High Prevalence of *Giardia* detected in cats by PCR

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Abstract

Microscopy, PCR and a *Giardia* CELISA test were used to determine the prevalence of *Giardia* in 40 faecal samples obtained from domestic cats in the Perth metropolitan area. A prevalence of 5, 80 and 60% was found by the tests, respectively. The results show that more sensitive techniques such as PCR may be necessary, and may yield more reliable results, in the detection of low levels of *Giardia* in domestic cats.

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1. Introduction

*Giardia* is a ubiquitous enteric protozoan affecting humans and a range of domestic and wild mammals (Filippich et al., 1998; Thompson et al., 2000). The parasite has a world-wide distribution and is commonly accepted as a zoonotic agent (Milstein and Goldsmid, 1997; Thompson et al., 2000). Importantly, *Giardia* is the most common parasitic disease of humans (Stevenson and Hughes, 1988) and is considered to be a re-emerging infection because of its association with outbreaks of diarrhoea in child care centres (Thompson, 1994; Thompson et al., 2000). Only the species *Giardia duodenalis* (also known as *Giardia intestinalis* and *Giardia lamblia*) has been recognised in cats (Swan and Thompson, 1986).

Most clinically apparent infections in cats occur in young animals, with the most consistent sign being diarrhoea. Asymptomatic infections are common and have been found to occur more frequently in mature animals (Barr and Bowman, 1994). The disease is usually self-limiting, however, the chronic form may last for years and is more common in immunocompromised cats.

Detection of cysts or trophozoites of *Giardia* is made by faecal examination using direct or concentration methods (Dubey, 1993). Since there is a periodicity of cyst excretion, multiple faecal samples should be examined; preferably a minimum of three samples on
alternate days (Sherding, 1983). For reliable diagnosis of intestinal parasites, a combination of several techniques has been recommended (Beaver et al., 1984). Molecular techniques such as the polymerase chain reaction (PCR) and coproantigen-capture ELISA assays are increasingly being used for the detection of Giardia (Rosoff et al., 1989; Barr and Bowman, 1994). Although Giardia is a common parasite in cats world-wide (Collins et al., 1987; Hill et al., 2000; Spain et al., 2001), results from a recent study suggests that its prevalence may have declined in Perth (Sargent, 1997, 0%) when compared to previous studies (Swan and Thompson, 1986, 14%). This was confirmed in a recent comprehensive survey of 418 domestic cats, all of which were found to be negative for Giardia by microscopy (McGlade, 2001). Therefore, the present study was conducted in which a subsample of 40 of the 418 cats were examined using microscopy, PCR and a coproantigen ELISA kit (CELISA Cellabs, Brookvale, Australia) to determine the prevalence of Giardia.

2. Materials and methods

Forty fresh faecal samples were randomly selected from 418 samples collected from cats in Perth. These 418 samples originated from five sources (a local cat boarding facility, cat refuges, pet shops, breeders, and private cat owners) so that the samples were representative of domestic cats in metropolitan Perth (McGlade, 2001).

2.1. Microscopy

Zinc sulphate flotation (Faust et al., 1938) was employed to detect the presence of Giardia cysts and trophozoites in the faeces. The preparations were examined under 100× magnification for 5–10 min for the presence of cysts and trophozoites. The identity of parasitic stages was confirmed by examining under 400× magnification.

