Detection of Cryptosporidium and Giardia (oo)cysts by IFA, PCR and LAMP in surface water from Rasht, Iran

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Received 8 January 2013; revised 25 April 2013; accepted 26 April 2013

Background: Cryptosporidium and Giardia in water supplies is acknowledged as a public health problem. In the present study, we applied immunofluorescence assay (IFA), PCR and loop-mediated isothermal amplification (LAMP) for the detection of the two protozoa.

Methods: Over a period of 12 months, surface water samples were collected from two rivers in the north of Iran, and filtrated by 142 mm membrane filters. At each sampling point 10 L water were used for IFA and the 10 L were analysed using molecular methods.

Results: In 15/40 samples, (oo)cysts were detected by one of the IFA, PCR or LAMP methods. Five samples that were Cryptosporidium-negative by IFA were positive by LAMP. A total of 10 out of 13 samples that were Giardia-positive by IFA were also positive by PCR. IFA revealed high levels of Giardia, with 1–1800 cysts and 1–16 Cryptosporidium oocysts detected per 10 L.

Conclusion: The study reveals that the investigated water supplies were contaminated by Cryptosporidium and Giardia. The LAMP assay has advantages for detection and screening of these protozoa at relatively low concentration in water samples. The three assays applied are complimentary but no single one will give the true prevalence of these parasites in surface water samples. However, each method has its own advantages and disadvantages dependent of the aim and the study design; a combination of detection methods should be applied to discover whether water is, or is not, contaminated with (oo)cysts. This is the first report on the occurrence of (oo)cysts in Iranian surface waters to compare the results of parasite detection obtained with the different methods.

Keywords: Water, Giardia, Cryptosporidium, PCR, LAMP, Iran

Introduction

The occurrence of the (oo)cysts of Cryptosporidium and Giardia in water supplies is globally acknowledged as a public health problem. There are numerous documented reports of water-borne outbreaks of giardiasis and cryptosporidiosis throughout the world.1 At least 199 outbreaks due to the waterborne transmission of parasitic protozoa were reported during the period from 2004-2010.2

A widely used method for detection of oocysts in environmental samples is immunofluorescence assay (IFA), which is time-consuming and subject to variations in sensitivity. Furthermore, this assay does not lend itself to batch processing of samples.3 Traditional phenotypic techniques meet with difficulties in the specific diagnosis of cryptosporidiosis, therefore new molecular tools must be applied. Identifying the species infecting humans and animals is important in determining the epidemiology of disease and likely transmission routes.4,5

Molecular-based methods have been increasingly used for detection, analysis and species identification of Cryptosporidium oocysts and Giardia cysts in water samples.6 Loop-mediated isothermal amplification (LAMP) is an emerging technology that is rapidly becoming recognised as a useful diagnostic tool in the field of parasitology. The successful use of LAMP has been reported for the diagnosis of protozoan parasites, including Cryptosporidium4,6,7 and Giardia.8 However, the application of most of these methods to detect Cryptosporidium and Giardia from environmental samples needs adapting for the specific investigation and water quality, as no standardized methods are available and the possibility to amplify these organisms from the environmental samples in vitro is limited to zero.

In many countries, such as Iran, there are no requirements for testing surface waters for the presence of these parasites. To the authors’ best knowledge, no cryptosporidiosis or giardiasis outbreaks associated with contaminated water consumption have
been described in Iran. No previous data are available on the occurrence of these organisms in water supplies in Iran. The numbers of clinical cases are probably underestimated in Iran due to the lack of systematic methods for the diagnosis of these parasites. The use of molecular tools for the detection and characterisation of Giardia and Cryptosporidium species have been widely applied in recent years, particularly for the detection and characterisation of species from water supplies. Recent reports have confirmed that LAMP can be used as an alternative assay that improves the detection of Giardia and Cryptosporidium species in water supplies.

In the present study, we applied IFA, PCR and LAMP assays for the comparative detection of Giardia and Cryptosporidium (oo)cysts in environmental water samples from Sefidrood and two branches of this river, namely Zarjoob and Goharrood, which pass Rasht city, the capital city of Gilan.

Materials and methods

General information on geography

Gilan is a province in the north of Iran that lies along the Caspian Sea. Gilan has a humid, temperate climate with more than 1200 mm annual rainfall and is known for its moderate, mild and Mediterranean-like climate. Large parts of the province are mountainous, green and forested. Thousands of tourists use the seashore and rivers for swimming and camping activities.

