Detection of *Giardia intestinalis* in water samples collected from natural water reservoirs and wells in northern and north-eastern Poland using LAMP, real-time PCR and nested PCR

Anna Lass, Beata Szostakowska, Krzysztof Korzeniewski and Panagiotis Karanis

ABSTRACT

Giardia intestinalis is a protozoan parasite, transmitted to humans and animals by the faecal–oral route, mainly through contaminated water and food. Knowledge about the distribution of this parasite in surface water in Poland is fragmentary and incomplete. Accordingly, 36 environmental water samples taken from surface water reservoirs and wells were collected in Pomerania and Warmia-Masuria provinces, Poland. The 50 L samples were filtered and subsequently analysed with three molecular detection methods: loop-mediated isothermal amplification (LAMP), real-time polymerase chain reaction (real-time PCR) and nested PCR. Of the samples examined, *Giardia* DNA was found in 15 (42%) samples with the use of LAMP; in 12 (33%) of these samples, *Giardia* DNA from this parasite was also detected using real-time PCR; and in 9 (25%) using nested PCR. Sequencing of selected positive samples confirmed that the PCR products were fragments of the *Giardia intestinalis* small subunit rRNA gene. Genotyping using multiplex real-time PCR indicated the presence of assemblages A and B, with the latter predominating. The results indicate that surface water in Poland, as well as water taken from surface wells, may be a source of *Giardia* strains which are potentially pathogenic for humans. It was also demonstrated that LAMP assay is more sensitive than the other two molecular assays.

Key words | environment, genotype, *Giardia*, LAMP, nested PCR, real-time PCR

Anna Lass
Beata Szostakowska
Department of Tropical Parasitology, Institute of Maritime and Tropical Medicine in Gdynia,
Medical University of Gdansk,
9b Powstania Styczniowego Str.,
Gdynia 81-519,
Poland

Anna Lass
Panagiotis Karanis (corresponding author)
State Key Laboratory of Plateau Ecology and Agriculture, Center for Biomedicine and Infectious Disease, Qinghai Academy of Animal Sciences and Veterinary Medicine,
Medical School of Qinghai University Xining,
1#Wei'er Road, Qinghai Biological Scientific Estate Garden,
Xining 810016,
P. R. China
E-mail: *panagiotis.karanis@uk-koeln.de*

Krzysztof Korzeniewski
Epidemiology and Tropical Medicine Department in Gdynia,
Military Institute of Medicine in Warsaw,
Grudzinskiego St. 4,
Gdynia 81-103,
Poland

INTRODUCTION

Giardia intestinalis is a protozoan parasite causing gastrointestinal diseases in humans and animals worldwide (Karanis & Ey 1998; Smith *et al.* 2007; Reynolds *et al.* 2008; Ryan & Cacciò 2013). It has been estimated that ingestion of as few as ten cysts can lead to the development of infection in humans and animals (Rendtorff 1954). The clinical manifestation of giardiasis includes severe diarrhoea, abdominal cramps, nausea, weight loss, and an acute phase which usually lasts a few weeks and which may develop into a chronic recurring disease (Wolfe 1992; Farthing 1996; Adam 2001; Lebwohl *et al.* 2003). The prevalence of *Giardia* infections in humans was previously estimated at 2–5% in industrialised countries and 20–30% in developing countries (Thompson & Monis 2004). However, regarding Europe, the epidemiology report from ECDPC in 2014 confirmed 17,278 giardiasis cases, reported by 23 countries in the EU/EEA, with the highest number of cases reported by Germany ($n = 4{,}011$), followed by the United Kingdom ($n = 3{,}628$), which accounted for 44% of cases (ECDPC 2015).

This parasite is transmitted by the faecal–oral route, through contaminated water and food (Karanis *et al.* 2007; Yoshida *et al.* 2007; Baldursson & Karanis 2011; Efstratiou *et al.* 2017). The presence of *Giardia* cysts in water is a problem throughout the world. They are resistant to

doi: 10.2166/wh.2017.039

unfavourable environmental conditions and can remain viable for almost two months at 0–2 °C (deRegnier *et al.* 1989). To date, at least 905 outbreaks associated with waterborne transmission of protozoan parasites have been reported, of which *G. intestinalis* has been responsible for 40.6% over the last 100 years (Karanis *et al.* 2007), 35.2% in the period 2004–2010 (Baldursson & Karanis 2011) and 37% in the period 2011–2116 (Efstratiou *et al.* 2017). In Europe, a major waterborne outbreak of giardiasis occurred in Bergen, Norway in 2004 (Nygård *et al.* 2006). Surface water such as rivers and lakes may be contaminated through the discharge of untreated and treated sewage and runoff of manure (Karanis *et al.* 1998, 2007; Hunter & Thompson 2005; Baldursson & Karanis 2011; Efstratiou *et al.* 2017). In terms of sources of contamination of water and food, a particularly important role is played by various hosts acting as reservoirs of infection, including farm, household, and wild animals. For example, semi-aquatic species such as beavers deposit their faeces in water, and commensal rodents (house mice, rats, muskrats) contribute to soil/food contamination affecting both humans and animals. Some species, e.g., birds or insects, may also act as vectors facilitating the mechanical transmission of cysts (Bajer 2008).

The distribution of *Giardia* infections in humans in Poland (1–9% in children; 3–7% adults) is similar to that noted in other European countries (Bajer 2008). However, knowledge about the occurrence of this parasite in surface water in Poland is fragmentary. So far, only a few studies have shown its prevalence in various bodies of water in this country. *Giardia* spp. cysts, among others, were microscopically detected in 85% of water samples taken from 13 WTPs located in eastern Poland (Sroka *et al.* 2013). Evidence of environmental contamination also derives from studies reporting the presence of this protozoan in surface water reservoirs and fresh fruits in different parts of Poland (Szostakowska *et al.* 2005; Jędrzejewski & Majewska 2007; Polus & Kocwa-Haluch 2014; Adamska 2015).

Differentiation of genotypes cannot be done on the basis of parasite morphology, but requires molecular methods (Karanis & Ey 1998; Plutzer *et al.* 2008; Plutzer & Karanis 2009). The aim of the study was to estimate the occurrence of *G. intestinalis*, including genotypes pathogenic to humans, in water samples collected from surface water reservoirs and wells in northern and north-eastern Poland using three molecular detection methods, namely loop-mediated isothermal amplification (LAMP), real-time polymerase chain reaction (real-time PCR) and nested PCR, and to compare the usefulness of these methods for screening environmental samples for *Giardia* cysts.