2.2. PCR

2.2.1. Collection and purification of cysts

Cysts were partially purified from faecal material by filtering through sterile gauze followed by phosphate-buffered saline (PBS)-ether sedimentation. Cysts were further purified using a modification of the method described by Lumb et al. (1988). Modifications included a reduction in the number of Ficoll gradients from 6 to 4. Approximately 2 g of fresh faeces was transferred into a 10 ml centrifuge tube using a wooden applicator stick. The tube was then filled to the 2 ml mark with PBS and mixed thoroughly. The liquid was pipetted through two layers of sterile surgical gauze and all liquid was squeezed into a clean 10 ml centrifuge tube. The tube was then filled to the 2 ml mark with PBS and mixed thoroughly. The liquid was pipetted through two layers of sterile surgical gauze and all liquid was squeezed into a clean 10 ml centrifuge tube. Tubes were centrifuged at 3500 rpm at 10 °C for 5 min. The supernatant was removed and the pellet was resuspended in PBS to the 1 ml mark. One millilitre of liquid was then added to a 10 ml centrifuge tube containing 6 ml PBS and 2 ml ether. The lids were replaced tightly and the tubes were shaken. The tubes were centrifuged at 3000 rpm at 10 °C for 8 min, with the samples being shaken after the initial 30 s and then recentrifuged to ensure complete mixing of the PBS, ether and sample. The supernatant was removed and the pellet was resuspended in PBS to a final volume of 2 ml. Two millilitres of liquid was then slowly
added drop by drop to a 10 ml centrifuge tube containing ficoll gradient. The tubes were centrifuged at 3000 rpm at 10°C for 45 min. The interphase between the 0.5 and 1.0% ficoll layers was then added to a 10 ml centrifuge tube containing 6 ml PBS and the tubes were centrifuged at 3000 rpm at 10°C for 20 min. The supernatant was removed and discarded. The pellet was placed in a labelled 1.5 ml eppendorf tube and stored in the refrigerator at 4°C.

2.2.2. Isolation of DNA

DNA was extracted using Qiagen kit (Qiagen GMBH, Hilden, Germany) for PCR. The eppendorf tubes containing the purified samples were centrifuged at 14,000 rpm for 3 min. The supernatant was removed, discarded, and 80 µl of ATL tissue lysis buffer (Qiagen) added to each 20 µl sample and the tubes vortexed. The samples underwent freeze-thawing by placing the tubes in boiling water (100°C), followed by immediately placing the tubes in liquid nitrogen, followed by their immersion in boiling water. This was carried out six times per sample in order to rupture cysts and oocysts. The samples were then incubated in boiling water for a further 15 min. After this time, 180 µl of AL binding buffer (Qiagen) and 10 µl of Prep-A-Gene® Matrix (Biorad, Richmond, CA) were added to the samples and the tubes were vortexed. The tubes were then incubated at 72°C for 15 min DNA binding to the Prep-A-Gene® Matrix (Biorad). The tubes were then centrifuged at 14,000 rpm for 3 min and the supernatant removed and discarded. Seven hundred microlitres of AW wash buffer (Qiagen) was added to the samples and the tubes were vortexed and then centrifuged at 14,000 rpm for 3 min. The supernatant was removed and discarded and the washing step repeated. The pellets were then dried by placing the tubes with the lids open in a vacuum trap until dry. Once dry, 50 µl of AE elution buffer (Qiagen) was added to the samples and the tubes were vortexed and then centrifuged at 72°C for 15 min. The tubes were centrifuged at 14,000 rpm for 5 min and the supernatant was transferred to a fresh, labelled o-ring tube and stored at −20°C until required.

2.2.3. Giardia 18S-rDNA nested PCR

*Giardia* was detected by using specific primers and PCR conditions to amplify a ~130 bp product from the 18S-rDNA gene using the primers RH11 and RH4 as previously described (Hopkins et al., 1997) and the primers GiarF and GiarR for the secondary PCR as previously described (Read et al., 2002). PCR reactions were performed in a total volume of 25 µl, with the primary PCR reaction mixture containing 2.5 µl 10× DNA polymerase buffer (Biotech International), 1.5 mM MgCl2 (Biotech International), 200 µM dNTP's, 12.5 pmol of RH11 forward and RH4LM reverse external primers, 1.25 µl of dimethyl sulfoxide (DMSO), 0.1 unit of 7th Plus DNA polymerase (Biotech International) and PCR Grade Ultra Pure Water (Biotech International). For the primary reaction, 1.0 µl of DNA was added as the template. The secondary PCR reaction mixture consisted of the same reagents, in the same quantities, as described for the primary reaction mixture with two exceptions. First, the external primers were replaced with the addition of 12.5 pmol of GIAR18SIR forward and GIAR18SER reverse internal primers. Secondly, 0.1 unit of *Taq*Extender™ (Stratagene) was added to the secondary tubes instead of DMSO. After the first round of amplification of the primary tubes, a 1.0 µl aliquot from the primary tubes was added to the corresponding secondary tubes as the template and amplified in a final volume of 25 µl. PCR reactions were performed on a
Perkin-Elmer GeneAmp 2400 (Perkin-Elmer) thermocycler. The thermocycler conditions consisted of three cycles of 94 °C for 2 min, 53 °C for 1 min, 72 °C for 2 min followed by 50 cycles of 94 °C for 30 s, 53 °C for 20 s, 72 °C for 30 s and 1 cycle of 72 °C for 7 min. PCR products were subjected to electrophoretic separation in 1.0% (w/v) agarose (Promega, Madison, USA) gels in TAE buffer (40 mM Tris–HCl, 20 mM acetate; 2 mM EDTA; pH 7.0) stained with ethidium bromide (10 μg/ml). A 100-bp molecular mass ladder (BioLabs, New England) and positive and negative controls were included in each gel. The positive control was a *Giardia*-positive sample (BAH45) previously obtained from a human case and amplified in vitro in our laboratories; the negative control contained the PCR reagents but no DNA. Electrophoresis was performed in Minisub electrophoretic cells (Biorad) at 90 V for 30 min. The gels were illuminated on a UVP dual-density transilluminator. Gel photographs were taken using Molecular Analyst Version 1.4 (Biorad).

