Epidemiological survey in Łęczyńsko-Włodawskie Lake District of eastern Poland reveals new evidence of zoonotic potential of Giardia intestinalis

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Abstract

Faecal samples from 297 farm animals were collected from 18 households in distinct sites of the Łęczyńsko-Włodawskie Lake District of eastern Poland. They included samples from 86 cattle (Bos taurus), 84 pigs (Sus scrofa f. domestica), 81 sheep (Ovis aries), 10 horses (Equus caballus), and 36 dogs (Canis lupus familiaris). The samples were examined for the presence of Giardia intestinalis by the Direct Fluorescence Assay (DFA) and semi-nested PCR. All amplicons were sequenced on both strands. By DFA, cysts of Giardia spp. were detected in 66 of 297 faecal samples (22.2%). Positive specimens for Giardia spp. were derived from 29.8% of examined pigs, 21.0% of sheep, 18.6% of cattle, 10% of horses, and 19.4% of dogs. Based on the detection of the β-giardin gene by PCR, 39 (13.1%) of the 297 examined samples were recognized as positive. Detection of the presence of Giardia cysts by DFA test was overall significantly higher compared to PCR (p=0.0045). By PCR, Giardia was found in 28.1% of sheep, 11.6% of cattle, 10% of horses, 9.5% of pigs and 5.6% of dogs. Partial β-giardin gene sequences were obtained for 73.7% of the PCR positive samples. From sequenced samples derived from the studied animals, Giardia were identified as assemblage A (8 samples), B (1 sample) and E (18 samples). As assemblages A and B may be zoonotic, the farm animals living in eastern Poland could be regarded as a potential source of Giardia infection for humans.

Key words

Giardia intestinalis, farm animals, faecal samples, eastern Poland, Direct Fluorescence Assay, PCR

INTRODUCTION

Giardiasis is a common gastrointestinal infection, occurring worldwide in livestock, pets and wildlife [1, 2]. The parasite has two stages in the life cycle: infective cyst and trophozoite. The major sources and routes of cyst transmission are contaminated water and food or direct fecal-oral contact [3]. Non-specific symptoms, such as diarrhea, greasy stools, flatulence and abdominal cramps, might be noticed during the course of infection. As a high percentage of cases are asymptomatic, infection in young children may be the cause of poor cognitive function and stunted growth [4]. Basic diagnostic methods of G. intestinalis are relatively simple and commercial tests are widely available. The diagnostics involve light microscopy examination of stool samples, ELISA or Direct Fluorescence Assay (DFA). However, only the molecular characterization of G. intestinalis genotypes guarantees accurate identification of organisms and assessment of zoonotic transmission [5, 6, 7].

To date, eight assemblages of G. intestinalis have been identified. Generally, assemblages A and B infect humans and are potentially zoonotic [8]. There are numerous papers describing the occurrence of assemblages A and B among various mammals such as non-human primates, hoofed animals, beavers, marsupials, rodents, canids and felids [1, 9]. To date, it has been suggested that zoonotic transmission of Giardia can also occur between dogs and humans living in the same locality [10, 11]. Assemblages C-H appear to be host specific. Also, a difference in polymorphism among assemblages has been noticed. According to data, assemblage B is the most variable, which causes problems with data interpretation during molecular diagnostics [12, 13, 14].

Despite the number of conducted epidemiological surveys being high, data about the prevalence of G. intestinalis infection among farm animals in central and eastern Europe are fragmentary, and often involve only the microscopic or serological investigation without genotyping [13].

The aim of this study was to estimate the prevalence of G. intestinalis infection among selected species of farm and companion animals from Łęczyńsko-Włodawskie Lake District of eastern Poland, based on the examination of faeces with the use of DFA and PCR tests.

MATERIALS AND METHODS

Farm animals. Faecal samples from 297 animals were collected in 2013–2014. They included samples from 86 cattle (Bos taurus), 84 pigs (Sus scrofa f. domestica), 81 sheep (Ovis aries), and 10 horses (Equus caballus) bred in 18 households in Łęczyńsko-Włodawskie Lake District (Fig. 1). Faecal samples were collected from sites in the vicinity of animals. Thirty-six samples from dogs (Canis lupus familiaris) were also collected; the animals came both from urban as well as rural areas, including households, in which samples were acquired from other animals species. Samples were stored at 4–8 °C until further analysis.
During the survey, additional sampling (faecal samples) were collected from two farmers living in one of households.

