Determining the zoonotic significance of Giardia and Cryptosporidium in Australian dogs and cats

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Abstract

In a recent study of intestinal parasites in dogs and cats in Australia, Giardia was found to be the most prevalent parasite in dogs. The aim of the current study through the use of molecular tools was to determine the zoonotic significance of the Giardia and Cryptosporidium isolates recovered from dogs and cats during the Australian study. Of the isolates successfully amplified all but one of the Giardia from dogs was either Assemblage C and/or D, with one Assemblage A. Of the cat samples amplified all but one were Assemblage F, with one Assemblage D. We hypothesize that the lack of zoonotic Giardia Assemblages recovered is a result of their being a low prevalence of Giardia in the human population. The Cryptosporidium recovered from dogs and cats was determined to be C. canis and C. felis, respectively, a finding which supports growing evidence that Cryptosporidium in companion animals is of limited public health significance to healthy people.

Keywords: Giardia; Cryptosporidium; Dogs; Cats; Zoonotic; Zoonoses

1. Introduction

The occurrence of Giardia duodenalis and Cryptosporidium spp. in dogs and cats is of potential significance from both clinical and public health perspectives. We recently reported the results of a national study of intestinal parasites in healthy pet dogs and cats in Australia, where Giardia was found to be the most prevalent parasite in dogs (9.4%) (Palmer et al., 2008). In the same study 0.6% of dogs were found positive for Cryptosporidium, while the prevalence of Giardia and Cryptosporidium reported in cats was 2.0% and 2.4%, respectively.

Although infection with Giardia is common, most dogs and cats remain asymptomatic. Yet infection, regardless of the clinical manifestation, is usually associated with a degree of intestinal pathology, which can result in chronic malabsorption. Giardia has been reported to cause villus atrophy, diffuse shortening of microvilli, reduced disaccharidase activity, loss of epithelial barrier function, increased permeability and enterocyte apoptosis (Buret, 2007).

Cryptosporidium infection in dogs and cats does not appear to be common. However, oocyst shedding is more common in younger animals and stress can induce shedding in adult animals suggesting that chronic and
subclinical infections could be more common than surveys indicate (Thompson et al., 2005). Cryptosporidium infection is associated with villus atrophy, villus fusion and inflammation (Koudela and Jiri, 1997), which result in loss of absorptive surface area and impaired nutrient transport.

It is well known that dogs can harbour infections with either zoonotic or host-specific assemblages of Giardia (Caccio et al., 2005; Thompson et al., 2007), and this has been demonstrated in a number of recent studies in urban areas of Mexico, Brazil, Japan, Italy, Poland and Thailand (Berrilli et al., 2004; Itagaki et al., 2005; Lalle et al., 2005a; Eligio-Garcia et al., 2005; Zygnier et al., 2006; Volotao et al., 2007; Inpankaew et al., 2007). In a recent report from Germany it was found that of 60 Giardia positive samples collected from dogs in urban areas 60% were infected with zoonotic Giardia from Assemblage A, 12% with dog-specific Assemblages C and D and the remaining 28% harboured mixed infections (Leonhard et al., 2007).

Few studies have been undertaken in cats but Vasilopulos et al. (2007) examined 250 cats from Mississippi and Alabama, U.S.A and of 17 positive for Giardia found six infected with Assemblage A-I and 11 with Assemblage F (the cat genotype).

Recent molecular epidemiological studies of Cryptosporidium in dogs and cats have shown that they seem to be almost exclusively infected with the host-adapted species C. canis and C. felis, respectively (Xiao et al., 2007). The greatest zoonotic concern regarding these two species of Cryptosporidium is in immunocompromised persons. In human immunodeficiency virus (HIV) infected people, infections with C. canis and C. felis have been associated with chronic diarrhoea (Cama et al., 2007). Chronic diarrhoea in such individuals can lead to a wasting syndrome and eventually death (Cama et al., 2007).

