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Assessment of molecular methods as a tool for detecting pathogenic protozoa isolated from water bodies

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ABSTRACT

Several species belong to the *Cryptosporidium* and *Giardia* genus, the main parasitic protozoa occurring in water, but only some of them are infectious to humans. We investigated the occurrence of *Cryptosporidium* and *Giardia* and identified their species in the water samples collected from natural water bodies in north-western Poland. A total of 600 samples from water bodies used for bathing, sewage discharge, as drinking water sources and watering places for animals were screened. The samples were collected during a 3-year period in each of the four seasons and filtered using Filta-Max (IDEXX Laboratories, USA). Genomic DNA was extracted from all samples and used as a target sequence for polymerase chain reaction (PCR) and TaqMan real-time PCR, as well as for reverse line blotting (RLB) methods. PCR methods seem to be more sensitive to detect *Giardia* and *Cryptosporidium* DNA in water samples than RLB methods. All PCR products were sequenced and three were identified as *C. parvum* and four as *G. intestinalis*. The overall prevalence of *C. parvum* (0.5%) and *G. intestinalis* (0.6%) in the samples suggests that the risk of *Cryptosporidium* and *Giardia* infections in north-western Poland is minimal.

Key words | *Cryptosporidium parvum*, *Giardia intestinalis*, molecular methods, water-borne protozoa

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INTRODUCTION

Cryptosporidium and *Giardia* are widespread in the environment; aquatic and water contamination by protozoan pathogens from this genus can pose a significant threat for public health. It is well recognized that protozoan parasites of *Cryptosporidium* (Apicomplexa phylum) and *Giardia* species (Sarcomastigophora subphylum) are causes of widespread gastrointestinal disease and morbidity in humans, as well as decreases in farm animal production. These microorganisms infect the small intestine, occupying epicellular and extracellular intestinal niches, which has an influence on host–parasite interactions, pathophysiology and diarrhoea mechanisms (Graczyk *et al.* 1997; Reynolds *et al.* 2008; Ortega-Pierres *et al.* 2009). Both parasites are transmitted via the faecal–oral route, and the utilization of contaminated drinking water and use of recreational waterways are significant pathways of infection in developed countries. Cryptosporidiosis and giardiasis remain a public health concern, as demonstrated by continued outbreaks of these diseases attributable to waterborne transmission (Graczyk *et al.* 2010; Yoder & Beach 2010). In Poland, water contamination with *Cryptosporidium* and *Giardia* is not routinely monitored and waterborne outbreaks of cryptosporidiosis or giardiasis have not been reported in this country yet. Human cases of cryptosporidiosis in Poland and laboratory-confirmed cases were reported to the National Institute of Hygiene as single cases only, while the average annual number of reported cases of giardiasis is around 3,000. The first cases of cryptosporidiosis in HIV-positive patients in Poland were described in 1998 (Majewska *et al.* 1998).

Despite progress in the development of molecular methods for detection and genetic characterization of *Cryptosporidium* and *Giardia*, identification of their different species and strains in water still remains difficult, because the routine methods require further improvement (Karanis

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et al. 2006; Skotarczak 2010). For environmental samples, the detection of *Cryptosporidium* and *Giardia* (oo)cysts DNA with the molecular methods is often hampered by the presence of organic and inorganic substances, that can potentially inhibit trials based on the nucleic acids, and the efficiency of DNA extraction methods is determined by DNA recovery rate and polymerase chain reaction (PCR) inhibitor fall during the DNA extraction. Studies by many authors have revealed that PCR inhibitors occurring in water samples suppress or reduce PCR amplification (Kreader 1996; Jiang *et al.* 2005; Skotarczak 2009; Plante *et al.* 2011; Schriewer *et al.* 2011). Additional difficulties are that in water samples (oo)cysts occur in very low concentration and the exceptionally robust nature of the (oo)cyst cell wall requires more stringent treatments for disruption for recovery of DNA (Jiang *et al.* 2005). Considering the limitations mentioned above, in our earlier studies we made comparison of the efficiency of various DNA extraction and amplification methods for DNA of *C. parvum* oocysts and *G. intestinalis* cysts (Adamska *et al.* 2010, 2011a, 2011b, 2012), as well as application of molecular methods for environmental monitoring studies. In the present study, we examined surface water from north-western Poland with some molecular methods, in order to estimate the occurrence of *C. parvum* and *G. intestinalis*, species infectious to humans, in water samples of different origin collected during a 3-year period over four seasons, which was done for the first time in this part of Poland.