Sampling sites

During 2009–2010, a total of 20 surface water samples were collected from the Sefidrood River and two of its branches, Zarjoob and Goharrood in Rasht city (Table 1). Zarjoob and Goharrood flow into the internationally significant Anzali Wetland and to the Caspian Sea in Gilan province.

At each sampling point, a 20 L water sample was collected and divided in two equal portions; 10 L were used for IFT and the other part was analysed using molecular methods. The water was collected in sterile plastic tanks and transferred to the laboratory for parasitological analysis. The samples were analysed in the laboratory of the Protozoology Unit in the Department of Parasitology and Mycology in the School of Medicine at Shahid Beheshti University of Medical Sciences, Tehran, Iran and in the Laboratory of Molecular and Medical Parasitology in the Medical School of the University of Cologne, Germany.

| Table 1. Summarised results of Giardia duodenalis and Cryptosporidium spp detection in Rasht surface water, obtained using the IFA, PCR and LAMP methods |
|---|---|---|---|---|---|---|
| Sample code | Origin of the sample | IFA | PCR | LAMP |
| | | C | G | C | G | C | G |
| 1 | Sefidrood River | 2 | 180 | – | + | – | + |
| 2 | Goharrood River | 3 | 150 | – | + | – | + |
| 3 | Sefidrood River | – | 27 | – | + | – | + |
| 4 | Eaynak lagoon | 10 | 340 | – | + | – | + |
| 5 | Goharrood River | 16 | 1800 | + | + | + | + |
| 6 | Zarjoob River | – | 41 | + | + | + | + |
| 7 | Zarjoob River | 1 | 1 | – | + | + | + |
| 8 | Sefidrood River | – | – | – | – | – | – |
| 9 | Zarjoob River | – | 6 | – | + | + | + |
| 10 | Zarjoob River | – | 6 | – | – | – | – |
| 11 | Goharrood River | – | 5 | – | – | – | – |
| 12 | Sefidrood River | – | – | – | – | – | – |
| 13 | Emmzadehashem Dam | 2 | 1 | – | – | + | + |
| 14 | Bijar Dam | – | 1 | – | + | + | + |
| 15 | Sangar Dam | – | 1 | – | + | + | + |
| 16 | Emmzadehashem Dam | – | – | – | – | – | – |
| 17 | Emmzadehashem Dam | – | – | – | – | – | – |
| 18 | Emmzadehashem Dam | – | – | – | – | – | – |
| 19 | Sefidrood River | – | – | – | – | – | – |
| 20 | Sefidrood River | – | – | – | – | – | – |
| Total | 6/20 | 13/20 | 2/20 | 10/20 | 11/20 | 8/20 |

–: negative; +: positive; C: Cryptosporidium spp; G: Giardia duodenalis.
centrifuged at 1500 g for 15 min. The supernatant was removed, and the pellet (1–2 ml, depending on water turbidity) was subjected to sucrose flotation.

Detection and enumeration of (oo)cyst by IFA

A 25-μl aliquot of each suspension was placed on a glass–well microscope slide for the enumeration of Cryptosporidium oocysts and *Giardia* cysts. The samples were mounted onto slides, fixed with methanol and stained with fluorescein isothiocyanate (FITC)-conjugated anti-Cryptosporidium spp. and anti-*Giardia* spp. monoclonal antibodies (MAB). Each slide was scanned completely for cysts and oocysts with an immunofluorescent microscope. Organisms that met the criteria for *Giardia* cysts and Cryptosporidium oocysts, including the properties of size, shape, and fluorescence under a total magnification of ×200 to ×400, were labelled as presumptive (oo)cysts.