MATERIALS AND METHODS

Sampling

Sampling sites and study area

In total, 36 environmental water samples were collected in 2012–2014 from the area of two neighbouring provinces in Poland, Pomerania (in the north) ($n = 16$) and Warmia-Masuria (north-eastern part of the country) ($n = 20$). In total, 26 water samples were taken from 13 surface water reservoirs and ten water samples from five surface wells 5–7 m in depth (Table 1, Figure 1). Surface water samples originated from 11 lakes, including Choczewskie, Żarnowieckie, Wysockie, Sitno, Dłużecko, Osowskie, Dobre, Rydzówka, Mamry, Jeziorak, and Pozorty, a pond, and the Vistula Lagoon (Figure 1). Most of the lakes selected for investigations are often used as recreational sites for tourists and local inhabitants, with watering places, guesthouses and campgrounds in the vicinity. The lakes differ in terms of surface area and water quality. The Vistula Lagoon, also used for water sports and recreation, is a brackish-water lagoon on the Baltic Sea roughly 90 km long and 10 to 19 km wide, separated from Gdańsk Bay by the Vistula Spit. The lagoon serves as the mouth of several branches of the Vistula River and is connected to Gdańsk Bay via the Strait of Baltiysk. All wells, as well as the pond, examined in this study are located in homesteads (gardens/yards) situated in small villages in Warmia-Masuria province. According to the owners, water from these wells is not used for drinking; however, it might be used to water plants in kitchen gardens.

Water samples were collected twice from the same sampling sites. One sample was taken between May and June (when the water temperature is relatively cool), the other between August and October (when the water is warmer).

Table 1 | Summary results of detection of *Giardia intestinalis* DNA in different water samples collected from surface water reservoirs and wells in the Pomerania province and Warmia-Masuria province, Poland using three assays: LAMP, real-time PCR and nested-PCR

		Positive results of molecular investigations					
		LAMP		Real-time PCR		Nested PCR	
Type of sampling site	Number of samples investigated	No.	%	No.	%	No.	%
Surface water	26	10	38.46	8	30.76	7	26.92
Lakes	22	7		5	22.72	5	22.72
Pond	2	1		1		1	
Lagoon	2	2		2		1	
Wells	10	5	50	4	40	2	20
Total	36	15	41.66	12	33.33	9	25

Sampling system

Fifty-litre samples of surface water and water from wells were collected and filtered on site with the use of a portable filtration set consisting of clean 10-L polythene vessels (used for taking water from the source), a peristaltic pump head (Masterflex L/S Easy-Load II) attached to the handle of a cordless drill/driver (DeWalt DC980 Type 10), an Envirochek capsule membrane filter (Pall-Gelman Laboratory, USA), a flow meter (PTFE, Aalborg, USA), a manometer, and elastic pipes. Water from lakes, the pond and the lagoon was taken 1–3 m from the shore. If necessary, in order to remove larger particles (for example, leaves), surface water was first filtered with a sieve (pore size 1 mm).

Water was filtered according to US EPA Method 1623 (United States Environmental Protection Agency) with the Envirochek capsule filters. Filtration was performed at a flow rate of 0.7 L/min, until about 500 mL of water was left in the vessel. Then, 200 mL of PET buffer [0.01% Tween 80, 0.02% PPi, 0.03% EDTA] was added to wash

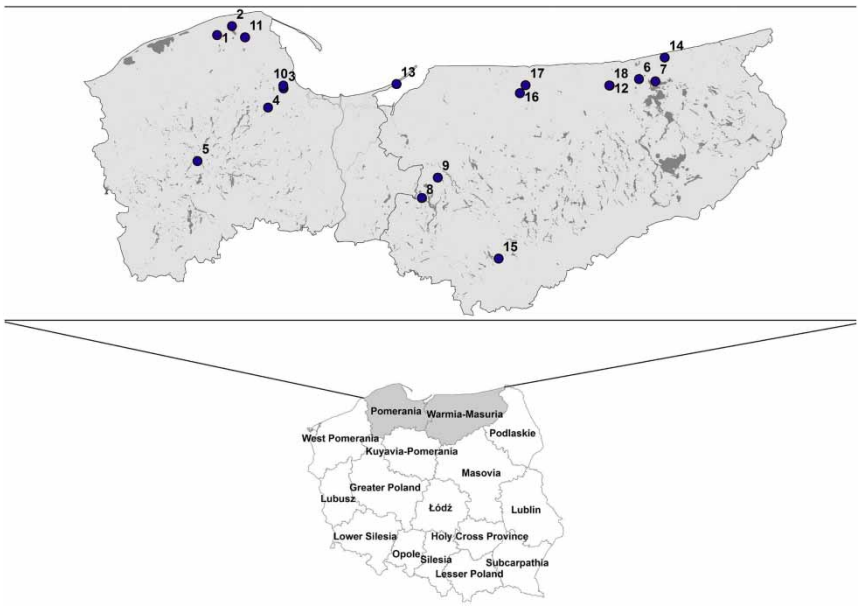

Figure 1 | Origin of water samples analyzed in the Pomerania province and Warmia-Masuria province, Poland. Black dots and numbers represent localisations of sampling sites: 1, Choczewskie Lake; 2, Żarnowieckie Lake; 3, Wysockie Lake; 4, Sitno Lake; 5, Dłużecko Lake; 6, Rydzówka Lake; 7, Mamry Lake; 8, Jeziorak Lake; 9, Pozorty Lake; 10, Osowskie Lake; 11, Dobre Lake; 12, pond; 13, Vistula Lagoon; 14, well (Ołownik); 15, well (Jankowice); 16, well (Zielenica); 17, well (Wiewiórki); 18, well (Radosze).

the vessel walls and filtration was continued. Following filtration, the capsule filter was filled with 100 mL of PET buffer, transported to the laboratory and kept at 4°C.

Each water sample was filtered with changing the pipe section to a new one between the water sample and the filter to avoid contamination.

Removal and concentration of *Giardia* cysts from filters

Following completion of the filtration procedure, the filter was left for 15 min in a vertical position, then inverted and left for 15 min in the inverted position. Then, the first fraction of washings was placed in a 225-mL conical tube; the filter was filled again with 100 mL of PET buffer and shaken in a horizontal position for 15 min using an automatic shaker (CAT S50, Germany). Then, the filter was rotated (180°) and shaken again for 15 min. The second fraction of washings was added to the conical tube containing the first fraction, mixed, and centrifuged for 30 min at 1,250 × *g* and 4°C without braking, using a Hermle Z383 K centrifuge (Labortechnik, Germany). Next, the supernatant was removed and the obtained pellet (≤0.25 mL) was suspended in 2 mL of PBS buffer [1% BSA, 0.05% Tween 20] (larger pellets were suspended in 4 mL of PBS buffer) and stored for further analysis.

DNA extraction

Prior to DNA extraction, the material obtained from filters was frozen three times at −70°C and thawed at 30°C in a water bath to disrupt the cyst walls and improve the efficiency of DNA extraction, which was performed using a Sherlock AX kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. All of the PCR templates were treated with an Anty-Inhibitor Kit (A&A Biotechnology) which removes polyphenolic PCR inhibitors using specific absorption particles, thereby removing factors that could interfere with the PCR. The PCR templates were stored at −20°C.

