

Detection of *Giardia intestinalis* Assemblages A, B and D in Domestic Cats from Warsaw, Poland

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Abstract

Giardia intestinalis is a complex species divided into 7 assemblages (A – G). Two of them (A and B) are infective for both humans and animals. In cats four assemblages can occur: A, B, D, and F. Assemblages A and B infect either cats, dogs and humans, assemblage D infects cats and dogs and assemblage F only cats. The purpose of this study was to determine the prevalence and genotypes of *G. intestinalis* in cats from Warsaw. From November 2006 to March 2007 a hundred sixty samples of stool were collected and examined by light microscopy. *G. intestinalis* cysts were detected in 3.75% of samples. DNA extracted from positive samples was used as template for PCR-RFLP using *Giardia* specific primers and the amplicons were sequenced. A comparison of the obtained DNA sequences with the *Giardia* sequences in the GeneBank database revealed assemblage A in 1.25% of the investigated cats, assemblage B in 1.25% and D in 1.25%.

Key words: *Giardia intestinalis*, assemblage in cats, genotype, PCR-RFLP, zoonosis

Introduction

The protozoan parasite *Giardia intestinalis* infects vertebrates including humans, domestic and wild animals. This parasite can cause gastrointestinal infections ranging from mild to severe as well as chronic diseases. *Giardia* is one of the most common causes of diarrhoea in humans and the most frequent parasite of companion animals. Usually symptoms occur in six to fifteen days after infection. In the acute stage symptoms can last from two to four days, and after that a chronic phase can appear and can last from a few weeks to several years. However the disease is usually self-limiting and asymptomatic infections are common (Flanagan, 1992; Farthing, 1997; Singer and Nash, 2000; Adam, 2001). The potential health risk to humans from gastrointestinal parasites remains a significant problem throughout the world (Schantz, 1994). The main origin of infection is water contaminated with *Giardia* cysts (Karanis *et al.*, 2007). However, food, especially raw vegetables, may be also contaminated with *Giardia* cysts. In addition, infection may be transmitted by direct person-to-person or person-to-animal contact, especially in communities

with poor standards of hygiene (Hunter and Thompson, 2005). The prevalence of human giardiasis in the world ranges from 0.004% to almost 100%, and the most prevalent infections are detected in children < 2 years in developing countries. The mean prevalence in Europe, North America and Australia is 0–32%, depending on region and age group. In Poland the prevalence of *Giardia* infection observed in humans is 0.04–9%, but children are infected more frequently than adults. In cats the prevalence of infection observed in the world is 0.6 to 80%, but in Poland the prevalence is rather low (1.3%). However only a few studies were performed (Zygner and Wędrychowicz 2008; Bajer *et al.*, 2009).

The species *G. intestinalis* includes seven assemblages A-G, that can be characterized using, for example, the glutamate dehydrogenase (gdh), small-subunit (SSU) rRNA, and triosephosphate isomerase (tpi) genes (Monis *et al.*, 1999; van Keulen *et al.*, 2002; Read *et al.*, 2004; Caccio *et al.*, 2005; Papini *et al.*, 2007). Assemblages A and B infect humans and other hosts, including cats (Monis *et al.*, 1998; Monis *et al.*, 1999; Thompson *et al.*, 2000; van Keulen *et al.*, 2002; Monis *et al.*, 2003). Assemblage C infects only dogs, D infects

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dogs and cats and assemblage F infects cats alone (Santini *et al.*, 2006; Souza *et al.*, 2007; Palmer *et al.*, 2008). Zoonotic transmission of *G. intestinalis* is still under consideration despite increasing knowledge of the molecular identification of *Giardia* from different hosts (Monis *et al.*, 2003; Thompson, 2004; Hunter and Thompson, 2005). Although Majewska (1994) showed zoonotic potential of *Giardia*, the reservoir of infection for humans is still unknown. In Poland assemblages A and B were detected in faecal samples from humans. However, in that study *Giardia* cysts were not detected in humans who had had permanent contact with animals (Solarczyk *et al.*, 2010).