The aim of this current study was to determine the zoonotic significance of the Giardia and Cryptosporidium isolates recovered during a national survey of intestinal parasites in dogs and cats in Australia.

2. Material and methods

2.1. Samples collected

Single faecal samples were collected from dogs and cats from across Australia from both urban and rural locations. A total of 1400 canine and 1063 feline faecal samples were collected from 59 veterinary clinics and 26 refuges: refuges (dogs n = 590, cats n = 491) and dogs/cats presented to veterinary clinics without gastrointestinal (GI) complaints (dogs n = 810, cats n = 572). On the day of collection faecal samples were preserved separately in 10% formalin for microscopic screening and 20% dimethyl sulfoxide (DMSO) for molecular screening. There was a time lag of up to 3 months before samples were sent to the laboratory. Once at the laboratory samples were refrigerated at 4 °C and the time taken before samples were processed was highly variable, ranging from immediately to 6 months.

Different host factors were recorded for each animal sampled, for these details refer to Palmer et al. (2008).

2.2. Parasitological procedures

Formalised faecal samples were screened using a standard sedimentation in water technique, a Malachite Green stain for Cryptosporidium oocysts (Elliot et al., 1999) followed by saturated salt and D-glucose centrifugal flotation and microscopy, as previously described by Henriksen and Christensen (1992).

2.3. Molecular methods

Genotyping of Giardia was initially performed at the 18S rDNA locus for a subset of 88 of the 131 microscopy positive dog samples. This subset consisted of 43 of the 44 positive samples from veterinary clinics and 45 randomly selected samples of the 87 positive samples from refuges. A decision was made not to amplify all of the microscopy positive samples due to limited time and expense, and because we were finding very consistent results. For confirmation of the assemblages determined using this locus, a selection of these dog samples was also amplified at the beta giardin (β-giardin) locus.

Because the products produced by the primers used for the 18S rDNA are identical for both Assemblage A and Assemblage F, all of the Giardia positive cat samples were only amplified at the β-giardin locus.

PCR amplification of the actin gene locus was performed for all of the samples found positive for Cryptosporidium on microscopy.

2.3.1. DNA extraction

Two hundred micrograms of faeces was suspended in 1.4 ml ATL tissue lysis buffer (Qiagen, Hilden, Germany). This suspension was then subjected to five cycles of freeze-thawing at liquid nitrogen temperatures followed by boiling for 10 min. DNA was then isolated from the supernatant using the QIamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Final elutions of DNA were made in
2.3.2. PCR amplification and sequencing of the Giardia 18SrDNA

A nested PCR was used for amplification at the 18SrDNA locus. For the primary reaction, primers RH11 and RH4 (Hopkins et al., 1997) were used and for the secondary reaction primers GiarF and GiarR (Read et al., 2002) were used. The amplification conditions for both sets of primers were the same as those described by Hopkins et al. (1997).

Products were isolated from agarose gel using a DNA purification kit (Mo Bio, UltraClean GelSpin, U.S.A.) as per manufacturer’s instructions, except for a reduced elution volume. Sequencing reactions were performed without DMSO, using the Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer’s instructions. PCR products were sequenced in the reverse direction only (GiarR). Reactions were electrophoresed on an ABI 3730 48 capillary machine. Sequences were analysed using Finch TV Version 1.3.1 (Geospiza Inc.) and isolates were grouped into their genetic Assemblies based on their polymorphisms within the 130 base pair sequence (Hopkins et al., 1997; Monis et al., 1999).