DNA amplification

For specific detection of *G. intestinalis* DNA, nested PCR, real-time PCR and LAMP assays, developed by Hopkins *et al.* (1997), Guy *et al.* (2003) and Plutzer & Karanis (2009), respectively, were used in this study as described below.

Detection with nested PCR

Nested PCR was performed with the use of a set of the primers GIAF, GIAR (outer primers) and RH4, RH11 (inner primers) specific to the small subunit (SSU) rRNA gene of *G. intestinalis* developed by Hopkins *et al.* (1997). The amplification reaction mixture consisted of 12.5 μL of the standard and ready-to-use PCR mixture 2xPCR Mix Plus High GC (A&A Biotechnology) containing recombinant Taq polymerase, PCR buffer, magnesium chloride, nucleotides, stabilisers, and gel loading buffer, 0.25 μM of each primer (Metabion, Germany), and 3 μL of DNA in the first PCR and 1 μL of DNA in the second PCR, respectively, in a 25 μL reaction volume. Amplifications were performed with an initial polymerase activation step (10 min at 95°C), followed by 35 cycles of denaturation (45 s at 96°C), annealing of primers (30 s at 55°C for the outer and 5 s at 9°C for the inner PCR), strand extension (30 s at 72°C), and final extension (4 min at 72°C). Nested PCR reactions were performed using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, USA). The PCR products (292 bp) were analysed using a GelDoc-It Imaging System (UVP, USA) following electrophoresis on a 2% gel agarose, which was stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany).

Detection with real-time PCR

Real-time PCR was performed with the use of the β-giardin P241 primer-TaqMan probe set specific to a fragment of the *G. intestinalis* β-giardin gene (Guy *et al.* 2003). The amplification reaction mixture consisted of 12.5 μL of Real-Time 2x HS-PCR Master Mix Probe (A&A Biotechnology), 300 nM of each primer (Metabion), 200 nM of the hydrolysis probe (Metabion), and 3 μL of template DNA in a 25 μL reaction volume. The amplification programme, consisting of initial denaturation (10 min at 95°C), 45 cycles of denaturation (15 s at 95°C), and annealing and elongation (1 min at 60°C), was performed in an

Mx3005P thermocycler (Stratagene, USA). PCR products were analysed using MxPro QPCR Software. The cycle threshold (CT) value, determining the cycle number at which the reporter's fluorescence exceeded the threshold value, was recorded. A sample was considered positive if the CT value was <45.

Detection with LAMP

The LAMP assay was performed using a Loopamp DNA amplification kit (Eiken Chemical, Tokyo, Japan) based a set of primers specific to the EF1-α-gene of *G. intestinalis* and thermal conditions described by Plutzer & Karanis (2009). The reaction mixture consisted of 12.5 μL of 2× reaction mix buffer, 40 pmol of each of the primers FIP and BIP, 20 pmol of each of the primers LF and LB, 5 pmol of each of the primers B3 and F3 (GENEWIZ SZ, Suzhou, China), 1 μL of Bst DNA polymerase (8 U), and 2 μL of the extracted DNA in a 25-μL reaction volume. LAMP reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA). The amplified products were analysed using a WD-9413B gel imaging analysis system (Beijing Liuyi Biotechnology, China) following electrophoresis on agarose gel (Biowest Regular Agarose G-10, Gene Company) stained with ExRed nucleic acid electrophoresis dye (Beijing Zoman Biotechnology, China).

All PCR/LAMP experiments were performed including *Giardia* positive controls (genomic DNA extracted from trophozoites of a *Giardia* strain cultured axenically in the Department of Tropical Parasitology, Medical University of Gdańsk, Poland) to ensure the correct functioning of the reaction and negative controls (water template) to eliminate contamination of the PCR components.

All of the samples found to be negative were re-tested for the presence of PCR inhibitors by mixing 4 μL of DNA template and 1 μL of the *Giardia* positive control.

Identification of *Giardia* assemblages A and B using real-time PCR

Real-time PCR was carried out on an Mx3005P thermocycler (Stratagene, USA) with the *Giardia*-specific β-giardin primer-probe sets P434 (P-1), designed based on the Portland 1 sequence of the *G. lamblia* β-giardin gene (assemblage A), and P434 (H3), designed based on the H3 sequence of the *G. lamblia* β-giardin gene (assemblage B), according to Guy *et al.* (2003, 2004). The amplification reaction mixture and programme were identical to those used for the β-giardin P241 primer-TaqMan probe set described above.

Sequencing

The final nested PCR products of the positive samples, after being cleaned with a Clean-Up kit (A&A Biotechnology), were sequenced. The products of the sequencing reaction, performed with the primers described by Hopkins *et al.* (1997), were cleaned with an ExTerminator kit (A&A Biotechnology) and subjected to analysis using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, USA). The results were analysed using ABI PRISM DNA Sequencing Analysis version 3.7 for the Windows NT platform (Applied Biosystems) and GeneStudio™ Professional (GeneStudio, Inc., USA).

RESULTS

In total, 36 water samples collected from surface water reservoirs and wells in northern and north-eastern Poland were examined for *G. intestinalis* using three different DNA-based molecular assays: LAMP, real-time PCR and nested PCR. *Giardia* DNA was detected in 15 (42%) samples tested with LAMP, 12 (33%) tested with real-time PCR and nine (25%) tested with nested PCR (Table 1, Figures 2–4).

In three of the water samples (templates 1S2, 3S2 and 18 S2) positive results were obtained only with the LAMP assay. The remaining water samples, which were positive in LAMP, were also positive by at least one additional method: real-time PCR or nested PCR (Table 2).

Among the 13 different surface waters investigated, *Giardia* DNA was detected in three lakes (Choczewskie, Wysockie and Osowskie) and the Vistula Lagoon located in Pomerania province, and in two lakes (Jeziorak and Pozorty) and one small pond located in the Warmia-Masuria province (Table 2, Figure 1). Water samples collected from