The aim of this study was to analyse the genetic diversity of *Giardia* isolates from clinical cases among cats in Warsaw, Poland by *gdh* PCR-(RFLP) assay and the single *gdh* gene PCR assays. The different sequences were used to construct a database so it was possible to compare the result of this study with sequences previously published and available in the GenBank database.

Experimental

Materials and Methods

Giardia cysts were identified in the Division of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, Warsaw University of Life Sciences. Fecal samples were collected from November 2006 to May 2007 in the Small Animal Clinic, Department of Clinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences. In total 160 cat stool samples were collected. Giardiasis was diagnosed by detection of cysts in fecal samples using Meridian MeriFluor® *Cryptosporidium/Giardia* test according to manufacturer procedure (Meridian Diagnostics Inc.).

DNA was extracted from all positive fecal samples using a stool extraction kit (QIAamp DNA stool kit, QIAGEN). A DNA fragment (about 770 bp) of the *gdh* gene was amplified using PCR-RFLP with primer GDH1 (5' ATC TTC GAG AGG ATG CTT GAG 3') and GDH4 (5' AGT ACG CGA CGC TGG GAT ACT 3') as reported Homan *et al.* (1998). This method allowed to distinguish between assemblages A and B by RFLP analysis. Amplification was performed on a total reaction volume of 50 µl, containing template DNA and the following PCR mixture: 10 × *Taq* Reaction buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 1.25 units of *Taq* DNA polymerase (Fermentas) and 0.5 µM of each primer. The conditions of PCR were as follows: initially 94°C for 3 min, then 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s, and finally, after these cycles, 72°C for 10 min (Homan *et al.*, 1998). The reactions were performed in a PTC 200 Thermal Cycler (MJ Research).

The PCR products were visualized by electrophoresis in 1% agarose gel with ethidium bromide. In all cases, the PCR products were gel purified using a gel extraction kit (Macherey – Nagel), and sequenced using an AbiPrism 3100 and GeneScan Analysis Software. The PCR products were sequenced in both directions using either GDH1 or GDH4 primers. Results were compared with sequences available in the GenBank database.

The PCR products were purified using a gel extraction kit (Macherey – Nagel), and then digested with *DdeI* in a reaction mixture of 2 µl of 10 × buffer, 1 µl of *DdeI*, 5 µl purified PCR products and distilled water to a final volume of 20 µl at 37°C for 1 hr. The digested mixtures were analysed by electrophoresis in 2% agarose gel with ethidium bromide (Homan *et al.*, 1998).

To be able to amplify and distinguish all assemblages, a distinct fragment of the *gdh* gene (220 bp) was amplified. The *gdh* gene fragment was amplified using the forward primer GDHF3 (5'-TCC ACC CCT CTG TCA ACC TTT C-3') and the reverse primer GDHB5 (5'-AAT GTC GCC AGC AGG AAC G-3') as reported Itagaki *et al.* (2005). PCR reaction mixtures consisted of 0.5 µM of each primer, 0.2 mM of each dNTP, 2 mM MgCl₂, 1 unit of *Taq* DNA polymerase (Fermentas) and 10 × *Taq* Reaction buffer (Fermentas). The reactions were performed on a total reaction volume of 25 µl. The conditions of the PCR were as follows: initially 94°C for 3 min, then 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, and finally, after all these cycles, 72°C for 10 min (Itagaki *et al.*, 2005). The reactions were performed in a PTC 200 Thermal Cycler (MJ Research). The products of PCR were visualized by electrophoresis in 2% agarose gel with ethidium bromide. The PCR products were gel purified using the same kit mentioned above and sequenced using an AbiPrism 3100 and GeneScan Analysis Software. PCR products were sequenced in both directions using either GDHF3 or GDHB5. The results were compared with sequences available in the GenBank database.