Detection of (oo)cysts by PCR

DNA extraction

After sucrose flotation of the river water concentrated samples, DNA was extracted using the QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instructions with the addition of 10 time freeze–thaw as has been described previously.5

Detection by PCR

A nested–PCR was performed to detect Cryptosporidium oocysts. PCR primers 5′-TTCTAAGCTAACTAGCC-3′, 5′-CCCATTTTCTTC GAAACAGGA-3′ for primary PCR and 5′-GGAAGGCTTGATTATT AGATAAAG-3′, 5′-CTCATAGGCTGAGGAAGTA-3′ for secondary PCR that have been previously described were used to amplify a 825-bp fragment of Cryptosporidium oocyst 18s RNA.9 Both PCRs were performed in standard mixtures of 50 μl containing 1.25 μl primer mixtures (200 nmoles of each primer), dNTP mix 8 μl (200 mM dNTP of each dNTP) (Finnzymes, Espoo, Finland), 1 x PCR buffer containing 1.5 mM MgCl2 (Qiagen) 5 μl; 3 mM MgCl2 (Qiagen) 3 μl; 0/5 μl of 2.5 U HotstarTaq DNA polymerase (Qiagen) and 2 μl bovine serum albumin (acetylated, 10 mg/ml) (Promega, Madison, WI, USA), 3 μl DNA and 27.25 μl distilled water.

For primary PCR, the amplification reactions were run according to the following PCR program: an initial step at 94 °C, 3 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min; then 72 °C for 7 min and a final hold at 4 °C. For secondary PCR, each reaction was prepared as for primary PCR, but F2 and R2 primer were used, and the following PCR program was run: 94 °C, 3 min; 35 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min; then 72 °C for 7 min and a final hold at 4 °C. Semi-nested-PCR was performed using the primers GDHeF: 5′-TCAACGTYA VCY GYG GYT GCC GT-3′, GDHiF: 5′-CAG TAC AAC TAC GCC GTG GCC-3′ in primary PCR and GDHiR: 5′-GTT RTC CTT GCA CAT CTC C-3′ in secondary PCR, as published by Read et al.10 to amplify a 432-bp fragment of the *Giardia* glutamate dehydrogenase gene (GDHi).

The PCR was performed in a standard mixture of 25 μl containing 200 nmol of each primer, 0.2 mM dNTP, 1.5 mM MgCl2, 2.5 U Taq DNA polymerase (Qiagen), and 2 μl bovine serum albumin (BSA, 10 mg/ml) plus 10X Perkin-Elmer PCR buffer. The templates were subjected to an initial denaturation at 94 °C for 2 min followed by 35 cycle of 94 °C for 2 min, 55 °C for 10 seconds, 72 °C for 30 seconds and a final extension at 72 °C for 5 min. A secondary PCR was run using the following conditions: an initial step at 96 °C for 4 minutes, followed by 35 amplification cycles 94 °C for 20 s, 53 °C for 20 s, 72 °C for 40 s, and a final extension at 72 °C for 5 min. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.6 mg/ml) and visualised with the Gel Doc device.

Detection by LAMP

Cryptosporidium LAMP assay: primer sets targeting the S-adenosyl-l-methionine synthetase (SAM-1) gene were used for *C. parvum*, *C. meleagridis* and *C. hominis*.7

*Giardia* LAMP assay: the elongation factor 1α (EF1α) gene from *G. duodenalis* Assemblage A and B was used for detection, as described.8

The LAMP reaction was performed in a reaction mixture of 25 μl containing 1.3 μM of primer mixture (40 pmol each of the FIP and BIP primers, 20 pmol each of the LF and LB primers and 5 pmol each of the F3 and B3 primers), 12.5 2 x reaction buffer (40 mM Tris-HCl, 20 mM KCl, 16 mM MgSO4, 20 mM [NH4]2SO4, 0.2% Tween 20, 1.6M betaine and 2.8 mM each deoxynucleoside triphosphate), 1 μl (8 units) Bst DNA polymerase, 2 μl DNA, and 8.2 μl distilled water. Samples were incubated at 63 °C for 60 min in the case of Cryptosporidium LAMP and for 120 min in the case of *Giardia* LAMP. The samples were heated at 80 °C for 7 min to terminate the reactions. Magnesium pyrophosphate, a by-product of the amplification reaction, is produced in proportion to the amount of amplified products and results in turbidity of the solution. Because LAMP amplification can produce extremely large amounts of amplified products, white turbidity can be visually observed. Based on this feature, the presence of turbidity can indicate the presence of the target gene, and visual detection can be achieved. In each trial, a positive and a negative control were included.6