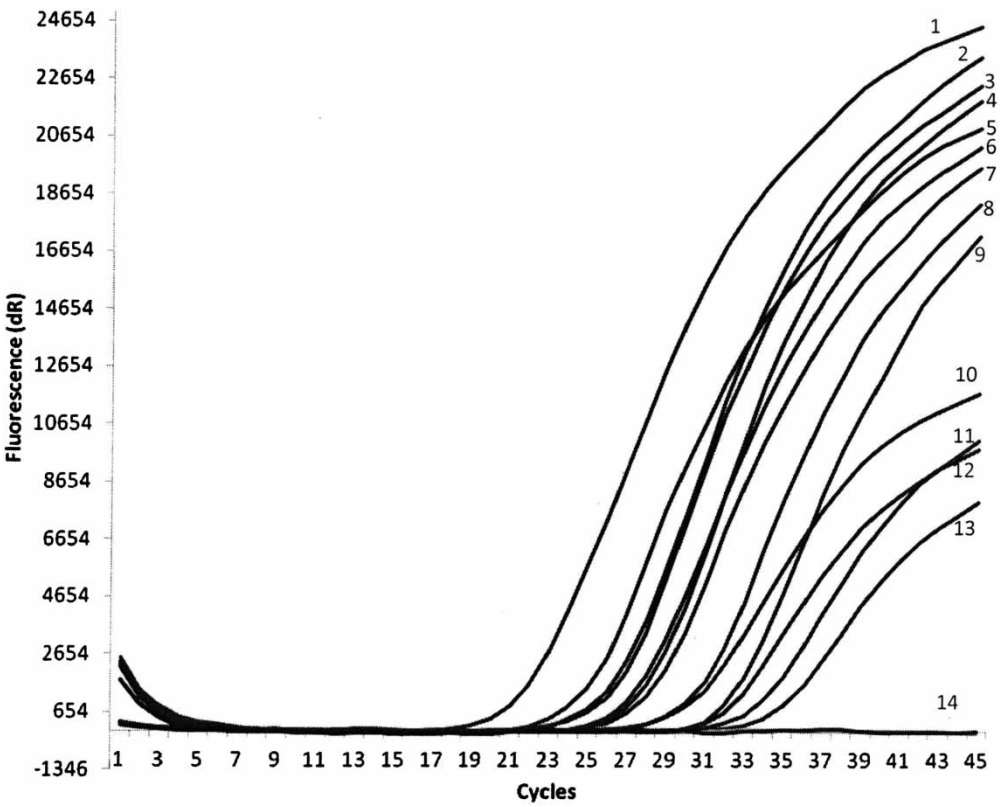

Figure 2 | Results of the *Giardia intestinalis* DNA detection in environmental water samples using real-time PCR: line 1, positive control; line 2, Jeziorak Lake (S1); line 3, Choczewskie Lake; line 4, pond; line 5, Pozorty Lake; line 6, Vistula Lagoon (S1); line 7, Osowskie Lake; line 8, Jeziorak Lake (S2); line 9, Vistula Lagoon (S2); line 10, well Ołownik; line 11, well Radosze; line 12, well Jankowice; line 13, well Zielenica; line 14, negative control.

the remaining six water bodies, all lakes (Żarnowieckie, Dłużecko, Sitno and Dobre in Pomerania province; Rydzówka and Mamry in Warmia-Masuria province) tested negative for *Giardia* DNA (Table 2).

Among five wells examined, *Giardia* DNA was detected in four, that is, in five of ten water samples collected (Tables 1 and 2).

Among all water samples collected in the period between May and June, nine (60%) samples were positive for *Giardia* spp. Of the samples collected during this period, samples (four lakes, the pond and Vistula Lagoon) from six natural water bodies ($n = 13$) and from three wells ($n = 5$) were positive. In water samples from the same sites collected between August and October, *Giardia* DNA was amplified in six (40%) of them (Table 2).

In the case of one well (located in the village of Radosze), two lakes (Choczewskie and Jeziorak) and the Vistula Lagoon, a positive result for *Giardia* DNA amplification was noted in both water samples collected (S1 and S2) from the investigated sampling sites. For water samples taken from the remaining sampling sites, one of two collected samples was positive (Table 2).

According to real-time PCR analysis, genotyping was successful in ten water samples. Both of the *G. intestinalis* genotypes in question (assemblages A and B) were recorded in two water samples, which were collected from Osowskie (lake) and the Vistula Lagoon. In the remaining water samples, only assemblage B of *G. intestinalis* was detected (Table 2, Figure 5(a) and 5(b)).

Sequencing of nested PCR products was successful in six water samples. Comparison of the obtained sequences with the *Giardia* sequences available in the GenBank confirmed that the detected PCR products were fragments of the *Giardia intestinalis SSU* rRNA gene. Derived sequences are deposited in GenBank under accession numbers KY489978–KY489983.

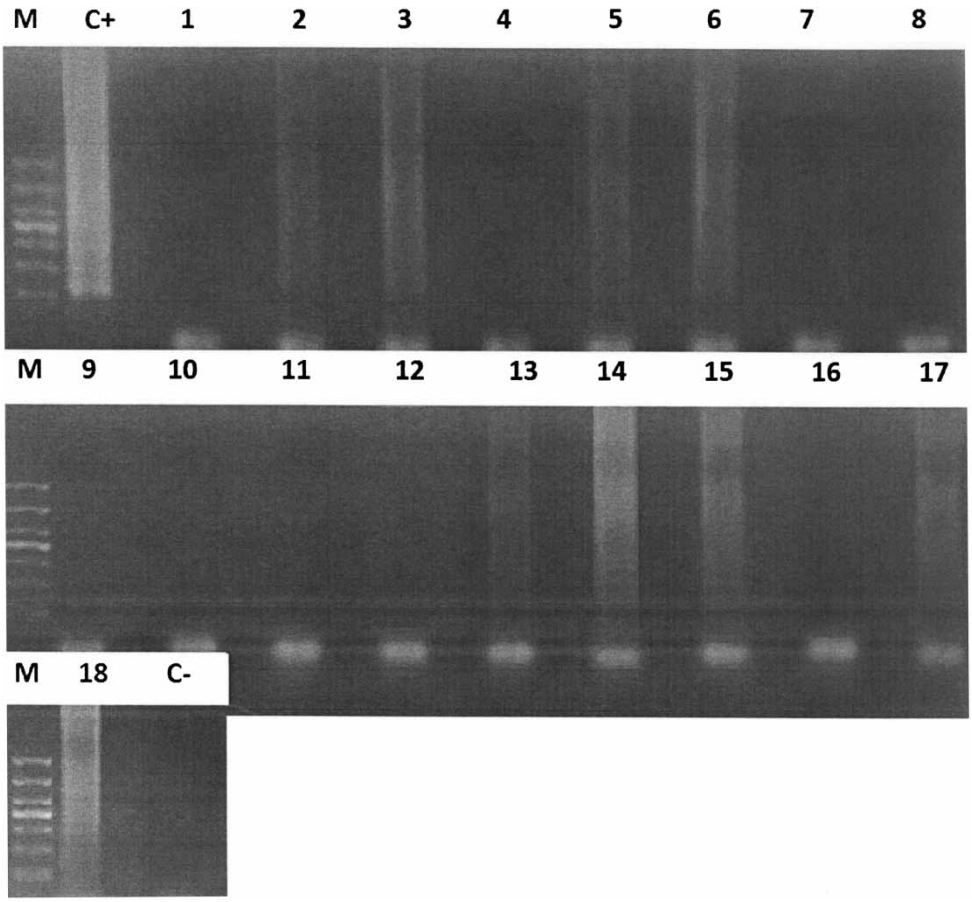

Figure 3 | Results of the *Giardia intestinalis* DNA detection in environmental water samples using LAMP: M, molecular weight marker (2,000 bp, Takara, Japan); C +, positive control; C −, negative control; line 1, well Ołownik (S1); line 2, well Jankowice (S1); line 3, well Zielenica (S1); line 4, well Wiewiórki (S1); line 5, well Radosze (S1); line 6, Choczewskie Lake (S1); line 7, Żarnowieckie Lake (S1); line 8, Wysockie Lake (S1); line 9, Sitno Lake (S1); line 10, Dłużecko Lake (S1); line 11, Rydzówka Lake (S1); line 12, Mamry Lake (S1); line 13, Jeziorak Lake (S1); line 14, Pozorty Lake (S1); line 15, Osowskie Lake (S1); line 16, Dobre Lake (S1); line 17, pond; line 18, Vistula Lagoon (S1).