2.2.4. *Giardia* CELISA

The samples were assayed following the Cellabs *Giardia* CELISA test directions. The samples were prepared by diluting the samples and negative control 1:10 in 10% formalin and allowing to stand overnight at 4 °C. One hundred microlitres of the diluted supernatant, 100 μl of positive control and 100 μl of diluent buffer was dispensed in the corresponding microwells. The microwell plate was covered with foil and incubated in a humidified chamber for 10 min at 37 °C. The test solutions were then flicked out and the wells rinsed four times with wash buffer. Fifty microlitres of diluted 1:200 anti-*Giardia* antibody was dispensed in each microwell and incubated for 1 h at 37 °C as described previously. The solution was then flicked out and the wells rinsed four times with wash buffer. Equal volumes of the chromogenic substrate 3,3′,5,5′-tetramethyl benzidine (TMB) substrate Solution A and B were combined and mixed. Fifty microlitres of the solution was dispensed in each microwell and incubated for 20 min at room temperature in the dark. Fifty microlitres of stopping solution was then added to each well. The results were read visually.

2.2.5. Sequencing of *Giardia* positive samples

PCR products were sequenced, with each reaction containing 2 μl Dye Terminator (ABI Prism™ Dye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City California), 2 μl Half-Term (Life Technologies), 1.25 pmol reverse primer, 5.5 μl PCR product. The total reaction volume was 10 μl. Thermocycler conditions consisted of one cycle of 96 °C for 2 min 20 s followed by 48 cycles of 96 °C for 10 s, 53 °C for 5 s, 60 °C for 4 min. Sequencing reactions were ethanol precipitated according to the following: 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol were added to the DNA suspension and incubated on ice for 60 min. These were then centrifuged at full speed for 30 min, the supernatant removed, and the pellet washed with two volumes of 70% ethanol. After a further centrifugation at full speed for 5 min, the supernatant was removed and the pellet vacuum dried. Once dried, the pellet was stored at −20 °C prior to application to a sequencing gel.

2.2.6. Sequence analysis and genotyping

The SeqEd program V1.0.3 (Applied Biosystems) was used to edit sequences. Miscalled bases were corrected by analysing chromatogram peaks and comparing these to published sequences. The unknown sequences were aligned with known published sequences of the
major genotypes using the alignment program CLUSTAL X (Thompson et al., 1997). The genotypes of samples were determined based on this comparison.

2.2.7. Statistical analysis
Data was analysed using Statistix (Statistix for Windows, Version 7.0, Analytical Software, Tallahassee, FL). The prevalence and 95% confidence interval (CI) was calculated. The different prevalences detected by the three techniques were statistically compared using the chi-square ($\chi^2$) test for independence.

3. Results

The prevalence of Giardia obtained using the three different techniques is shown in Table 1. The prevalence from the nested PCR (80%) was significantly higher ($P < 0.0001$) than that detected by microscopy (5%) (Fig. 1). The CELISA detected 60% of samples to be positive for Giardia and this was significantly higher ($P < 0.0001$) than that detected by microscopy. No samples found negative by PCR were positive using the CELISA (Table 2).