2.3.3. PCR amplification and sequencing of the Giardia β-giardin gene

The amplification of the β-giardin gene was performed using a nested PCR protocol. In the primary PCR reaction a fragment was amplified using the forward primer G7 and the reverse primer G759, as previously described (Caccio et al., 2002). In the nested PCR reaction, the primers described by Lalle et al. (2005b) were used. Amplification conditions were modified for both the primary and secondary reactions. In both reactions 1 µl of extracted DNA was added to 1.5 mM MgCl₂, 10 pmol of each primer, 200 µM of each dNTP, 0.5 units of Tha Plus DNA polymerase (Biotech International, Perth, Australia), 1 × reaction buffer (67 mM Tris–HCl, 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/ml gelatin) and H₂O to 25 µl total. Reactions were denatured at 94 °C for 2 min followed by 40 cycles in the primary and 35 cycles in the secondary, each consisting of 30 s at 94 °C, 30 s of annealing (65 °C for the primary and 55 °C for the nested) and 60 °C at 72 °C, followed by a final extension of 7 min at 72 °C.

Products were isolated and sequenced and analysed as per the 18SrDNA locus above. Sequencing was performed with both the forward and reverse primers from the nested PCR. Sequences were aligned with each other as well as previously published sequences for G. duodenalis isolates using Clustal W (Thompson et al., 1994).

2.3.4. PCR amplification and sequencing of the Cryptosporidium actin gene

PCR amplification of the actin gene locus was performed using a nested PCR as previously described (Ng et al., 2006). Sequencing was performed with both the forward and reverse primers from the nested PCR. Products were isolated, sequenced and aligned as described above.

2.4. Statistical analysis

The methods used to determine any association between parasitism and host factors are outlined in Palmer et al. (2008).

3. Results

3.1. Microscopy

For the majority of microscopy positive Giardia and Cryptosporidium samples the quantity of cysts/oocysts was low. Frequently there was less than one cyst/oocyst recovered per 40X field of view.

3.2. Risk factor analysis

Multivariate analysis revealed that dogs from refuges and dogs, which were less than one year of age were at greatest risk of Giardia infection. Statistical analysis was not performed on the dogs positive for Cryptosporidium and the cats positive for either Giardia or Cryptosporidium, as there were too few positives.

3.3. Molecular characterization of Giardia and Cryptosporidium isolates

It should be mentioned that genotyping of the microscopy positive Giardia samples was initially attempted at the triose phosphate isomerase (tpi) gene and the glutamate dehydrogenase (gdh) gene, using primers previously described by Sulaiman et al. (2003) and Read et al. (2004), respectively. Unfortunately, all attempts at these two loci were unsuccessful. Amplification of the tpi gene resulted in non-specific banding patterns, while the gdh primers were unable to amplify
any of the samples apart from the positive control (axenic culture of the *G. duodenalis* Portland 1 strain).

Of the 88 *Giardia* positive dog isolates sequenced at the 18SrDNA locus, 46.6% (41) were Assemblage C, 50% (44) were Assemblage D, 2.2% (2) were mixed genotype C and D and 1.1% (1) was Assemblage A. A subset of the isolates (*n* = 12) was also genotyped at the β-giardin locus and confirmed the 18S results.

Of the 21 *Giardia* positive cat samples 8 were successfully sequenced at the β-giardin locus, 7 of which were Assemblage F and one was Assemblage D.

Phylogenetic analysis for subgrouping genotypes was determined not to be necessary as no *Giardia* Assemblages A or B were recovered using the β-giardin gene. Although 1 dog isolate was found to be Assemblage A using the 18Sr DNA locus, we were unable to amplify this sample at the β-giardin gene and the 18S fragment is too small to perform phylogenetic analysis on.

Four of the 8 *Cryptosporidium* positive dog specimens were successfully sequenced and were found to be *C. canis*. Eighteen of the 26 positive cat isolates were sequenced and all were identified as *C. felis*.

4. Discussion

Risk factor analysis revealed that dogs from refuges and dogs, which were less than one year of age were at the greatest risk of *Giardia* infection. Refuge dogs are at more risk of infection due to the direct contact they have with other dogs and their excrement, environmental contamination and the immunosuppressive effects of stress. Younger animals are at risk of infection due to their immature immune systems.

