RESEARCH NOTE

Multilocus genotyping of *Giardia duodenalis* isolates from red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) from Poland

Piotr Solarczyk¹, Anna C. Majewska¹, Bożena Moskwa², Władysław Cabaj², Mirosława Dabert³, Piotr Nowosad¹

¹Department of Biology and Medical Parasitology, Poznań University of Medical Science, Faculty of Medicine I, 10 Fredry Street, 61-701 Poznań, Poland;

²Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Twarda 51/55, Warsaw, Poland;

³Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland

Abstract: A total of 181 faecal samples were collected from wild cervids in two regions of Poland. *Giardia* cysts were detected in one faecal specimen from red deer and in two samples from roe deer. Fragments of the β -giardin (*bg*) triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes were successfully amplified from the *Giardia* isolate obtained from red deer, whereas only amplicons of *bg* and *gdh* were obtained from *Giardia* isolates derived from two roe deer. The result of genotyping and phylogenetic analysis showed that the *G. duodenalis* isolate from red deer belonged to sub-assemblage AIII, which has never been identified in humans, whereas isolates from roe deer clustered within zoonotic sub-assemblage AI. Further studies are necessary to explain which *Giardia* assemblages and/or sub-assemblages occur in wild cervids in various regions of the world. Moreover, the impact of *Giardia* infection on the health of wild cervids should also be elucidated.

Keywords: *Giardia*, genotypes, molecular epidemiology, phylogeny, zoonoses

The cosmopolitan flagellate *Giardia duodenalis* (Lambl, 1859) (syns. *G. intestinalis*, *G. lamblia*) is one of the most common intestinal protozoan parasites of humans and many species of animals (Thompson 2004, Lasek-Nesselquist et al. 2010, Yang et al. 2010). This species exhibits great genetic heterogeneity and eight major genetically distinct assemblages (A–H) have been recognized. These assemblages differ in host specificity. *Giardia duodenalis* isolates belonging to assemblages A and B are found in both humans and animals, whereas the rest of the assemblages (C–H) are more host-adapted: C and D occur in canids, E in livestock, F in cats, G in rodents, H in marine mammals (Sprong et al. 2009, Lasek-Nesselquist et al. 2010). However, further genotyping studies at different levels of resolution have indicated greater genetic variability among some assemblages than was previously found (Cacciò and Ryan 2008, Sprong et al. 2009). Besides that, new *G. duodenalis* genotypes are still being identified, mostly in wild animals (Lalle et al. 2007, Gaydos et al. 2008, Lebbad et al. 2010, Thompson et al. 2010).

Such considerable genetic variability among *G. duodenalis* isolates obtained from different hosts and from various geographic regions complicates determination of the role of animals as a source of human infection. Whereas the role of pets and livestock in *Giardia* transmission has been studied intensively,

the role of wild animals, particularly artiodactyls, has only recently been taken into consideration (Trout et al. 2003, Lalle et al. 2007, Robertson et al. 2007, Kutz et al. 2008, Beck et al. 2010). Wild cervids might play a significant role in contamination of the environment with *Giardia* cysts because large populations occur worldwide, they excrete a relatively large volume of faeces, and their feeding ranges usually overlap with cattle pastures.

Thus, they might be potential reservoirs of *Giardia* infection both for humans and livestock, and might represent a public and veterinary health interest. However, there are few genotyping studies of *Giardia* isolates found in wild cervid animals. So far, sub-assemblage AI (with zoonotic potential) and AIII (which is mainly cervid-adapted), as well as non-zoonotic assemblage E, have been identified in cervids in different geographic regions (Trout et al. 2003, van der Giessen et al. 2006, Lalle et al. 2007, Robertson et al. 2007, Beck et al. 2010, Lebbad et al. 2010). Moreover, recent studies showed variations in dissemination of *G. duodenalis* genotypes among geographic regions and between farmed and wild ruminants (Feng and Xiao 2011). The aim of this study was a multilocus genotyping of *Giardia* isolates recovered from wild cervids.

A total of 181 faecal samples were collected from wild cervids in west-central and north-eastern regions of Poland. Specimens were taken from 65 fallow deer (*Dama dama*) (Linnaeus), 61 red deer (*Cervus elaphus*) Linnaeus, 50 roe deer (*Capreolus capreolus*) (Linnaeus), and five moose (*Alces alces*) (Linnaeus). Most of the faecal samples ($n = 174$) were taken from the hunter-killed animals during selective shootings, whereas only seven specimens were picked up immediately after excretion next to feeding sites during animal observation.

Each faecal sample was concentrated using the sucrose gradient centrifugation technique, with the final sediment being examined using a light microscope. Wet and trichrome stained smears were microscopically examined for the presence of *Giardia* cysts.