METHODS

Selection of water bodies

Surface water samples were collected from 50 sites of 36 water bodies during a 3-year period: in winter 2009, in spring, summer, autumn and winter 2010 and 2011 and in spring, summer and autumn 2012. The examined water bodies and sites, as well as the number of samples, are given in Table 1.

Except for one river, all examined sites are used for bathing during summer months. Two lakes and one river serve as drinking water sources for people, and 11 lakes and one river are watering places for wild and domestic animals. Four lakes, two rivers and, partially, the sea (two sites) are situated near farms and cultivated fields. Six lakes, five rivers and the sea (two sites) also receive sewage discharge.

Table 1 | Number of water bodies and sites and number of samples during 3 years and four seasons

	Lake water	River water	Sea water	Total
Number of bodies	27	8	1	36
Number of sites	30	12	8	50
Number of samples	360	144	96	600

Collection and preparation of samples

The 50-l water samples were collected from each site by passing through separate compressed-foam depth filters (Idexx Laboratories, USA) with the use of a pump (Grundfoss, Denmark) with a flow rate of 4 l/min. Filtration and elution procedures were carried out in accordance with the manufacturer's instructions. The water samples were prepared with the use of a Manual Filta Max® Wash Station (Idexx Laboratories, USA) and auxiliary equipment.

Microscopic examination

To detect *Cryptosporidium* sp. oocysts in water samples, the modified Kinyoun's acid-fast stain was used (Garcia 2007). For the recovery of *Giardia lamblia* cysts, Lugol solution was used for direct staining of wet preparations.

DNA extraction

For DNA extraction, 300 μl of each eluate were collected using QIAamp DNA Tissue Mini Kit (Qiagen, Germany). Before using the kit, three cycles of liquid nitrogen/water bath incubation (100 °C), each for 2 minutes, were applied in order to destroy (oo)cysts walls, as well as the overnight incubation with proteinase K (Adamska *et al.* 2010, 2011a, 2011b, 2012). Further DNA extraction was performed according to the manufacturer's instructions.

Nested and semi-nested PCR amplification

A region of the 18S rDNA gene was amplified in the nested PCR protocol in order to detect *Cryptosporidium* DNA. The

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method involves the amplification of an approximately 1,325 bp long primary product using the primers CX1F 5'-TTCTAGAGCTAATACATGCG-3' and CX1R2 5'-CCCTAATCCTTCGAAACAGGA-3', followed by a secondary amplification of an internal fragment with a length of approximately 840 bp using the primers CX2F 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and CX2R 5'-AAGGAGTAAGGAACAACCTCCA-3' (Xiao *et al.* 2001; Adamska *et al.* 2011b, 2012). To detect *G. intestinalis* DNA, a region of the *β*-giardin gene was amplified by semi-nested PCR protocol (Cacciò *et al.* 2002). Amplification of an approximately 753 bp-long primary product, followed by a secondary reaction of an internal fragment with a length of approximately 384 bp was performed. For the I. PCR step, a PCR product was amplified with the use of primers G7 5′-AAGCCC GACGACCTC ACCC GCAGTGC-3′ and G759 5′-GAGGCC GCCCT GGATCTTC GAGACGAC-3′. For the II. PCR step, a PCR product was amplified with the use of primers G376 5′-CATAACGACGCCATC GCGGCTCTC AGGAA-3′ and G759 5′-GAGGCC GCCCT GGATCTTC GAGACGAC-3′ (Cacciò *et al.* 2002; Adamska *et al.* 2010, 2011a).

Each PCR mixture (total volume of 10 μl) contained 5 pM of each primer (Genomed, Poland), 0.3 nM of each deoxynucleotide triphosphate (Novazym, Poland), 1 μl of 10 × PCR buffer, 30 mM $MgCl_2$, 0.5 U of Taq polymerase (Novazym, Poland), and 1 μl of DNA template. Bovine serum albumin (BSA) was added to the PCR mix at concentration 15 ng/μl in order to increase the reaction sensitivity (Adamska *et al.* 2011a, 2012). The reactions were performed in a DNA thermal cycler (Biometra and Eppendorf, Germany). As a positive control, DNA isolated from *C. parvum* oocysts and *G. intestinalis* cysts, obtained from Bulk Stock Live (BTF Biomerieux, Arustralia) was used. Negative control reaction mixtures contained sterile distilled water in place of DNA template. Thermal-time profiles in the first and second PCR for *C. parvum* and *G. intestinalis* were the same as described by Xiao *et al.* (2001) and Castro-Hermida *et al.* (2008), respectively. All analyses were carried out in two replicates. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide.