DISCUSSION

Giardia intestinalis is responsible for symptomatic giardiasis, which has been noted in about 200 million people worldwide (Carmena 2010). Water and food, among other routes of infection, are the most common transmission vehicles. To date, *Giardia* has been isolated worldwide from aquatic environments, including natural surface water reservoirs (rivers, lakes), treated drinking water supplies, bodies of recreational water (swimming pools and water parks) and wells (Karanis *et al.* 1996a, 1996b, 1996c, 1998). Concentrations of *Giardia* cysts are commonly reported in the range of 0.01–150 per litre; however, they may reach higher values in agricultural runoff and urban wastewater effluent. Cysts can survive for 2–3 months in an aquatic environment; their viability decreases with increases in temperature and at extremely low temperatures (Carmena 2010).

Detection of small numbers of organisms in water is difficult and requires very sensitive techniques, such as molecular methods which additionally enable genotyping of *G. intestinalis* isolates, which is impossible using microscopy. Many molecular assays, usually based on gene markers such as 18S rDNA, glutamate dehydrogenase, triose phosphate isomerase, β-giardin, and elongation factor 1α (EF1α), have been developed to detect *Giardia* isolates in clinical and environmental samples with high specificity and sensitivity. The methods include variants of PCR and nested PCR as well as the recently

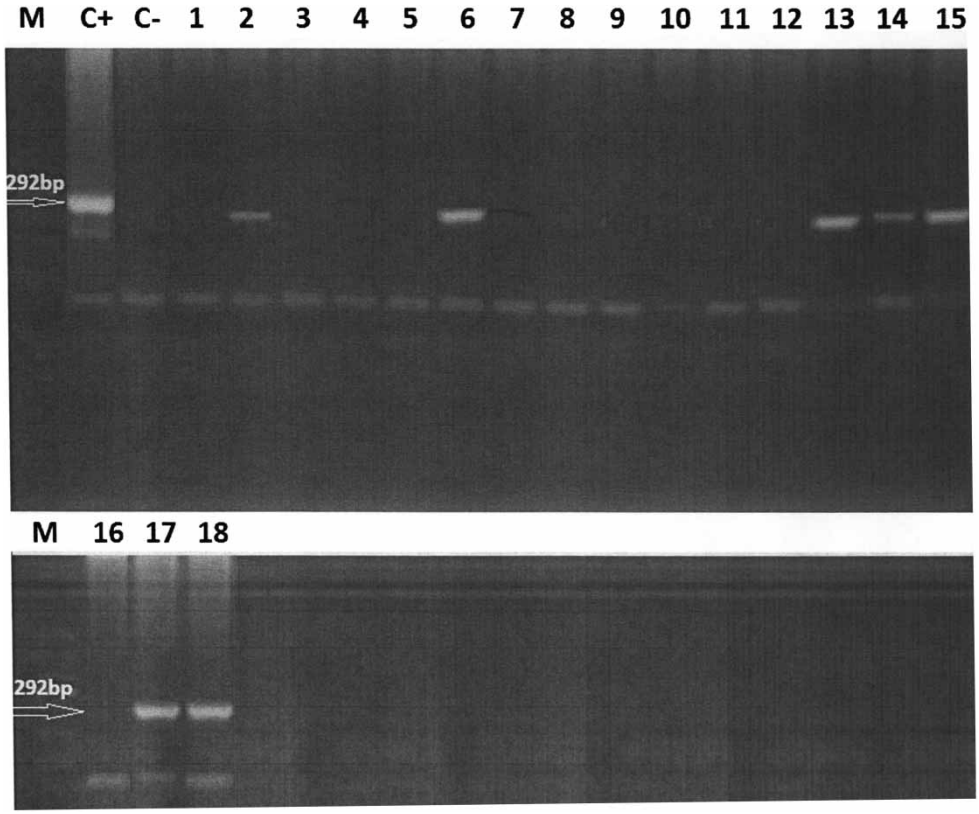

Figure 4 | Results of the *Giardia intestinalis* DNA detection in environmental water samples using nested PCR: M, molecular weight marker (1,000 bp, Takara, Japan); C+, positive control; C−, negative control; line 1, well Ołownik (S1); line 2, well Jankowice (S1); line 3, well Zielenica (S1); line 4, well Wiewiórki (S1); line 5, well Radosze (S1); line 6, Choczewskie Lake (S1); line 7, Żarnowieckie Lake (S1); line 8, Wysockie Lake (S1); line 9, Sitno Lake (S1); line 10, Dłużecko Lake (S1); line 11, Rydzówka Lake (S1); line 12, Mamry Lake (S1); line 13, Jeziorak Lake (S1); line 14, Pozorty Lake (S1); line 15, Osowskie Lake (S1); line 16, Dobre Lake (S1); line 17, pond; line 18, Vistula Lagoon (S1).

developed LAMP assay (Karanis & Ey 1998; Guy *et al.* 2003, 2004; Caccio *et al.* 2005; Plutzer & Karanis 2009).

In this report, we present the results of detecting *Giardia* DNA in concentrates from 50-L samples from several bodies of water located in northern and north-eastern Poland for the presence of *G. intestinalis* using three different molecular detection methods: LAMP, real-time PCR and nested PCR. Positive DNA amplification was noted in 42%, 33% and 25% of the water samples, respectively. Therefore, all three methods detected the presence of *Giardia* DNA in extracts from particles recovered from environmental water samples. However, from the point of view of higher sensitivity, LAMP has the advantage. The results of our findings confirmed that *G. intestinalis* is present in various water sources, including surface-water reservoirs and wells in Pomerania and Warmia-Masuria provinces, Poland. Moreover, the results of multiplex real-time PCR showed that the detected strains of *G. intestinalis* belong to assemblages A and B, with genotype B predominating. This indicates that water in the investigated area may be a source of giardiasis in humans, who use contaminated water bodies for recreational activities and water from contaminated surface wells for gardening.

