ORIGINAL ARTICLE

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

Krzysztof Stojecki[1], Jacek Sroka[1,2], Tomasz Cencek[1], Jacek Dutkiewicz[2]

[1] Department of Parasitology, National Veterinary Research Institute, Pulawy, Poland
[2] Department of Biological Health Hazards and Parasitology, Institute of Rural Health, Lublin, Poland

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.

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.

Annals of Agricultural and Environmental Medicine 2015, Vol 22, No 4

595

Krzysztof Stojecki, Jacek Sroka, Tomasz Cencek, Jacek Dutkiewicz. Epidemiological survey in Łęczyńsko-Włodawskie Lake District of eastern Poland reveals new …
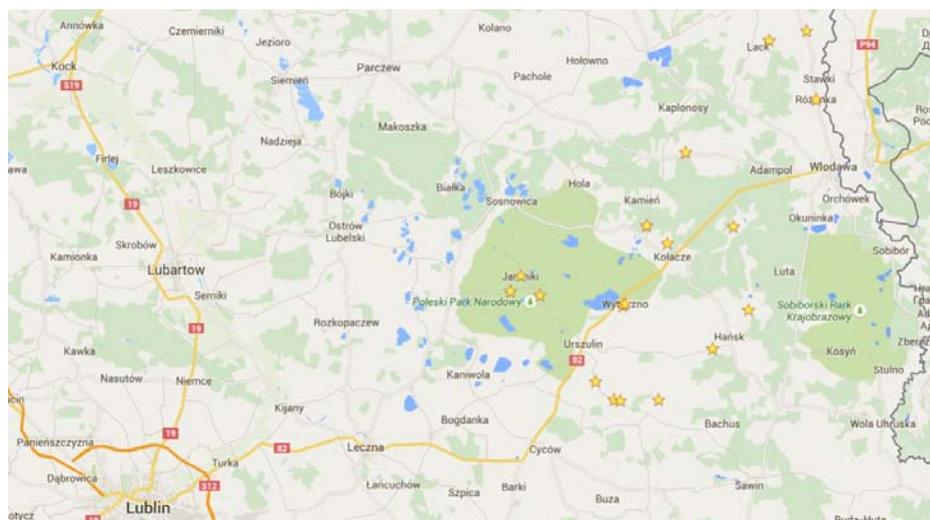
Figure 1. Map of sampling sites marked with stars (reprinted from Google Maps, 2015)

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 $NaNO_3$ 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 4'6-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 $MgCl_2$, 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 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Chromatograms were examined using BioEdit (http://www.mbio.ncsu.edu/BioEdit/). Nucleotide sequences were compared with those available in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

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,

596

Annals of Agricultural and Environmental Medicine 2015, Vol 22, No 4

Krzysztof Stojecki, Jacek Sroka, Tomasz Cencek, Jacek Dutkiewicz. Epidemiological survey in Łęczyńsko-Włodawskie Lake District of eastern Poland reveals new …

KT731987; horse – KT731988; human – KT731989; pigs – KT731990 – KT731994; sheep – KT731995 – KT732004, KT732007 – KT732011, KT732013.