Sequencing

All nested and semi-nested PCR products obtained from surface water samples were sequenced (Macrogen, Korea) in order to check the protozoan species and the reaction specificity. Obtained sequences were initially compared with homology sequences deposited in GenBank database using the Basic Local Alignment Search Tool at the National Centre for Biotechnology Information.

Taqman nested real-time PCR and TaqMan real-time PCR

A region of the small subunit of the rRNA gene of *C. parvum* and *G. intestinalis* was used as a target sequence for nested real-time PCR. To detect *C. parvum* DNA, we used the outer primers CPrI and CPrII, amplifying a 676 bp fragment, the inner primers, CPrF3 and CPrR3, amplifying a 118 bp fragment and a TaqMan probe, CPrP3-1 (Bialek *et al.* 2002; Minarovicova *et al.* 2009; Adamska *et al.* 2011b, 2012). A region of the small subunit rRNA gene of *G. intestinalis* was used as a target sequence for real-time PCR. Primers Gd-80F and Gd-127R amplifying a 62-bp fragment and the probe Gd-FT (Haque *et al.* 2007; Adamska *et al.* 2010, 2011a) were used to detect *G. intestinalis* DNA.

Each PCR mixture for *C. parvum* (total volume 10 μl) contained 8 pM of each outer primer and the probe (Genomed, Poland), 0.9 nM of each dNTP (Novazym, Poland), 1 μl of 10 × PCR buffer, 25 mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Novazym, Poland) and 2 μl of DNA template. Each PCR mixture for *G. intestinalis* contained 14 pM of each primer, 8 pM of the probe Gd-FT (Genomed, Poland), 0.5 nM of each dNTP (Novazym, Poland), 15 mM MgCl2, 1.5 U Taq DNA Polymerase (Novazym, Poland) and 2 μl of DNA template. BSA was added to PCR mix at concentration 5 ng/μl in order to increase the reaction sensitivity (Adamska *et al.* 2011a, 2012). Reactions were performed in Rotor Gene 6000 (Corbett Research, Sydney, Australia). All oligonucleotide sequences and thermal profiles were as described previously (Haque *et al.* 2007; Minarovicova *et al.* 2009). The threshold cycle (C_T) was calculated for individual samples using the software of the cycler with the manual threshold set at a fluorescence value of 10^{-2}. Appropriate positive and negative controls were included in each PCR run. All analyses were carried out in two replicates.

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Reverse line blotting

The macroarray method (reverse line blotting (RLB)) was also used in order to detect more protozoan species. Two 5′ biotinylated starters, complementary to 18S rDNA gene of Sporozoa protozoa (*Cryptosporidium parvum*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, *Sarcocystis hominis* and *Isospora belli*) and *Balantidium coli* were designed. The sequence of the forward starter was 5′–CCAGCAGCCGCGGTAATTC–3′ and the reverse starter 5′–AAGAATTTCACCTCTGA–3′. Probes with 5′ terminal aminogroup, specific for 18S rDNA gene of all protozoa mentioned above, were also designed: 5′–TTAAAGATTTTTATCTT–3′ for *C. parvum*, 5′– ATTTC TCCACACTTCTGT–3′ for *T. gondii*, 5′–TCATCCGG CCTTGCCCGTA–3′ for *C. cayetanensis*, 5′–TATTATYCY TAATATNAT–3′ for *S. hominis*, and 5′–CTTCGCACT TAACTG–3′ for *I. belli*, 5′–CTAGAATCTTGGTTAATTCT–3′ for *B. coli*. Owing to the low level of similarity between 18S rDNA sequences of *G. intestinalis* and other protozoa, *Giardia* was excluded from the RLB method. All reactions were performed using Miniblotter® MN45 System (Immunetics, Netherlands) and Mini-Hybridization Oven OV3 (Whatman Biometra, Germany). To detect the reaction products, we applied incubation in BM Blue POD substrate (Roche Applied Science, USA). The positive control was DNA extracted from: *C. parvum* and *G. intestinalis* (oo) cysts (Bulk Stock Live, BTF Biomérieux, Australia), *T. gondii* cysts (National Veterinary Research Institute, Pulawy) and stool of a pig infected with *B. coli* (West Pomeranian University of Technology, Szczecin). All analyses were carried out in two replicates.