Results

Natural samples: The study determined that most of the samples contained high concentrations of *Giardia* cysts and low concentrations of Cryptosporidium oocysts (Table 1). *Giardia* cysts were more prevalent (13 out of 20 samples) than Cryptosporidium oocysts (4 out of 20) based on the IFA method. Of the six samples that were Cryptosporidium-positive by IFA, one sample was also positive by PCR (Figure 1). A total of 10 out of 13 samples that were *Giardia*-positive by IFA were also positive by PCR (Figure 2). All samples that were *Giardia*-negative by IFA were also negative by PCR, whereas LAMP confirmed that 8 out of 20 samples were *Giardia*-positive, and 11 samples contained Cryptosporidium oocysts (Table 1; Figure 3). In 11/20 samples, both parasites were detected by at least one of the methods used. The Goharrood and Zarjooob Rivers were heavily contaminated, with concentrations ranging from 1–1800 cysts and 1–16 oocysts per 10 L. In the samples collected from sources of drinking water, the Sangar Dam, the Bijar Dam and the Emamzadeh-hashem Dam, only one *Giardia* cyst per 10 L was detected by IFA, but these samples were found positive for Cryptosporidium DNA according to the LAMP assay; in the
case of the Emamzadeh-hashem Dam, two Cryptosporidium oocysts were detected by IFA.

Discussion

Waterborne diseases occur worldwide, and outbreaks caused by the contamination of community water systems have the potential to cause disease in large numbers of consumers. In addition to outbreaks caused by contaminated potable water, there are outbreaks caused following the accidental ingestion of recreational (or other) waters.

Rapid and effective monitoring methods are needed at drinking water facilities to determine the occurrence of oocysts in source and treated water. The development of such methods would lead to better decisions concerning treatment, contamination, and public health risks. The rivers considered in the study flow into the Anzali Lagoon and the Caspian Sea, which will also be affected. In the Sangar Dam, the Bijar Dam and the Emamzadeh-hashem Dam, which are also sources of drinking water, only one Giardia cyst per 10 L water was detected by IFA, and these samples were also found to be positive for Cryptosporidium DNA by LAMP; in the case of the Emamzadeh-hashem Dam, two Cryptosporidium oocysts were detected.

The microscopic identification of the (oo)cysts was based on IFA analysis. Giardia cysts occurred more frequently (13/20) and at a higher density than Cryptosporidium oocysts (6/20) and in the investigated water samples by PCR results showed a similar trend. More frequent findings of Giardia cysts in untreated waters rely on the assumption that giardiasis is more widespread and occurs with greater intensity than cryptosporidiosis. Other studies reported this trend in surface waters in other countries. Furthermore, several characteristics of the oocysts could also cause their detection to be less than expected. Oocysts of Cryptosporidium are smaller in size (approximately 4–6 μm), and they are able to change shape, which could allow them to squeeze through the pores of the membrane filter during vacuum pump filtration.

For internal testing reasons (oo)cysts were obtained from the faeces of naturally infected calves and humans. Water samples (5 L) were seeded in duplicate at concentrations of 5 (oo)cyst/L and 10 (oo)cyst/L. The seeded samples were concentrated by filtration through a membrane filter and purified by sucrose flotation. The (oo)cysts were then counted microscopically by IFA using the Cryptosporidium/Giardia IFA cell test (Cellabs, Brookvale, NSW, Australia).
The mean recovery rate was 12% for Cryptosporidium and 18% for Giardia (oo)cysts and all seeded samples were also positive by molecular methods PCR and LAMP (data not shown).

The concentrations of (oo)cysts in surface water are usually low, ranging from 1–18 (oo)cysts/10 L. In the Netherlands, higher concentrations of (oo)cysts in surface water have been detected. The average concentration, corrected with the average recovery efficiency, was 4.5 and 5.4 oocysts/L and 22 and 95 cysts/L in rivers Rhine and Meuse, respectively. Natural surface water from rivers and reservoirs in northern Spain have been found with concentrations that reached 1767 Cryptosporidium oocysts and >25 000 Giardia cysts per 100 L. Surface water in Italy was contaminated with 0–5 Cryptosporidium oocysts and 6 × 10¹ – 8 × 10¹ Giardia cysts per/L. Cryptosporidium oocysts in surface waters draining from a livestock farm on a Warwickshire (UK) estate has shown a median concentration of 0.48 oocysts/L.