Results

Microscopic analysis of the 160 samples proved that only 6 (3.75%) were positive for *Giardia* cysts. In PCR-RFLP 770 bp products were obtained (Fig. 1a) in 4 cases. After RFLP analysis two of them were recognized as assemblage A and another two as assemblage B (Fig. 1b). A comparison of all four sequences from PCR-RFLP with those from the GeneBank database allowed to identify them as fragments of *G. intestinalis* *gdh* gene and confirmed RFLP analysis. The sequencing and genotyping of two amplicons (220 bp) obtained from PCR using starters GDHF3 and GDHB5 revealed assemblage D (Fig. 1c).

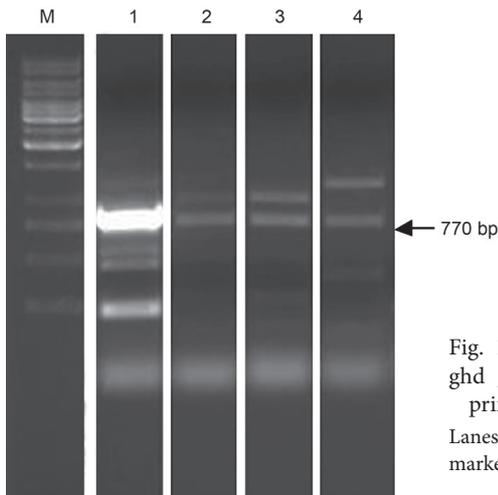


Fig. 1A. Detection of *Giardia* *ghd* gene by PCR-RFLP with primers GDH1 and GDH4.

Lanes: M - 1 kb molecular weight marker (Fermentas); 1-4 - cat samples 770 bp.

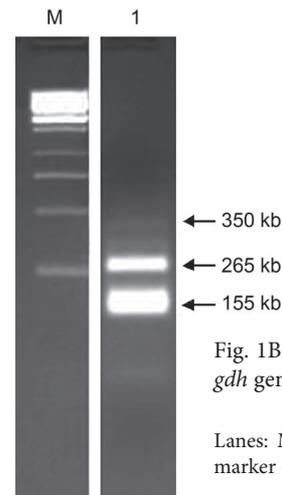


Fig. 1B. Restriction patterns of *gdh* gene amplified with GDH1 and GDH4.

Lanes: M, 1 kb molecular weight marker (Fermentas); lane 1, isolate (B) from cat.

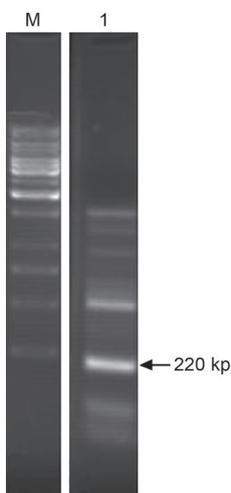


Fig. 1C. Restriction patterns of *gdh* gene amplified with GDH1 and GDH4.

Lanes: M, 1 kb molecular weight marker (Fermentas); lane 1 isolate (B) from cat.

Discussion

The potential risk to human health from *G. intestinalis* infections remains a meaningful problem all over the world. Recent studies on parasites of dogs and cats have demonstrated that the levels of *Giardia* infections were higher than expected (Johnson and Gasser, 1993; Bugg *et al.*, 1999; Itoh *et al.*, 2006). Previous studies on feline giardiasis suggested a significant problem for human health because of the potential risk for zoonotic transmission (Robertson *et al.*, 2000; Thompson *et al.*, 2000; van Keulen *et al.*, 2002; Read *et al.*, 2004; Thompson, 2004). However, many scientists claimed that most cat infections were caused by assemblages D or F, non-pathogenic for human (Monis *et al.*, 1998; Monis *et al.*, 1999; van Keulen *et al.*, 2002; Monis *et al.*, 2003). In European countries, the genotypic characterization of *G. intestinalis* infections in cats has received little attention and very few isolates have been characterized (Berrilli *et al.*, 2004; Lalle *et al.*, 2005; Papini *et al.*, 2007). In this study, four out of six *Giardia* positive cats had assemblages A or B potentially pathogenic for human. Therefore, the results of this study suggest that the population of Warsaw cats may pose a risk to

human health because of the possibility of zoonotic transmission.