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 sequences 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 this sequences. Among isolates from clade A, a high level of sequences variability also
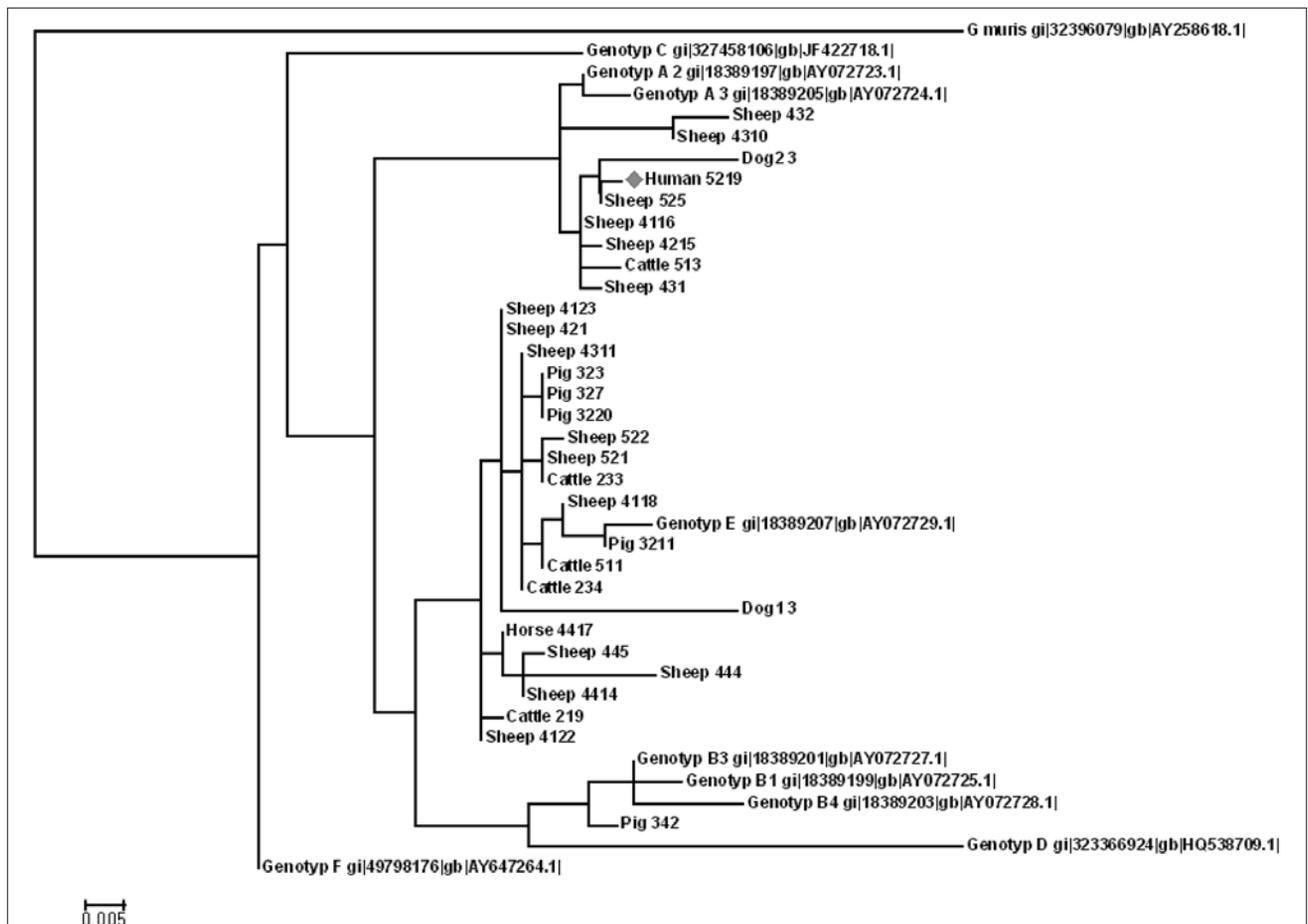

Figure 2. Phylogenetics tree – relationships between sequenced samples

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

Species	DFA	PCR	N (%) samples of sequenced [assemblages]
Cattle (*Bos taurus*)	16/86 (18.6%)	10/86 (11.6%)	4/86 (4.7%) [A, E]
Sheep (*Ovis aries*)	17/81 (21.0%)	18/81 (22.2%)	16/81 (19.8%) [A, E]
Horses (*Equus caballus*)	1/10 (10.0%)	1/10 (10.0%)	1/10 (10.0%) [E]
Pigs (*Sus scrofa* f. *Domestica*)	25/84 (29.8%)	8/84 (9.5%)	5/84 (6.0%) [B, E]
Dogs (*Canis lupus familiaris*)	7/36 (19.4%)	2/36 (5.6%)	2/36 (5.6%) [A, E]
Total	66/297 (22.2%)	39/297 (13.1%)	28/297 (9.4%)

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 also reported by researchers [27, 29, 30].