Total genomic DNA was directly extracted from faecal samples. A FastDNA kit (BIO101, Vista, California, USA) was used for extraction of the *Giardia* DNA based on a protocol described previously (da Silva et al. 1999). The eluted DNA was purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the kit instructions.

Fig. 1. Phylogenetic relationship of 17 *Giardia* isolates inferred by the neighbour-joining analysis of the glutamate dehydrogenase nucleotide sequences. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Isolates from this study: JC002 – from red deer, CC1 and CC2 – from roe deer. Reference human isolates: RB (EF685702), Portland 1 (EF685702), H8 (EF507652) H19 (EF507660) and AD-45 (AY178739), AD-28 (AY178738); swecat171 – cat isolate (EU769223); K4016 – cow isolate (DQ182607); AD-136 and AD-148 – reference dog isolates (U60986 and U60982); NLR118 – roe deer isolate (DQ100288); P-15 – reference pig isolate (AY178741); AD-155 – reference rat isolate (AY178745). *Giardia ardeae* (AY258618) represents an outgroup.

Three genes were used for genotyping: a 753-bp fragment within the β -giardin gene (*bg*) was amplified using the G7 forward primer and the G759 reverse primer, as previously described, a 530-bp fragment of the triose phosphate isomerase (*tpi*) gene was amplified using the AL3543 and AL3546 as well as AL3544 and AL3545G7 primers, and a 430-bp fragment of the glutamate dehydrogenase (*gdh*) gene was amplified using two forward GDHeF, GDHiF and one reverse GDHiR primers (Cacciò et al. 2002, Sulaiman et al. 2003, Read et al. 2004). Amplification involved use of a 25 μ l suspension of the following reagents: 1.5 mM $MgCl_2$, 0.6–1 μ M of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 0.5 U of AmpliTaq Gold DNA polymerase.

PCR was carried out using a GeneAmp 2400 thermocycler. In nested and semi-nested PCR 1 μ l of PCR mixture from the first reaction was used. A negative control consisting of a reaction mixture without the DNA template and a positive control (*Giardia* DNA extracted from cultured trophozoites of the Portland-1 reference strain) were included in each experiment. PCR products were analyzed in 1% agarose gel stained with ethidium bromide. The PCR products were sequenced in both directions with the same set of primers. Sequencing was performed with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, USA).

Trace files were checked and edited using FinchTV 1.3.1 (Geospiza Inc., Seattle, USA). Contigs were aligned and manually assembled in GeneDoc v. 2.7.000 (Nicholas and Nicholas 1997). Phylogenetic trees were constructed by the neighbor-joining algorithm using the program MEGA version 4.0 (Tamura et al. 2007). Distance-based analyses were conducted using Kimura 2-parameter distance estimates using alignments

obtained from ClustalW. Bootstrap proportions were calculated by the analysis of 1 000 replicates of the phylogenetic tree.

From amongst the four examined species of wild cervids, *Giardia* cysts were detected only in one red deer and in two roe deer, originating from west-central and north-eastern regions of Poland, respectively. From the *Giardia*-positive faecal samples total DNA was extracted. All three genes were successfully amplified from the *Giardia* isolate obtained from red deer. Amplicons of *bg* and *gdh* genes were obtained from *Giardia* isolates derived from two roe deer, whereas PCR at the *tpi* locus failed. Subsequent sequencing revealed no double peaks in the chromatograms at the tested loci, and that the tested *Giardia* isolates fall into assemblage A.

The sequence of the *gdh* gene from the red deer *Giardia* isolate also showed 99% similarity with the sequences of the same molecular marker from roe deer (DQ100288) and cattle (DQ182607) and differed at this locus from two and four single-nucleotide polymorphisms (SNPs), respectively (van der Giesen et al. 2006, Langkjaer et al. 2007).

It is interesting to note that the *tpi* gene sequences of the Polish and Croatian (HQ259661) *Giardia* isolates from red deer showed only 96% similarity and differed at 16 SNPs (Beck et al. 2010).

The result of genotyping two *Giardia* isolates from roe deer at the *gdh* gene showed 100% similarity to human *Giardia* isolates (Portland 1, EF685701; RB, EF685702), which were determined as the AI sub-assemblage (Lassek-Nesselquist et al. 2010), and to a cat isolate (C43, AB569376), which belonged to assemblage A (Suzuki et al. 2011).

The nucleotide sequence of the *bg* gene of roe deer isolates did not match any of the known *Giardia* sequences at this locus.

Fig. 2. Phylogenetic relationship of 19 *Giardia* isolates inferred by the neighbour-joining analysis of the β -giardin nucleotide sequences. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Isolates from this study (in bold): JC002 – from red deer, CC1 and CC2 – from roe deer. Reference human isolates: WB and H2-001, (X85958 and FJ009208); LD18, Nij5, (AY072727, AY072725); GF-2 and GF-3 – ferret isolates (AB469365 and AB508814); A101 and swecat171 – cat isolates, (AY647264 and EU769206); P15 – reference cow isolate, (AY072729); A29 and A21 – reference dog isolates (AY545646 and AY545644); white-tailed deer (AY302561), fallow deer (DQ650649); BG-cer1 and BG-cer3 – moose isolates (DQ648781 and DQ648779). *Giardia muris* (AY258618) represents an outgroup.

