevalence or assemblage composition of *Giardia* infections in dogs. These results thus support the global picture for *Giardia* in dogs compiled by Ballweber et al. (2010). Furthermore, our study found a higher frequency of *Giardia* infections with assemblage B than assemblage A, which has not been reported elsewhere. This suggests that in North America at least, we cannot assume that, as in Europe (Sprong et al. 2009) assemblage B has a predominantly human distribution. We also found the β-giardin subgroups, A2, A3, B1, B3 and B4 in dogs, which have been reported previously in humans (Lalle et al. 2005) but not before in dogs, thus highlighting their zoonotic potential.

The high frequency of multiple/mixed infections found in this study has been increasingly reported from multilocus studies in other countries in both humans and dogs (e.g., Hussein et al. 2009; Sprong et al. 2009). This may be due to meiotic recombination or preferential amplification of one assemblage over another in mixed infections (Caccio et al. 2005, Cooper et al. 2007, Teodorovic et al. 2007, Weilinga and Thompson, 2007; Lasek-Nesselquist et al. 2009). As regards true mixed infections, it raises the question as to their source. It is possible that some infections are more commonly acquired as puppies (e.g. the dog assemblages) and others possibly later in life as contact with other potential hosts, both dog and human, increases, as does exposure to varied environments. The occurrence of mixed infections also raises the intriguing question of how long do individual infections persist and whether one assemblage may eventually outcompete another?

Of the 238 samples that were found microscopy positive in ANTECH laboratories prior to shipment to Australia, 148 were positive by PCR, of which 128 samples provided interpretable sequence data. The lower number of PCR-positives compared to microscopy is probably a result of the poor condition of some samples following shipment. PCR-inhibition is also a possible reason for unsuccessful amplification, however, in this study DNA was extracted using the Maxwell® 16 SEV Instrument (Promega) with the final elution further diluted to dilute the inhibitors. This method was successful when tested on known positive samples in our laboratory.

Although PCRs for three loci were run on all samples, the glutamate dehydrogenase gene

provided limited results with genotyping data for only17 samples. Amplification of the 18S locus is robust and useful for determining the presence or absence of an infection, the short sequence does not provide sub-assemblage information, in contrast to β-giardin appeared to be more sensitive in amplifying single and multiple infections (Figure 1). We considered that all genotypes amplified were present in an individual sample whether it was supported or differed to another locus. We could not determine if the difference in the genotypes from the different loci was due to sensitivity or preferential amplification of a loci.

The results demonstrate that a significant proportion of asymptomatic dogs in urban areas of the USA harbour zoonotic *Giardia*, and should therefore be considered a potential reservoir for infection in humans. All assemblages can be transmitted in cycles where only dog to dog transmission occurs, but humans may be involved in a zoonotic cycle of transmission with assemblages A and B, either contracting infection from dogs, or acting as a source of infection for dogs.

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342

343 **Table 1.** GenBank accession numbers for contigs for the three loci.

344

18S rRNA gene	β-*giardin gene*	*Glutamate dehydrogenase gene*
AF199443-D	AY072728-B	AF069059-B111
AF199444-F	AY545643-A	AY178750-BIV
AF199445-A1	AY545645-A11	AY826193-B
AF199446-A11	AY545646-C	DQ414242-A1
AF199447-B (111)	AY545648-D	L40510-A11
AF199448-E	AY545649-A1	U60982-C
AF199449-C	AY545649-A8	U60986-D
AF199450-G	AY647266-B	
DQ157272-E	DQ466724-30-A1	
M54878-A1	EU189375-E	
U09491-B	FJ009206-C	
U09492-B	P201207-A2	

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352 **Table 2.** Assemblage and Sub-assemblage information for 128 samples for one, two or
353 three loci.