Although there are few articles bringing insights into *G. intestinalis* zoonotic potential, the topic is under discussion among scientists [31].

To-date, 8 genotypes of *G. intestinalis* has been established. Genotype A and B, has been found in a wide range of hosts, including humans [11]. This parasite is only occasionally found in pigs. In the presented study, similar to that by Farzan et al [32], genotypes B and E were detected among pigs. In general, humans are susceptible to infection by *G. intestinalis* genotypes A and B [31], which corresponds with the results from two people from farm in the current study. For 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

1. Thompson RCA, Smith A. Zoonotic enteric protozoa. Vet Parasitol. 2011; 182(1): 70–78.
2. Lane S, Lloyd, D. Current trends in research into the waterborne parasite Giardia. Crit Rev Microbiol. 2002; 28(2): 123–147.
3. Adam RD. Biology of *Giardia lamblia*. Clin Microbiol Rev. 2001; 14(3): 447–475.
4. Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet 2002; 359(9306): 564–571.
5. Plutzer J, Ongerth J, Karanis P. Giardia taxonomy, phylogeny and epidemiology: Facts and open questions. Int J Hyg Eviron Health 2010; 213(5): 321–333.
6. Guy RA, Payment P, Krull UJ, Horgen PA. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol. 2003; 69(9): 5178–5185.
7. Lasek-Nesselquist E, Welch DM, Sogin ML. The identification of a new *Giardia duodenalis* assemblage in marine vertebrates and a preliminary analysis of *G. duodenalis* population biology in marine systems. Int J Parasitol. 2010; 40(9): 1063–1074.
8. Inpankaew T, Traub R, Thompson RC, SukthanaY. Canine parasitic zoonoses in Bangkok temples. Southeast Asian J Trop Med Publ Health 2007; 38(2): 247–255.
9. Sroka J, Giżejewski Z, Wójcik-Fatla A, Stojecki K, Bilska-Zając E, Dutkiewicz J, Cencek T, Karamon J, Zając V, Kusyk P, Dąbrowska J, Kochanowski M. Potential role of beavers (*Castor fiber*) in contamination of water in Masurian Lakes District (north-eastern Poland) with

598

Annals of Agricultural and Environmental Medicine 2015, Vol 22, No 4

Krzysztof Stojecki, Jacek Sroka, Tomasz Cencek, Jacek Dutkiewicz. Epidemiological survey in Łęczyńsko-Włodawskie Lake District of eastern Poland reveals new …

protozoan parasites *Cryptosporidium* spp. and *Giardia duodenalis*. Bull Vet Inst Pulawy 2015; 59(2): 219–228.

10. Lebbad M, Mattsson JG, Christensson B, Ljungström B, Backhans A, Andersson JO, Svärd SG. From mouse to moose: Multilocus genotyping of *Giardia* isolates from various animal species. Vet Parasitol. 2010; 168(3): 231–239.

11. Ryan U, Cacciò SM. Zoonotic potential of *Giardia*. Int J Parasitol. 2013; 43(12): 943–956.

12. Sprong H, Cacciò SM, van der Giessen JW. Identification of zoonotic genotypes of *Giardia duodenalis*. PLoS Neglect Trop Dis. 2009; 3(12): e558.

13. Bajer A. *Cryptosporidium* and *Giardia* spp. infections in humans, animals and the environment in Poland. Parasitol Res. 2008; 104(1): 1–17.

14. Pertoft H, Warmegard B, Hook M. Heterogeneity of lysosomes originating from rat liver parenchymal cells. Metabolic relationship of subpopulations separated by density-gradient centrifugation. Biochem J. 1978; 174: 309–317.

15. Stojecki K, Sroka J, Karamon J, Kusyk P, Cencek T. The influence of selected stool concentration techniques for the effectiveness PCR examination in *Giardia lamblia* diagnostics. Pol J Vet Sci. 2014; 17: 231–237.