However, these sequences exhibited 99% similarity with those of the isolates grouped in AI sub-assemblage: GF2 (AB469365) and GF3 (AB508814) from ferret, H2-001 (FJ009208) from human as well as BGcer-3 (DQ648779) from moose (Robertson et al. 2007, Abe et al. 2010, Solarczyk et al. 2010). The roe deer *Giardia* isolates differed at the *bg* locus from the above-mentioned isolates by one to five SNPs. All the substitutions were synonymous. No sequence variations were found between the roe deer *Giardia* isolates at both examined loci.

Nucleotide sequences of *bg*, *tpi* and *gdh* genes of *Giardia* isolate derived from red deer (EU626198, HM150751, HM150750), and sequences of *bg* and *gdh* genes of two *Giardia* isolates obtained from roe deer (HQ538712, HQ538713, HQ538714, HQ538715) were deposited in GenBank (NCBI).

The phylogenetic analysis of *bg* and *gdh* nucleotide sequences showed that the *Giardia* isolate from red deer clustered within the cervid-specific sub-assemblage AIII, whereas *Giardia* isolates from roe deer clustered within sub-assemblage AI that grouped zoonotic isolates (Figs. 1, 2). Bootstrap analysis indicated a strong statistical support for this grouping.

Our genotyping and phylogenetic analysis showed that red deer and roe deer were infected with different *G. duodenalis* genotypes belonging to assemblage A, which are predominant in wild mammals (Feng and Xiao 2011). In red deer we found a *Giardia* isolate belonging to sub-assemblage AIII. The sequences of the studied molecular markers of the *Giardia* isolate detected in red deer were identical to the sequences of the same gene fragments of *Giardia* isolates from fallow deer, roe deer, moose and cat, which were earlier determined as the AIII

sub-assemblage (van der Giessen et al. 2006, Lalle et al. 2007, Robertson et al. 2007, Lebbad et al. 2010). Previous studies indicated that *Giardia* isolates from the AIII sub-assemblage are specific for cervids. However, it should be assumed that host specificity of the isolates is not a stable phenotypic feature, because *Giardia* isolates belonging to the AIII sub-assemblage were found in wild boar and cat (Cacciò and Ryan 2008, Beck et al. 2010, Lebbad et al. 2010). It should also be noted that a genotype belonging to sub-assemblage AIII has never been identified in humans (Sprong et al. 2009), whereas the *Giardia* genotype belonging to sub-assemblage AI, which we found in two roe deer, has zoonotic potential (Sprong et al. 2009). In this study, both roe deer *Giardia* isolates shared the same sequences at the *gdh* locus as those isolates that were obtained from humans and cat (Lassek-Neselquist et al. 2010, Suzuki et al. 2011), while from one to five SNPs were found at the *bg* locus compared to sequences from *Giardia* collected from ferret, moose, and humans (Robertson et al. 2007, Abe et al. 2010, Solarczyk et al. 2010).

Since no sequence variations were observed between the roe deer *Giardia* isolates at both tested loci, it should be assumed that the infection source was the same for both animals. This is likely because both of the roe deer were shot during the same hunt. Since feeding ranges of red deer and roe deer often overlap with livestock pastures and some wild animals also feed on cultivated fields and pastures fertilized with manure or liquid manure, transmission of *Giardia* cysts between domestic and sylvatic animals, and vice versa, may be important. Recently, an extensive molecular characterization of 2418 human and ani-

mal *Giardia* isolates at four loci has shown that sub-assemblage AI is usually found in livestock, domestic animals and wildlife, but less often in humans, whereas AIII is mainly detected in wild mammals and very rarely in companion animals and cattle (Sprong et al. 2009).

Moreover, this study found variation in the geographic distribution of sub-assemblage AI; it is predominantly found in humans in Asia and Australia, and less often in Europe. Nevertheless, hunters should practice proper hygienic measures during evisceration of shot animals and not to leave the viscera of these animals. Such procedures are necessary to reduce the risk of infection and transmission of *Giardia* cysts to other sylvatic animals.

The present study provides the first molecular characterization of the *Giardia* isolates from red deer and roe deer in Poland. Our data indicate that deer can be infected with various genotypes of *G. duodenalis* assemblage A. Further studies are necessary to explain which *Giardia* assemblages and/or sub-assemblages occur in wild cervids in various regions of the world. Moreover, the impact of *Giardia* infection on the health of wild cervids should also be elucidated.

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