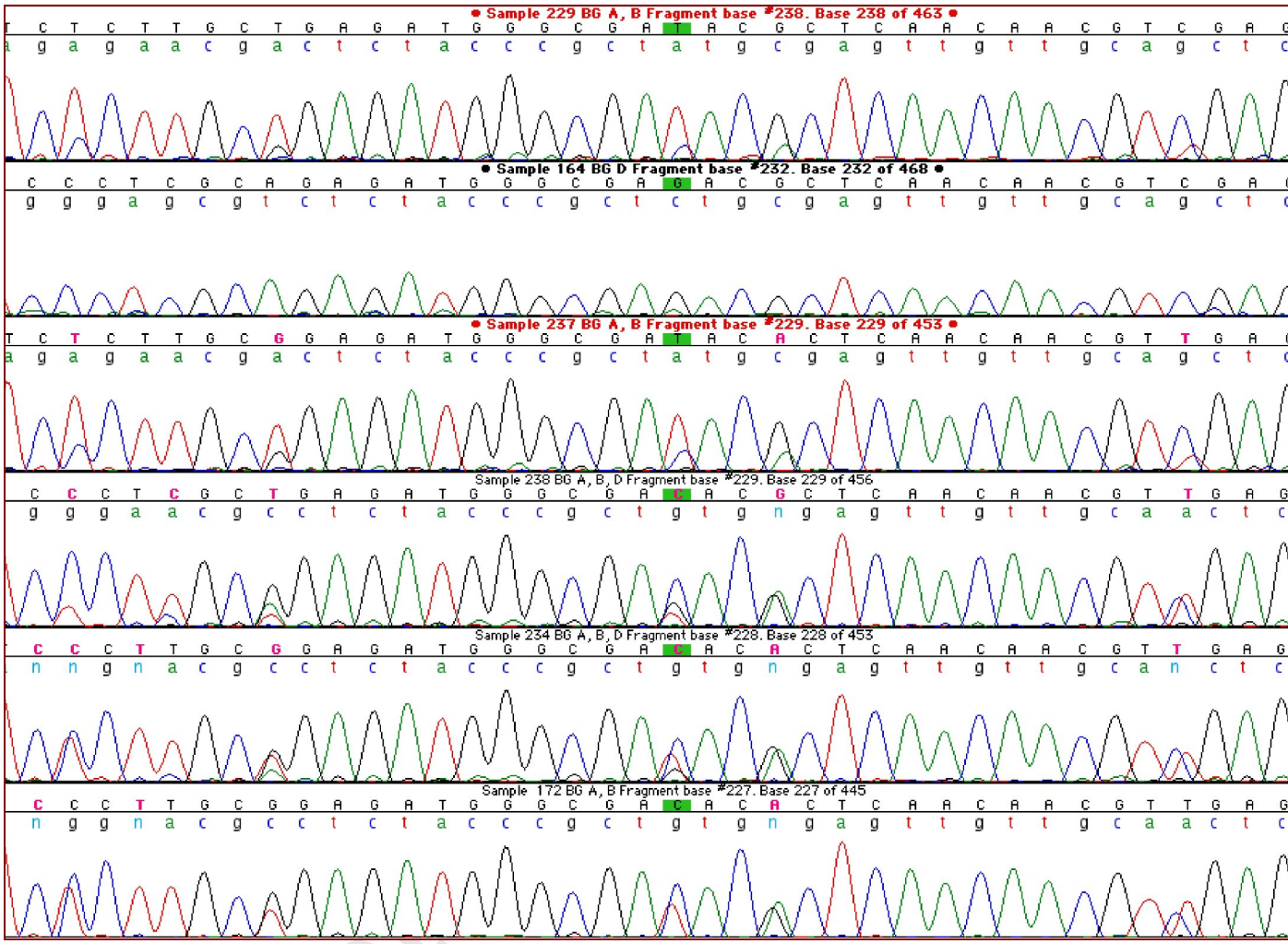
18S	GDH	ß *giardin*	*18S* and GDH	18S and ß giardin	*18S*, GDH and ß *giardin*
B (8)	C (5)	A2, A5 (1)	B, C/D - C/D (1)	B - A2 (2)	B - C - A2 (1)
B, C/D (1)		A2, B1 (6)	B - C (9)	B - A2, B1 (1)	
B, D (1)		A2, B3 (4)	B/C - B/C (1)	B - A5, B3 (1)	
C (3)		A2, B4 (9)	B/C – C (1)	B - B (1)	
C/D (1)		A3, B1 (2)		B - B1 (2)	
		A3, B4 (1)		B - D (1)	
		A5, B3 (1)		B, C/D - B (1)	
		A5, B4 (2)		C - A2 (1)	
		A8, B1 (1)		C - A2, B1 (2)	
		A8, B2 (1)		C - A2, B4 (3)	
		A8, B4 (1)		C - A3, B4 (2)	
		B1 (3)		C - A5, B4 (1)	
		B1, A2 (2)		C - A8, B3 (1)	
		B1, A8 (1)		C - D (1)	
		B1, B3 (1)		C - D/C, A2 (1)	
		B4, A5 (1)		C/D - A2, B3 (1)	
		B, A3 (1)		C/D - C/D, B3 (1)	
		C, A2, B1 (1)		D - A2, B3 (9)	
		C, A2, B4 (1)		D - A2, B4 (7)	
		D (2)		D - A3, B4 (1)	
		D, A2, B3 (1)		D - A5, B3 (1)	
		D, A2, B4 (1)		D - A8, B2 (1)	
		D, A3, B3 (1)		D - A8, B3 (1)	

D, A3, B4 (1)	D - B1 A2 (1)
D, A8 (1)	D - B1, B3 (1)
D, A8, B3 (1)	D - C, A2, B4 (1)
D, B1 (1)	D - D, B A2 (1)

Results in the columns labelled with the single locus are from samples which only amplified at this locus, for example the 18S column has results for 14 samples, which only amplified, at this locus (18S) and the GDH column has results for five samples which only amplified for GDH. Columns with two and three loci have results for samples with sub assemblage information at more than one locus, for example 18S and GDH or 18S and *ß giardin.* The dash between the letters separates the loci information. For example in the column labelled 18S and *ß giardin* the first sample is B-A2 (2), indicates this sample had assemblage B for 18S and sub-assemblage A2, for *ß giardin.* The 2 in parentheses indicate there were two samples with this result. A comma between letters e.g. A8, B2 indicates that both genotypes were found in a sample, results written C/D indicates it may have been either C or D.

Figure 1

Sequencing results for *ß giardin*. A single peak at a known SNP site indicates a single genotype and possibly a single infection, however two or three peaks may indicate the presence of multiple infections. Samples labeled 238, and 234 have three peaks at a single site.



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