**Stool samples preparation procedure.** One gram of faeces was mixed with 8 ml of 25% Percoll solution and filtered through a plastic sieve into a beaker. The suspension was poured into a 15 ml conical centrifuge tube, followed by centrifugation at 1,600 g for 5 min. Three volumes of saturated NaNO₃ were added to form a sediment and mixed, followed by centrifugation at 1,000 g for 10 min. Then, 200 µl solution from top layer was removed, placed in the new 15-ml conical centrifuge tube and washed by centrifugation (1,100 g × 10 min) in 13 ml re-distilled water. Sediment was stored at 4°C until further analysis [15].

**Direct Fluorescence Assay (DFA).** DFA was performed using commercial test Aqua-Glo™ G/C (Waterborne Inc., New Orleans, USA). Twenty-five microliters of sediment, obtained after performing the concentration methods, was placed on a microscopic slide and allowed to dry at room temperature. Next, samples were fixed by 50 µl of methanol and allowed to dry. After that, 50 µl of 4′-diamidino-2-phenyl indole (DAPI) in PBS (0.4 μg DAPI/ml) was dropped onto the slide and left for 4 min at room temperature. After removal of the DAPI, the slide was rinsed by adding 100 µl wash buffer and left for 1 minute. The wash buffer was then removed, and 50 µl of conjugate anti-*Giardia* with fluorescein isothiocyanate (FITC) placed in each well. The slides were placed in a humidified chamber and incubated at 37°C for 30 min. The washing step was performed (as described above). One drop of BlockOut™ counterstain was added to each well to reduce non-specific background fluorescence, and the slide incubated for 1 minute at room temperature. After washing (as described above) and drying the slide, a drop of Fade™ mounting medium was placed in each well, covered by glass, and viewed under epifluorescence microscope (×400). *Giardia* cysts were identified on the basis of their size shape, and structure according to guidelines described in method 1623 [16]. Positive and negative controls were used. The investigation was performed in a qualitative manner.

**DNA extraction and PCR amplification.** Two hundred microliters of sediment obtained by the stool concentration technique described above were subjected to DNA extraction. The samples underwent seven freeze-thawing cycles by placing the tubes in liquid nitrogen, followed by immediately placing the tubes in 70°C water.

DNA was extracted using QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol (samples were lysed with proteinase K overnight). The extracted DNA was stored at -20°C until PCR assay. The semi-nested PCR described by Caccio et al. [17] with slight modifications was performed. Each reaction mixture (50 µl) contained 10 pmol of each primer (G7 and G759 or G376 and G759), 0.2 mM of each dNTPs (Fermentas, Vilnius, Lithuania), 50 mM of KCl, 10 mM of Tris-HCl (pH 9.0), 2 mM of MgCl₂, 1.5 U of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany) and 1–2 µl of DNA. Amplification was performed using a TProfessional 48 thermal cycler (Biometra GmbH, Göttingen, Germany). Each semi-nested PCR product was subjected to electrophoresis (1.5% agarose gel stained with ethidium bromide). Negative and positive DNA samples were included in each PCR reactions.

**Sequences and phylogenetics analysis.** Suitable PCR products were purified by spin column (QiAquick PCR Purification Kit, Qiagen GmbH), and sequenced on both strands using a BigDye Cycle Sequencing Kit (Qiagen GmbH, Hilden, Germany) and 1–2 µl of DNA. Amplification was performed using a TProfessional 48 thermal cycler (Biometra GmbH, Göttingen, Germany). Each semi-nested PCR product was subjected to electrophoresis (1.5% agarose gel stained with ethidium bromide). Negative and positive DNA samples were included in each PCR reactions.

**Statistical analysis.** Differences between frequency of the detection of *Giardia* cysts by microscopy and PCR were assessed by Student’s t-test using STATISTICA v. 5.1 package (Statsoft, Tulsa, OK, USA).