RESULTS

The sequencing of all obtained nested and semi-nested PCR products revealed the presence of *C. parvum* and *G. intestinalis* (oo)cysts DNA in five examined sites with the use of nested PCR and semi-nested PCR, respectively (Table 2). *G. intestinalis* cysts DNA was detected in four samples, collected in spring 2011 (three samples) and in spring 2012 (one sample). All four positive samples were collected from four lakes used for bathing; two of them are also used for sewage discharge and one is a watering place for wild animals (Table 2). *Cryptosporidium* oocysts DNA was observed in three water samples collected in autumn 2010 from three lakes used for bathing; two of them are also used for sewage discharge and one of them is located near a farm as well (Table 2). The overall prevalence of *C. parvum* is 0.5% and *G. intestinalis* 0.6%, in all examined samples of water.

The presence of protozoan DNA was not detected using TaqMan nested real-time PCR. No (oo)cysts of *Cryptosporidium* and *Giardia* were detected during microscopic examination. We obtained one positive signal of the RLB method for *Cryptosporidium* only, in a water sample obtained from Dabie Duze Lake (Lubczyna) in autumn 2010.

DISCUSSION

To advance the monitoring of *Cryptosporidium* oocysts in water, the United States Environmental Protection Agency (USEPA) introduced the 1622 method and then the 1623 method used for concentration and detection of

Table 2 | Presence of *C. parvum* and *G. intestinalis* in examined water bodies

		Detected protozoan species		
Site	**Location**	**Autumn 2010**	**Spring 2011**	**Spring 2012**
Rusalka Lake (1)	Szczecin	*C. parvum*	*G. intestinalis*	none
Glebokie Lake (1, 2, 3)	Szczecin	*C. parvum*	none	none
Dabie Male Lake (1, 2)	Szczecin	none	*G. intestinalis*	none
Dabie Duze Lake (1, 2)	Lubczyna	*C. parvum*	none	*G. intestinalis* assemblage B
Weltynskie Lake (1, 4)	Wirow	none	*G. intestinalis*	none

1, site used for bathing; 2, site used for sewage discharge; 3, site located near farm; 4, watering place.

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Cryptosporidium oocysts and *Giardia* cysts in water samples. Both methods are used for determining the presence and concentration of (oo)cysts in water, and they consist of: filtration, concentration, immunomagnetic separation, contrast staining, fluorescent antibodies and microscopic detection, as well as calculation of (oo)cysts of these parasites (Skotarczak 2009).

Many laboratories not only in the USA, but also in Europe and around the world have adopted these methods to investigate the occurrence of (oo)cysts of these pathogens in water. However, it should be noted that the methods do not allow the identification of the species of *Cryptosporidium* or *Giardia*, or the origin of species infectious to humans. The result is that many authors have obtained very high percentages of samples positive for the presence of *Cryptosporidium* and *Giardia* (oo)cysts in water but without indication of species, only with a genus designation, which does not give grounds for the assessment for human risk.

For example, in the case of Gallas-Lindemann *et al.* (2013), the purpose of their study was to establish the prevalence of *Cryptosporidium* spp. and *Giardia* by detecting (oo)cysts from different water sources which were collected in Germany. (Oo)cysts were identified by immunofluorescence test (IFT) and out of 206 wastewater samples, 64 (31.1%) were found to be positive for *Cryptosporidium* oocysts and 134 (65.0%) positive for *Giardia* cysts. According to the authors, this study provides substantial evidence that *Cryptosporidium* spp. oocysts and *Giardia* cysts are able to enter and circulate in the aquatic environment with potential negative implications for public health. Similarly, studies from Spain (Castro-Hermida *et al.* 2010), Luxembourg (Helmi *et al.* 2011), Hungary (Plutzer *et al.* 2007), Poland (Bajer *et al.* 2012) and France (Mons *et al.* 2009) indicated the widespread incidence of (oo)cysts in a high percentage of samples of water from various sources, but these studies did not determine the species. The results in the later study of Plutzer *et al.* (2008) are a very good example, where samples were collected from several water bodies. The (oo) cysts were purified according to the USEPA 1623 method and they were detected using IFT and PCR products sequencing. Although 67% and 42% of the IFT-examined samples were *Cryptosporidium* and *Giardia* positive, respectively, PCR confirmed only half of them. Sequencing detected *G. intestinalis* in only 12 samples, in one sample *C. parvum* and in other separate sample *C. meleagridis*, showing a real threat to public health. Thus, only specific detection based on species determination allows the real evaluation of the presence of *Cryptosporidium* and *Giardia* (oo)cysts in water and the risk for human health.