G. intestinalis has been found in various water reservoirs worldwide. In Poland, there have been very few studies concerning detection of *G. intestinalis* in water sources. *Giardia* spp. cysts, among others, were found using microscopic methods in 85% of water samples taken from 13 sewers located in eastern Poland (Sroka *et al.* 2013). The presence of *G. duodenalis* was also confirmed in recreational water reservoirs in north-eastern Poland with the use of direct fluorescence assays (46%) and molecular methods (48%) (Sroka *et al.* 2015). Another study by

Table 2 | Comparison of the occurrence of *Giardia intestinalis* DNA in surface water and well samples collected in different seasons from the Pomerania province and the Warmia-Masuria province, Poland

Sampling site		Results of molecular investigations regarding two sampling periods[#]			Molecular detection results			Genotyping
No.	Type	Samples	May–June	August–October	LAMP	Real-time PCR	Nested PCR	Assemblage
1	Choczewskie Lake	S1	+		+	+	+	B
		S2		+	+	−	−	
2	Żarnowieckie Lake	S1	−		−	−	−	
		S2		−	−	−	−	
3	Wysockie Lake	S1	−		−	−	−	
		S2		+	+	−	−	
4	Sitno Lake	S1	−		−	−	−	
		S2		−	−	−	−	
5	Dłużecko Lake	S1	−		−	−	−	
		S2		−	−	−	−	
6	Rydzówka Lake	S1	−		−	−	−	
		S2		−	−	−	−	
7	Mamry Lake	S1	−	−	−	−	−	
		S2	−	−	−	−	−	
8	Jeziorak Lake	S1	+		+	+	+	B
		S2		+	+	+	+	B
9	Pozorty Lake	S1	+		+	+	+	B
		S2		−	−	−	−	
10	Osowskie Lake	S1	+		+	+	+	A, B
		S2		−	−	−	−	
11	Dobre Lake	S1	−		−	−	−	
		S2		−	−	−	−	
12	Pond Radosze	S1	+		+	+	+	ND
		S2		−	−	−	−	
13	Vistula Lagoon	S1	+		+	+	+	A
		S2		+	+	+	−	B
14	Well Ołownik	S1	−		−	−	−	
		S2		+	+	+	+	B
15	Well Jankowice	S1	+		+	+	+	B
		S2		−	−	−	−	
16	Well Zielenica	S1	+		+	+	−	ND
		S2		−	−	−	−	
17	Well Wiewiórki	S1	−		−	−	−	
		S2		−	−	−	−	
18	Well Radosze	S1	+		+	+	−	B
		S2		+	+	−	−	

[#]Results obtained for all three detection methods performed; (+) positive result obtained with at least one detection method; (−) negative result obtained with all three detection methods. S1, first water sample collected; S2, second water samples collected; NI, not investigated.

Bajer *et al.* (2012), performed using IFA and nested PCR assays, showed the frequent occurrence (61%) of this parasite in surface water in Poland. Moreover, Polus & Kocwa-Haluch (2014) found *Giardia* DNA in four of 17 water bodies investigated in the vicinity of Cracow (southern Poland). The presence of this protozoan has also been confirmed using molecular assays in several water bodies used for recreational activities in north-western Poland (Adamska

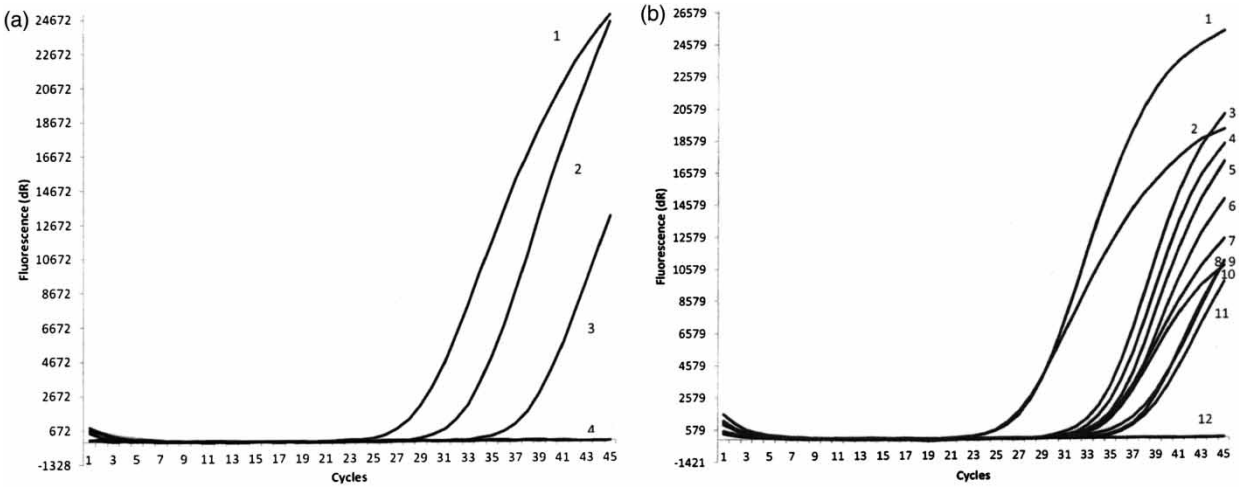

Figure 5 | Results of detection of *Giardia intestinalis* assemblage A and B in positive environmental water samples using real-time PCR. (a) Results for *G. intestinalis* assemblage A: line 1, positive control; line, 2 Vistula Lagoon (S1); line 3, Osowskie Lake (S1); line 4, negative control. (b) Results for *G. intestinalis* assemblage B: line 1, positive control; line 2, Choczewskie Lake; line 3, Jeziorak Lake (S1); line 4, Vistula Lagoon; line 5, Osowskie Lake; line 6, well Radosze; line 7, Pozorty Lake; line 8, well Ołownik; line 9, Jeziorak Lake (S2); line 10, well Zielenica; line 11, well Jankowice; line 12, negative control.

2015). Earlier studies performed in the Tri-City area (northern Poland) using the FISH/IF method also demonstrated the presence of *Giardia* cysts in three of seven examined lakes (Szostakowska *et al.* 2005).

It is known that genotypes A and B of *G. intestinalis* are responsible for infections in humans (Karanis & Ey 1998). Genotype A is associated with a mixture of human and animal isolates, while B tends to be predominantly associated with human isolates (Carmena 2010). Genotyping performed in our studies using real-time PCR showed that most of the investigated samples were contaminated with *G. intestinalis* assemblage B. These results are in accord with other studies performed in Poland showing the occurrence of both zoonotic assemblages in various water sources, with a higher frequency of genotype B (Adamska 2015; Sroka *et al.* 2015).

We found more positive water samples among those collected during spring and early summer as opposed to late summer (nine of 16) and autumn (six of 16). These results are in accord with the findings of Sroka *et al.* (2015), who detected *Giardia* cysts in samples collected from recreational water reservoirs in the Warmia-Masuria province in spring with a frequency approximately three times higher than that in autumn. This may result from changes in the prevalence of infection in hosts which excrete cysts of the parasite and contaminate the environment, as well as from the fact that the viability of *Giardia* cysts decreases with increasing water temperatures.