**Nucleotide sequence accession numbers.** All nucleotide sequences were submitted to the National Centre for Biotechnology Information (NCBI) GenBank database under the following accession numbers: cattle – KT731976-KT731978, KT731980, KT731982; dogs – KT731986,

RESULTS

Farm animals – DFA. Cysts of Giardia spp. were detected in 66 of 297 faecal samples (22.2%). Positive specimens for Giardia spp. were derived from 29.8% of examined pigs, 21.0% of sheep, 18.6% of cattle and 10% of horses. Analysis of the 36 samples derived from dogs resulted in the detection of 7 (19.4%) Giardia positive stool samples.

The number of cysts varied, from single to several hundred per slide, but a connection between number of cysts and species of host was not observed.

Positive results were obtained for pigs bred in 6 households, cattle from 6 households, sheep from 4 households and horses from one household.

PCR. Based on the detection of β-giardin gene of Giardia, 39 (13.1%) of the 297 examined samples were positive. According to data, detection of the presence of Giardia cysts by PCR was overall significantly less sensitive compared to DFA test (p=0.0045). Giardia was found in 22.2% of sheep, 11.6% of cattle, 10% of horses, 9.5% of pigs and 5.6% of dogs.

Comparison of the DFA and PCR positive results showed that they were similar for sheep (21.0% vs. 22.2%; p=0.853) and horses (10.0% vs. 10.0%; p=1.000). For pigs, the number of positive results in PCR was significantly lower in comparison to DFA (9.5% vs. 29.8%; p=0.0011) (Tab. 1). The numbers of positive results in PCR for cattle and dogs were lower compared to DFA (11.6% vs. 18.6%, and 5.6% vs. 19.4%, respectively) but the differences did not attain significance level (p=0.2016, and p=0.081, respectively).

People. By DFA and PCR, two Giardia positive samples were found from two persons who worked on the sheep farm, where simultaneously parasite was found in sheep and dogs. Sequence analysis of human and animals samples showed the occurrence of assemblage A.

Phylogenetics analysis. The relations between the obtained sequences are shown by a phylogenetic tree (Fig. 2) Sequences obtained in the study were distributed among three clades representing genotypes A, B and E; each group was represented by 10, 1 and 18 samples, respectively. On the 3 farms, the occurrence of 2 genotypes was reported. The variability of the parasite population was shown by occurrence of several SNPs. The consensus sequence obtained from the sequences assigned to clade E showed the highest similarity to P15 isolate (GenBank:AY072729) derived from pig. Nevertheless, the sequence comparison resulted in the detection of 5 SNPs. The single isolate, described as genotype B, showed the highest similarity to the LD18 isolate sequence (GenBank:AY072726) which originated from humans. There were 6 SNPs in the alignment of these sequences. Among isolates from clade A, a high level of sequences variability also
occurred. The sequence of B7V_oct’11 (GenBank: KF963547) isolate from water were highly similar to consensus sequences obtained from studied isolates assigned as assemblage A. The prevalence of *Giardia* infection and the distribution of the assemblages among all of the samples are shown in Table 1.

**Table 1. Prevalence of *G. intestinalis* among livestock and pets in Łęczyńsko-Włodawskie Lake District**

<table>
<thead>
<tr>
<th>Species</th>
<th>DFA</th>
<th>PCR</th>
<th>N (%) samples of sequenced assemblages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle (<em>Bos taurus</em>)</td>
<td>16/66 (16.6%)</td>
<td>10/86 (11.6%)</td>
<td>4/86 (4.7%) [A, E]</td>
</tr>
<tr>
<td>Sheep (<em>Ovis aries</em>)</td>
<td>17/81 (21.0%)</td>
<td>18/81 (22.2%)</td>
<td>16/81 (19.8%) [A, E]</td>
</tr>
<tr>
<td>Horses (<em>Equus caballus</em>)</td>
<td>1/10 (10.0%)</td>
<td>1/10 (10.0%)</td>
<td>1/10 (10.0%) [E]</td>
</tr>
<tr>
<td>Pigs (<em>Sus scrofa f. Domestica</em>)</td>
<td>25/84 (29.8%)</td>
<td>8/84 (9.5%)</td>
<td>5/84 (6.0%) [B, E]</td>
</tr>
<tr>
<td>Dogs (<em>Canis lupus familiaris</em>)</td>
<td>7/36 (19.4%)</td>
<td>2/36 (5.6%)</td>
<td>2/36 (5.6%) [A, E]</td>
</tr>
<tr>
<td>Total</td>
<td>66/297 (22.2%)</td>
<td>39/297 (13.1%)</td>
<td>28/297 (9.4%)</td>
</tr>
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</table>