16. U.S. Environmental Protection Agency. 2001. Method 1623: *Cryptosporidium* and *Giardia* in water filtration/IMS/FA. EPA 821-R-01–025. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.

17. Cacciò SM, De Giacomo M, Pozio E. Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol 2002; 32: 1023–1030.

18. Bajer A, Bednarska M, Siński E. [*Cryptosporidium* spp. and *Giardia* spp. – environmental studies in Poland]. Wiad Parazytol. 2009; 55: 301–304.

19. Thompson RCA, Monis PT. Variation in *Giardia*: implications for taxonomy and epidemiology. Adv Parasitol. 2004; 58: 69–137.

20. Huetink REC, Van der Giessen JWB, Noordhuizen JPTM, Ploeger HW. Epidemiology of Cryptosporidium spp. and *Giardia duodenalis* on a dairy farm. Vet Parasitol. 2001; 102: 53–67.

21. Traub RJ, Monis PT, Robertson I, Irwin P, Mencke N, Thompson RCA. Epidemiological and molecular evidence supports the zoonotic transmission of Giardia among humans and dogs living in the same community. Parasitology 2004; 128: 253–262.

22. Olson ME, Thorlakson CL, Deselliers L, Morck DW, McAllister TA. Giardia and Cryptosporidium in Canadian farm animals. Vet Parasitol. 1997; 68: 375–381.

23. Giangaspero A, Paoletti B, Iorio R, Traversa D. Prevalence and molecular characterization of *Giardia duodenalis* from sheep in central Italy. Parasitol Res. 2005; 96: 32–37.

24. Maddox-Hyttel C, Langkjær RB, Enemark HL, Vigre H. Cryptosporidium and Giardia in different age groups of Danish cattle and pigs—occurrence and management associated risk factors. Vet Parasitol. 2006; 141: 48–59.

25. Feng Y and Xiao L. Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. Clin Microbiol Rev. 2011; 24(1): 110–140.

26. Barr SC, Bowman DD, Heller RL, Erb HN. Efficacy of albendazole against giardiasis in dogs. Am J Vet Res. 1993; 54: 926–928.

27. Monis PT, Thompson RCA. Cryptosporidium and Giardia-zoonoses: fact or fiction? Infect Genet Evolution. 2003; 3: 233–244.

28. Johnson E, Atwill ER, Filkins ME, Kalush J. The prevalence of shedding of Cryptosporidium and Giardia spp. based on a single fecal sample collection from each of 91 horses used for backcountry recreation. J Vet Diag Invest. 1997; 9: 56–60.

29. Diaz V, Campos M, Lozano J, Manas I, Gonzalez J. Aspects of animal giardiosis in Granada province (southern Spain). Vet Parasitol. 1996; 64: 171–176.

30. Vasilopulos RJ, Rickard LG, Mackin AJ, Pharr GT, Huston CL. Genotypic analysis of *Giardia duodenalis* in domestic cats. J Vet Intern Med. 2007; 21: 352–355.

31. Cacciò SM, Ryan U. Molecular epidemiology of giardiasis. Mol Biochem Parasitol. 2008; 160: 75–80.

32. Farzan A, Parrington L, Coklin T, Cook A, Pintar K, Pollari F, Friendship R, Farber J, Dixon B. Detection and characterization of *Giardia duodenalis* and Cryptosporidium spp. on swine farms in Ontario, Canada. Foodborne Pathog Dis. 2011; 8: 1207–1213.

33. Stojecki K, Sroka J, Caccio SM, Cencek T, Dutkiewicz J, Kusyk P. Prevalence and molecular typing of *Giardia duodenalis* in wildlife from eastern Poland. Folia Parasitol. 2015; 62: 042.

34. Robertson LJ, Campbell AT, Smith HV. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl Environ Microbiol. 1992; 58(11): 3494–3500.

35. Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Mégraud F. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol. 1997; 35(4): 995–998.

36. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors–occurrence, properties and removal. J Appl Microbiol. 2012; 113(5): 1014–1026.