Feline giardiasis has been found all over the world and detection rates in particular regions fluctuate from 0.58% to 60%. In USA, 0.58% of cats out of 631021 examined possessed *Giardia* infection (De Santis-Kerr *et al.*, 2006), in Japan 40% of cats were infected out of 600 examined (Itoh *et al.*, 2006), in Turkey 22.4% of cats had it out of 100 examined (Cirak and Bauer, 2004). The prevalence may depend to a high degree on the method used for diagnosis. For instance, in Australia 5.6% or 60% of cats out of 40 were reported to be infected, using PCR and ELISA respectively (McGlade *et al.*, 2003). Also, in the Czech Republic 0.74% or 56.9% out of 107 investigated cats were found to be infected using conventional microscopic techniques or ELISA, respectively (Svobodova *et al.*, 1995). These differences result from the different specificity and sensitivity of the tests. Last study showed that specificity and sensitivity of an ELISA test compared with fluorescent antibody test amounted 0.96 and 0.51 respectively. However, positive predictive value was rather poor at prevalence rates 10% or less (Rishniw *et al.*, 2010). Thus, it is highly probable that the results of that test may be false positive rather than true positive. Moreover, Cirak and Bauer (2004) showed that another ELISA test was more often positive in microscopically *Giardia*-negative fecal samples in which *Isospora* spp. oocysts were detected than in samples without any parasites. This result may indicate cross-reactions in ELISA tests used in animals.

The sensitivity and specificity of light microscopy used in this study highly depends on the experience and knowledge of technician or researcher, but PCR tests are more objective, highly sensitive and specific (Prichard and Tait 2001; Barr, 2006; Allenspach and Gaschen, 2008). Nantavisai *et al.* (2007) showed that the sensitivity and specificity of PCR method for detection of *Giardia* DNA is 97.3% (95% confidence interval, 87.9–99.9%) and 100% (95% confidence interval,

91.3–100%), respectively. However, sensitivities of different PCR tests are also variable. This depends on primers used for amplification of different target gene locus. Nantavisai *et al.* (2007) showed that PCR test with primers compatible for SSU rRNA gene fragment detected *Giardia* DNA in concentration of 10 pg/μl DNA per PCR mixture while PCR test with primers compatible for Triosephosphate isomerase gene fragment detected *Giardia* DNA in minimal concentration of 1000 pg/μl. The primers compatible for glutamate dehydrogenase gene fragment used in this study were moderately sensitive. This primer set allows to detect *Giardia* DNA in minimal DNA concentration of 1000 pg/μl, but minimal *Giardia* cyst concentration detected by this primer set was 337 cysts/ml of fecal sample, while primers compatible for Triosephosphate isomerase gene fragment allowed to detect *Giardia* DNA when minimal cyst concentration was 3368 cysts/ml.

The results of this study differ from the results of previous studies. In Japan and Australia all or all but one cats were infected with non-pathogenic for humans assemblage F (Itagaki *et al.*, 2005, Palmer *et al.*, 2008). However, in Brazil 42.1% *Giardia* infections were caused by assemblage A, and the remaining cats were infected with assemblage F (Souza *et al.*, 2007). In Italy Papini *et al.* (2007) detected only assemblage A in all examined samples of cat feces. These differences can result from the fact that there are few researches on genotyping of *G. intestinalis* infections in cats. The importance of Warsaw cats in the transmission of *G. intestinalis* to humans cannot be finally evaluated because of the small number of positive samples. However, the results of this work and that from Brazil (Souza *et al.*, 2007) show there is a potential risk of human infection. Therefore, it is necessary to assume that a cat infected with *Giardia* possesses potentially zoonotic assemblages.

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