**DISCUSSION**

This study has provided new data about the prevalence of *Giardia* in eastern Poland (Łęczyńsko Włodawskie Lake District), especially with respect to the role of farm animals as a source of the parasite. Genotyping provided the data to conduct analysis of the spreading of the parasite between hosts. The region of the study – the Łęczyńsko Włodawskie Lake District – is an example of a rural area rich in swamps and watercourses, thus representing an environment suitable for the spread of the studied protozoa. The study concentrated on the prevalence of *Giardia* in livestock and pets, provided insights into the prevalence and genotypes of the parasite in selected hosts. According to the obtained results, the prevalence of *G. intestinalis* among farm animals in the Łęczyńsko-Włodawskie Lake District is relatively high. In Poland, the survey focused on the prevalence of *Giardia* in sheep and cattle, showed 1.3% and 2–14% prevalence respectively [18]. The study conducted among dogs resulted in a broad spectrum of prevalence, from 5% – 36% [13]. Worldwide, the results were even more dispersed and the occurrence of the parasite on each farm varied [19]. Furthermore, in previous surveys, *Giardia* was also found in a single animal as well as in all examined animals, which is corresponding with data in the presented study [20, 21, 22]. Similar results were also obtained in this study concerning the prevalence of *Giardia* in sheep and cattle, which ranged in previous studies from 1.5% – 38% in sheep and from 3.7% – 57.8% in cattle. However, in the current study, among pigs the incidence of *G. intestinalis* infection was higher than previously described (up to 31.1%) [22, 23, 24, 25]. In contrast, the prevalence of *Giardia* in dogs was lower than some data presented in literature where authors found the parasite in each studied dog [26, 27]. There are only few data about prevalence of *Giardia* in horses [28]; unfortunately, during the presented study it was possible to collect only 10 samples, which needs continuation. The high percentages of infected goats and cats (species not included in this study), has been previously described (up to 31.1%) [22, 23, 24, 25]. In contrast, the incidence of *G. intestinalis* infection was higher than in previous studies from 1.5% – 38% in sheep and from 3.7% – 14% in cattle [28]. In dogs, the assemblages A, B, C and D are infectious. In the presented study, analysis of stool samples derived from sheep, dogs and humans from one farm was conducted, and the same genotype (assemblage A) was found among all hosts, which strongly suggests cross-infection between them.

Assemblage B was widespread among various hosts, and it is possible that it can transfer between different environments – forest and agricultural. In a previous study by the authors of the presented article, assemblage D was found in wolves, but in the current study was not detected among pets and livestock [33]. However, the occurrence of genotype E was also found, which is characteristic for livestock.

Overall, in the presented study, DFA seems to be more sensitive than PCR, which corresponds with previous findings by the authors [33]. In contrast to PCR assay, DFA examination was more sensitive in the case of samples from pigs, cattle, and dogs, and less sensitive for samples from sheep. The lower sensitivity of PCR could be caused by the presence of empty cells of *Giardia* which might still contain the residual body and considered as dead [34]. Inhibitors of enzymatic reactions frequently occurring in stool could directly affect the success of efficient PCR reaction, and might be a cause of false-negative results [35, 36].

The high molecular variability in this study suggest a mixed population of the parasite occurring in the studied region, and among each host. Further studies on cross-infection could produce additional evidence of *Giardia* zoonotic or reverse-zoonotic transmission, and elucidate the direct routes of infection.

**REFERENCES**