It was disappointing that neither of the primer sets for the *Giardia* tpi and gdh genes was able to successfully amplify any of the isolates. The sensitivity of the β-giardin locus was also very poor as evident by the few cat isolates we were able to amplify. In contrast, we had great success with the 18SrDNA locus and according to Wielinga and Thompson (2007) the 18SrDNA locus is well suited for routine genotyping from environmental samples due to its high copy number, whereas the other structural and metabolic genes are estimated to be single or low copy numbers. Environmental samples are typically low in target DNA and contain many PCR inhibitory factors (Wielinga and Thompson, 2007). Castro-Hermida et al. (2007) reported that when the quantity of cysts was low (less than one cyst per field of view), PCR was unsuccessful at the gdh and β-giardin loci, which fit with our current findings.

Although it was expected that animals from refuges would be predominantly infected with *Giardia* host-specific genotypes, the low prevalence of zoonotic genotypes was unexpected in animals from households. Thompson et al. (1999) found that in the domestic, urban environment of Perth Western Australia, genotypes from Assemblage A and C were both equally common in the dogs sampled. It has been suggested that in environments where the infection pressure is low, such as in domestic households in urban settings, dogs are just as likely to harbour genotypes from Assemblage A as they are their own host-specific genotype (Assemblage C and D) (Thompson and Monis, 2004). In contrast, in Aboriginal communities in Australia, where *Giardia* infection is highly endemic for both the human and dog populations it was found that the dog genotype predominated in infected dogs (Hopkins et al., 1997). It is most probable that the dogs in these communities would be exposed to both the dog-specific as well as the zoonotic genotypes of *Giardia*, however, the dog-specific genotypes, which are probably ‘better’ host adapted are more likely to predominate through competitive exclusion (Thompson, 2000). Traub et al. (2004) reported that all of the dogs infected with *Giardia* in a remote tea-growing community in Assam, India were infected with *Giardia* from Assemblage A. The difference between Assam and the findings of Hopkins et al. (1997) in the Aboriginal Communities may reflect a closer association between individual dogs and their owners in Assam and the frequency with which dogs are able to eat human faeces in these communities, as well as the territorial nature of the dogs with little dog to dog contact (Traub et al., 2004). A possible explanation for the findings in the household animals in the current study, which was in contrast to the findings of Thompson et al. (1999), is that the dogs and cats in the current study had not been exposed to the zoonotic genotypes because the human population had a low prevalence of *Giardia* infection. Surveys that simultaneously target both humans and their companion animals with regards to risk factors for the prevalence of zoonotic versus dog-specific genotypes of *Giardia* will shed further light on this matter.

Unexpectedly, one of the *Giardia* isolates from a cat was identified as Assemblage D, an Assemblage usually regarded as dog-specific. In the literature there is only one other reference to such an occurrence; Mcglade et al. (2003) reported finding *Giardia* Assemblage D in 13 of 14 cat isolates sequenced. We were unable to ascertain whether the cysts present in the faeces in the
cat in our study were the result of mechanical transmission or a patent infection.

Future research into the possibility of correlating Giardia Assemblage type with differences in pathogenicity is needed. As a starting point samples should be taken from dogs and cats with giardiasis to see if they are mostly infected with a particular Giardia genotype.

The finding of C. canis and C. felis in dogs and cats, respectively, was not surprising and supports the limited number of studies conducted previously (Morgan et al., 1998; Abe et al., 2002; Fayer et al., 2006; Xiao et al., 2007). It would thus appear that globally, pet dogs and cats do not represent a public health risk with respect to the transmission of Cryptosporidium to humans unless they are immunocompromised.

In conclusion, although Giardia is the most prevalent parasite in dogs in Australia (Palmer et al., 2008) the zoonotic risk seems to be low. However, the option of genotyping Giardia positive specimens is not readily available to veterinarians in practice; consequently all positives should be assumed zoonotic. Therefore, treatment is necessary regardless of whether the animal is asymptomatic, given the zoonotic potential of this parasite and because they are a source of infection for other animals.

References