Our findings show that *Cryptosporidium* and *Giardia* (oo)cysts in surface water bodies in north-western Poland were not numerous and occurred randomly in the samples, without seasonal regularities, so the risk of infection during recreational activities connected with these water bodies is low. However, there is a risk of an outbreak even if only 10–30 *Cryptosporidium* oocysts are present in 100 l of water and *Giardia* may cause disease at a level as low as 3–5 cysts/100 L (Plutzer *et al.* 2007), so the possibility of infection should be taken into consideration.

In our previous studies, we compared the efficiency of various DNA extraction methods from *Cryptosporidium* and *Giardia* (oo)cysts because of the very robust nature of their walls (Adamska *et al.* 2010, 2011b). We carried out attempts to assess the effectiveness of recovery of *C. parvum* oocysts and *G. intestinalis* cysts from spiked environmental and distilled water samples. To remove inhibitors, PCR reactions were carried out with addition of BSA in 10 different concentrations. Effectiveness of oocysts recovery and sensitivity of PCR with each BSA concentration were measured with nested PCR signal and threshold cycle (C_T) of TaqMan nested real-time PCR (Adamska *et al.* 2011a, 2012). In our present study, we have employed our earlier practice for (oo)cysts recovery as well as DNA extraction and detection using different PCR methods. In addition, (oo)cysts were identified with microscopic examination and we also made attempts to adapt the RLB method to waterborne protozoa detection and differentiation of their species.

The RLB method was first described as a reverse dot blot assay for the diagnosis of sickle cell anemia, based on the hybridization of PCR products to specific probes immobilized on a membrane in order to identify differences in the amplified sequences (Gubbels *et al.* 1999). This method was initially used for the identification of *Streptococcus* serotypes (Kaufhold *et al.* 1994), followed by an RLB for *Mycobacterium tuberculosis* strain differentiation (Kamerbeek *et al.* 1997) and is not a new method in the diagnostics of parasitic protozoa; at the end of the 1990s it was adapted for the detection and differentiation of *Babesia* and

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Theileria species (Gubbels *et al.* 1999). Hybridization with the RLB technique allows simultaneous detection of many species and strains of investigated organisms on one membrane, which has the character of a macromatrix. Binding the PCR product with the probe occurs only when there is 100% complementarity between the probe and the amplicon. As a result, accurately designed probes enable interspecies differentiation and the possibility to detect microheterogenetic differences within the species, the so-called inner-species variability of investigated organisms. Thanks to these features, the RLB technique enables identification of organisms from the genus, through species, up to the specific strain. The advantage of RLB is an option to use it for samples in which there is a probability of presence of a bigger number of detectable organisms, for example environmental samples; hence we tested this assay for water samples in order to detect five Sporozoa protozoa and *Balantidium coli*.

Although we amplified successfully all three positive *C. parvum* samples with nested PCR primers and with RLB primers, the RLB hybridization reactions gave only one positive result (for *C. parvum*), the signal was very weak and this finding was not repetitive, so PCR seems to be more sensitive than RLB hybridization for detection of *C. parvum*. What is more, the RLB method is as time consuming as PCR and sequencing but it is more expensive. So, this technique is not an improvement on the methods used in Sporozoa differentiation or determination of their pathogenicity.

CONCLUSIONS

Identification of different *Giardia* and *Cryptosporidium* protozoa in water still remains problematic. The USEPA method is widely used for the detection of these parasites in water, but this method does not allow identification of the *Cryptosporidium* and *Giardia* species and does not differentiate between pathogenic and non-pathogenic types. Therefore, the number or percentage of *Cryptosporidium* or *Giardia* positive water samples and the level of public health threat might be actually lower than have been reported in numerous studies. Nested PCR and semi-nested PCR methods seem to be more sensitive to detect *Giardia* and *Cryptosporidium* DNA in water samples than real-time PCR and RLB methods. The low rates of *C. parvum* and *G. intestinalis* prevalence in the examined water samples from north-western Poland might not pose a significant risk to human health. Nevertheless, the identification of these parasite species indicates that the microbial quality of the examined water has been impacted by humans and livestock.

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