The source of contamination of water may vary, but it is always connected with the excretion of cysts by infected hosts (humans or animals). The prevalence of *Giardia* infections in the Polish population ranges between 1 and 8.8% in healthy children and 3.1 and 6.5% in healthy adults (Bajer 2008; Korzeniewski *et al.* 2014, 2016). *Giardia* spp. infections appear to be rare in farm animals in Poland (cattle 2.2–14%; sheep 1.3%) and more prevalent in wildlife. This parasite has been found in a range of host species, being especially common in rodents (including species of semi-aquatic rodents, e.g., European beavers and muskrats, with frequencies of 7.7% and 87%, respectively) and wolves (20–46%, depending on the method of detection), and less prevalent in other species such as European bison, roe deer and red deer (Karanis *et al.* 1996b, 1996c; Paziewska *et al.* 2007; Bajer 2008). *Giardia* cysts were also detected in house animals: in 6–36% of dogs, and in a small percentage of investigated cats (Bajer 2008; Jaros *et al.* 2011).

The source of contamination of water investigated in our study may differ depending on the localisation and character of particular sampling sites. Choczewskie is a very clean, flow-through lake situated in the Choczewo-Salino Protected Landscape Area (Pomerania province), a popular

site for tourism and recreation. Here, we detected *G. intestinalis* assemblage B, which may derive mainly from wildlife; however, a human origin cannot be excluded. The lakes Wysockie and Osowskie are small ribbon lakes. Low quality water from Wysockie (Gdańsk district), showing progressed eutrophication induced by the discharge of sewage into the aquatic system, is used mainly for factories and irrigation of agricultural areas. Osowskie, situated on the border of the Kashubian Lake District, is cleaner that Wysockie, but also polluted with waste coming from local farms as well as from a storm water drain. In these lakes, the occurrence of *G. intestinalis* assemblages A and B may have a human or mixed host origin. Jeziorak, a large ribbon lake, located in Warmia-Masuria province, has a rich lakeside and is surrounded by the marvellous forests of Iława Lake District Landscape Park. In this lake, we found DNA of *G. intestinalis* assemblage B, the source of which may be both human (i.e., waste may derive from certain yachts whose owners avoid treatment stations in marinas) and animal (wildlife from the surrounding landscape park and domestic animals from local homes). In contrast to Jeziorak, Pozorty is a small lake, surrounded by forests and fields, where the contamination most probably originates from wildlife and domestic animals. The Vistula Lagoon has poor quality water due to waste from sewage treatment plants and several rivers flowing into the lagoon. Therefore, the contamination with *Giardia* is probably of human origin. We also found *Giardia* DNA in wells located on private property (kitchen gardens/yards) in small poor villages in Warmia-Masuria province. The homes are located in an agricultural area, surrounded by forests and fields, and inhabited and/or visited by both domestic animals (dogs, cats) and wildlife. Although it is difficult to indicate the exact source of contamination in this case, the results are similar to the findings of other researchers who also detected *Giardia* and/or other parasites in these wells (Sroka *et al.* 2006; Alam *et al.* 2014).

The present study supplements previous information for Poland on the distribution of *Giardia* in surface waters, including five lakes, one pond and a lagoon in northern and north-eastern Poland. It provides a comparison of molecular detection methods (LAMP, RT-PCR and nPCR) for *Giardia* in concentrates recovered from filtering water. Management of sources contamination and investigations of possible giardiasis cases in the affected areas in association with control measures may effectively support control programme measures against transmission routes of *Giardia* in humans and animals in Poland.

CONCLUSIONS

In the present study, we detected DNA of *G. intestinalis* in surface water samples taken from lakes, a pond and a lagoon, as well as from wells located in Pomerania and Warmia-Masuria provinces, Poland. These findings indicate a potential risk for people using contaminated water. Moreover, we have demonstrated that the LAMP assay was the most sensitive of the three molecular detection methods used for screening of the environment for *Giardia* species.

ACKNOWLEDGEMENTS

We acknowledge the funding support from the MN 01-0188/08/772 project and One Thousand Talents Plan of the Chinese Government (No. WQ2013630172). The manuscript does not contain clinical studies or patient data. The authors declare that they have no conflict of interest.

REFERENCES

Adam, R. D. 2001 Biology of *Giardia lamblia*. *Clinical Microbiology Review* **14**, 447–475.

Adamska, M. 2015 Molecular characterization of *Cryptosporidium* and *Giardia* occurring in natural water bodies in Poland. *Parasitology Research* **114**, 687–692.

Alam, M. S., Khan, S. U., Ayaz, S., Akbar, N., Khan, M. A., Ahmad, I., Idrees, M. & Waqar, M. 2014 Molecular detection of *Giardia lamblia* and *Cryptosporidium parvum* in different water sources of district bannu, Khyber Pakhtunkhwa, Province of Pakistan. *British Microbiology Research Journal* **4**, 80–88.

Bajer, A. 2008 *Cryptosporidium* and *Giardia* spp. infections in humans, animals and the environment in Poland. *Parasitology Research* **104**, 1–17.

Bajer, A., Toczyłowska, B., Bednarska, M. & Siński, E. 2012 Effectiveness of water treatment for the removal of

Cryptosporidium and *Giardia* spp. *Epidemiology and Infection* **140**, 2014–2022.

Baldursson, S. & Karanis, P. 2011 Waterborne transmission of protozoan parasites: review of worldwide outbreaks – an update 2004–2010. *Water Research* **45**, 6603–6614.

Caccio, S. M., Thompson, A. R. C., McLauchlin, J. & Smith, H. W. 2005 Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends in Parasitology* **21**, 431–437.

Carmena, D. 2010 Waterborne transmission of *Cryptosporidium* and *Giardia*: detection, surveillance and implications for public health. In: *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* (A. Méndez-Vilas, ed.). Formatex Research Center, Spain, pp. 3–14.

deRegnier, D., Cole, L., Schupp, D. G. & Erlandsen, S. L. 1989 Viability of *Giardia* cysts suspended in lake, river and tap water. *Applied and Environmental Microbiology* **55**, 1223–1229.

ECDPC 2015 *Annual Epidemiological Report. European Centre for Disease Prevention and Control*. http://ecdc.europa.eu/en/healthtopics/giardiasis/Pages/Epidemiological-data.aspx.

Efstratiou, A., Ongerth, J. & Karanis, P. 2017 Waterborne transmission of protozoan parasites: review of worldwide outbreaks – an update 2011–2015. *Water Research* **114**, 14–22.

Farthing, M. J. 1996 Giardiasis. *Gastroenterology Medical Clinics of North America* **25**, 493–515.

Guy, R. A., Payment, P., Krull, U. J. & Horgen, P. A. 2003 Real-time PCR for quantitation of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Applied and Environmental Microbiology* **69**, 5178–5185.

Guy, R. A., Xiao, C. & Horgen, P. A. 2004 Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. *Journal of Clinical Microbiology* **42**, 3317–3320.