3.1. Sequencing of Giardia positive samples

Fourteen of the 32 isolates were sequenced. Sequence analysis revealed that all but one isolate most closely resembled G. duodenalis dog genotype Group 4 (Hopkins et al., 1997) (Fig. 2). The NCBI (http://www.ncbi.nlm.nih.gov) blast result for the isolate D78 showed

Table 1
Comparison of positive results and techniques used

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<th>Microscopy</th>
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<td>CELISA</td>
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Fig. 1. Agarose gel electrophoresis of PCR products for Giardia stained with ethidium bromide. Lanes 1 and 24, 100-bp ladder (BioLabs); lanes 2–21, sample numbers 1–20; lane 22, positive control (BAH45); lane 23, negative control.
Table 2
Comparison of PCR and the CELISA

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No samples found negative by PCR were positive using the CELISA.

Fig. 2. Partial phylogenetic analysis of Giardia isolates (tree generated using PHYLIP (Version 3.5c, 1993; J. Felsentein, Department of Genetics, University of Washington, Seattle, WA) and TreeView (Page, 1996)).
a 98% base pair sequence similarity over the 130 bp to G. duodenalis dog genotype Group 4 (Dog 6 isolate; accession number AF199443).

4. Discussion

The high prevalence of G. duodenalis detected by PCR (80%) in cats in the present study is surprising for a number of reasons. Studies conducted overseas have recorded prevalences of 2.4 and 7.3%, respectively, in cats (Hill et al., 2000; Spain et al., 2001). Similarly, in Australia Swan and Thompson (1986) found a prevalence of 14% in their study in Western Australia and a prevalence of 16% was recorded by Collins et al. (1987) in Sydney. Interestingly, Sargent (1997) did not detect Giardia in cats from refuges and veterinary clinics in Perth using microscopy. In the current study, only two (5%) cats were positive by microscopy and only one cyst was found on each slide.

Sequence analysis showed that all but one isolate most closely resembled G. duodenalis dog genotype Group 4. This genotype was previously thought to be an animal-specific genotype restricted to dogs (Thompson et al., 2000) having previously been detected only in dogs from Western Australia and South Australia (Hopkins et al., 1997; Monis et al., 1998). Previously, cats have been shown to be susceptible to type 1 and 2 sequences (Thompson et al., 2000). To the authors’ knowledge, this is the first time the dog genotype has been detected in another species of host. It is possible that the host range is wider than previously thought and may extend to include humans, raising the possibility that this genotype may have zoonotic potential.

The high levels of PCR positives and lack of confirmation by microscopy may have arisen from contamination. However, this was considered unlikely since a nested PCR procedure and appropriate controls were used. The second possible explanation was that low numbers of cysts were present in the faeces and microscopy was not sensitive enough to detect these low levels. Similar observations have been reported by Collins et al. (1987). The limitation of microscopy techniques, with respect to sub-clinical levels of Giardia infection, was illustrated in the current study when a skilled microscopist detected only 2 Giardia-positive samples out of 32 samples positive for Giardia using PCR. For both samples positive by microscopy, there was only one cyst detected on the slide.

The 40 samples were tested for Giardia using a coproantigen technique (Rosoff et al., 1989; Barr and Bowman, 1994) in order to determine the validity of the PCR results. The prevalence obtained by the CELISA verified the high prevalence detected by PCR. The prevalence detected by the CELISA was less than that obtained by PCR and indicated that the PCR used in this study was likely to be more sensitive than the CELISA. However, it is important to note that the CELISA test used in this study was designed for detecting Giardia in humans. The CELISA test is not considered optimal for dogs (Hopkins et al., 1997) and the same may apply to cats.

Although traditional techniques, such as microscopy, have been shown to be effective in detecting infections of Giardia in cats and other animals, the results obtained in this study show that more sensitive techniques such as PCR may detect low levels of Giardia infection in domestic cats. The results suggest that cats may be carrying low levels of infection or may not be shedding cysts and that certain factors may pre-dispose cats to such infections
becoming clinically significant. This may also be the case in other animals such as dogs. It is possible that domestic cats are a potential source of environmental contamination in metropolitan Perth.

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References