Hopkins, R. M., Meloni, B. P., Groth, D. M., Wetherall, J. D., Rrynoldson, J. A. & Thompson, R. C. 1997 Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. *Journal of Parasitology* **83**, 44–51.

Hunter, P. R. & Thompson, R. C. A. 2005 The zoonotic transmission of *Giardia* and *Cryptosporidium*. *International Journal for Parasitology* **35**, 1181–1190.

Jaros, D., Zygner, W., Jaros, S. & Wędrychowicz, H. 2011 Detection of *Giardia intestinalis* assemblages A, B and D in domestic cats from Warsaw, Poland. *Polish Journal of Microbiology* **60**, 259–263.

Jędrzejewski, S. & Majewska, A. C. 2007 Contamination of fresh food products with dispersive stages of intestinal parasites. *Wiadomosci Parazytologiczne* **53**, 104.

Karanis, P. & Ey, P. 1998 Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitology Research* **84**, 442–449.

Karanis, P., Schoonen, D. & Seitz, H. M. 1996a *Giardia* and *Cryptosporidium* in backwash water from rapid sand filters used for drinking water production. *International Journal of Medical Microbiology* **284**, 107–114.

Karanis, P., Opiela, K., Renoth, S. & Seitz, H. M. 1996b Possible implication of muskrats to contaminate surface water supplies with *Giardia* sp. cysts. *International Journal of Medical Microbiology* **284**, 302–306.

Karanis, P., Opiela, K., Arousi, H. M. & Seitz, H. M. 1996c A comparison of phase contrast microscopy and an immunofluorescence test for the detection of *Giardia* spp. in faecal specimens from cattle and wild rodents. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 250–251.

Karanis, P., Schoenen, D. & Seitz, H. M. 1998 Distribution and removal of *Giardia* and *Cryptosporidium* in water supplies in Germany. *Water Science and Technology* **37** (2), 9–18.

Karanis, P., Kourenti, C. & Smith, H. 2007 Water-borne transmission of protozoan parasites: a review of world-wide outbreaks and lessons learnt. *Journal of Water and Health* **5**, 1–38.

Korzeniewski, K., Augustynowicz, A. & Lass, A. 2014 Intestinal parasites in Polish community on the example of military environment. *International Maritime Health* **65**, 216–222.

Korzeniewski, K., Konior, M., Augustynowicz, A., Lass, A. & Kowalska, E. 2016 Detection of *Giardia intestinalis* infections in Polish soldiers deployed to Afghanistan. *International Maritime Health* **67**, 243–247.

Lebwohl, B., Deckelbaum, R. J. & Green, P. H. 2003 Giardiasis. *Gastrointestinal Endoscopy* **57**, 906–913.

Nygård, K., Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N., Hausken,, T. & Aavitsland, P. 2006 A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. *BMC Public Health* **6**, 141.

Paziewska, A., Bednarska, M., Niewęgłowski, H., Karbowiak, G. & Bajer, A. 2007 Distribution of *Cryptosporidium* and *Giardia* spp. in selected species of protected and game mammals from North-Eastern Poland. *Annals of Agricultural and Environmental Medicine* **14**, 159–167.

Plutzer, J. & Karanis, P. 2009 Rapid identification of *Giardia duodenalis* by loop-mediated isothermal amplification (LAMP) from faecal and environmental samples and comparative findings by PCR and real-time PCR methods. *Parasitology Research* **104**, 1527–1533.

Plutzer, J., Karanis, P., Domokos, K., Törökné, A. & Márialigeti, K. 2008 Detection and characterisation of *Giardia* and *Cryptosporidium* in Hungarian raw, surface and sewage water samples by IFT, PCR and sequence analysis of the SSUrRNA and GDH genes. *International Journal of Hygiene and Environmental Health* **211**, 524–533.

Polus, M. & Kocwa-Haluch, R. 2014 Occurrence of *Cryptosporidium, Giardia* and *Toxoplasma* in surface waters in the area of Cracow. *Environment Protection Engineering* **40**, 105–113.

Rendtorff, R. C. 1954 The experimental transmission of human intestinal protozoan parasites. II. *Giardia lamblia* cysts given in capsules. *The American Journal of Tropical Medicine and Hygiene* **59**, 209–220.

Reynolds, K. A., Mena, K. D. & Gerba, C. P. 2008 Risk of waterborne illness via drinking water in the United States. *Reviews of Environmental Contamination and Toxicology* **192**, 117–158.

Ryan, U. & Cacciò, S. M. 2013 Zoonotic potential of *Giardia*. *International Journal for Parasitology* **43**, 943–956.

Smith, H. V., Caccio, S. M., Cook, N., Nichols, R. A. B. & Tait, A. 2007 *Cryptosporidium* and *Giardia* as foodborne zoonoses. *Veterinary Parasitology* **149**, 29–40.

Sroka, J., Wójcik-Fatla, A. & Dutkiewicz, J. 2006 Occurrence of *Toxoplasma gondii* in water from wells located on farms. *Annals of Agricultural and Environmental Medicine* **13**, 169–175.

Sroka, J., Stojecki, K., Zdybel, J., Karamon, J., Cencek, T. & Dutkiewicz, J. 2013 Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in effluent from sewage treatment plant from eastern Poland. *Annals of Agricultural and Environmental Medicine* **1**, 57–62.

Sroka, J., Gizejewski, Z., Wojcik-Fatla, A., Stojecki, K., Bilska-Zajac, E., Dutkiewicz, J., Cencek, T., Karamon, J., Zajac, V., Kusyk, P., Dabrowska, J. & Kochanowski, M. 2015 Potential role of beavers (*Castor fiber*) in contamination of water in the Masurian Lake District (north-eastern Poland) with protozoan parasites *Cryptosporidium* spp. and *Giardia duodenalis*. *Bulletin of the Veterinary Institute in Pulawy* **59**, 219–228.

Szostakowska, B., Kruminis-Łozowska, W., Graczyk, T. K. & Myjak, P. 2005 Prevalence of Cryptosporidium oocysts in surface waters of Tri-city and its vicinity. In: *Abstracts of International Conference 'Biological and Chemical Factors of Environmental Contamination'*, Warsaw, Poland, 23–25 June, p. 21.

Thompson, R. C. A. & Monis, P. T. 2004 Variation in *Giardia*: implications for taxonomy and epidemiology. *Advances in Parasitology* **58**, 69–137.

Wolfe, M. S. 1992 Giardiasis. *Clinical Microbiology Reviews* **5**, 93–100.

Yoshida, H., Matsuo, M., Miyoshi, T., Uchino, K., Nakaguchi, H., Fukumoto, T., Teranaka, Y. & Tanaka, T. 2007 An outbreak of cryptosporidiosis suspected to be related to contaminated food, October 2006, Sakai City, Japan. *Japanese Journal of Infectious Disease Impact and Description* **60**, 405–407.

First received 10 February 2017; accepted in revised form 13 April 2017. Available online 14 June 2017