In a study of water supplies in southern Russia and Bulgaria, 16 out of 166 samples (9.6%) were positive for Giardia and 30 (18.1%) positive for Cryptosporidium. In the present study the Goharrood and Zarjoob Rivers were heavily contaminated with cysts (1–1800 per 10 L) and oocysts (1–16 per 10 L). According to the mean recovery of seeded (oo)cysts’ experiments in this study the average concentration will be more than these numbers in the recovery trials as mentioned above. The reasons for these high concentrations are probably because Goharrood and Zarjoob Rivers cross throughout Rasht city, and, therefore they are exposed to agricultural and urban wastes. After passing through the city, these two rivers provide water to farms that produce rice and vegetables. The flow of these rivers to Bandar Anzali Lagoon will result in pollution of this coastal lagoon and of the Caspian Sea in the northern Iranian province of Gilan. This lagoon occupies 15 000 hectares, and has been registered as an international wetland by the 1975 Ramsar Convention.

Several studies have shown that nested PCR appears to be more sensitive than microscopy for detecting Cryptosporidium and Giardia in water concentrates. In our study, only one sample out of six that were Cryptosporidium–positive by IFA and 10 out of 13 samples that were Giardia–positive by IFA were also positive by PCR. This could be due to the high concentration of PCR inhibitors or the presence of empty oocysts in the investigated water samples, which would prevent their detection by molecular-based methods. Additionally, these results may be due to the low concentration of DNA in IC and the uneven distribution of template DNA, especially in samples containing low numbers (1–3) of oocysts. We used non-acetylated bovine serum albumin in all primary PCRs to neutralise or inhibit PCR inhibitors.

IFA was the more sensitive technique in our studies than the PCR applied, however, analysis of PCR products can be used to determine the species and genotypes of an isolate. PCR confirmed the presence of Giardia in two samples, but LAMP did not. Application of the LAMP assay illustrates an important feature of LAMP: with the use of six primers to recognise eight distinct regions, only the target gene is amplified. The reaction is specific, and LAMP (primers FIP and BIP) has the ability to discriminate single-nucleotide differences (SNP). Thus, in cases where PCR products sequenced detected G. duodenalis and LAMP did not, it is possible that Giardia DNA in the samples present SNPs. The variation among the molecular methods results is probably due to stochastic sampling of low-concentration DNA and the uneven distribution of template DNA in each sample. However, another current limitation of the LAMP assay is the requirement for primer design, which is based on the available published gene sequences. Variations in primer binding regions would affect the ability of the G. duodenalis–specific LAMP primers to amplify the target region of the DNA from all subgenotypes and genotypes in the G. duodenalis–complex. This problem could be resolved in the future as more representative sequences become available. At present, the use of the LAMP assay for the detection of G. duodenalis Assemblage A and B is a complementary tool to PCR detection because the LAMP reaction is not affected by the presence of inhibitors. Therefore, LAMP may be used to “filter” false-negative reactions.

In the case of Cryptosporidium, 6/20 samples were positive by IFA, and one of these IFA-positive samples was positive by PCR. For four of the IFA-positive samples and five of the IFA-negative samples, Cryptosporidium were found using the LAMP method, indicating that the LAMP method is not affected by inhibitors, and the LAMP reaction was more sensitive than PCR. This has previously been demonstrated in applications of LAMP for Giardia and Toxoplasma detection in water samples.

In a previous study by Xiao et al., using the same primer set used in present study, the high sensitivity of the PCR approach was demonstrated when oocysts were purified by IMS. In the present study, the detection sensitivity of this 18S RNA primer set was much lower than the IFA and LAMP assays. The reason for this discrepancy may be the different methods of oocyst purification (e.g., sucrose flotation instead of IMS). The discrepancies caused by IFA–positive–PCR–negative results could be due to the tendency of the IFA reagents to cross–react with non–target organisms, such as algae, or due to the inhibitory effects on PCR enzymes caused by interfering substances, such as humic acids. It is important to note that because of the specificity of the targeting to actual genetic sequences that are unique to a given organism, the PCR method is less likely to show cross–reactivity. This allows for a distinct advantage over the IFA method. At present, empty oocysts cannot be detected by molecular–based methods, which lead to an underestimation of oocyst contamination. Microscopic examination adds significant value to PCR–negative results. The LAMP assay for the detection of Giardia and Cryptosporidium (oo)cysts is an excellent tool to complement the results of PCR because PCR is strongly inhibited by various substances that are often present in the samples, which have no effect on the LAMP assay. Therefore, by means of LAMP, it is possible to increase the methodological sensitivity and the number of positive results.

As mentioned above, each method has advantages and disadvantages, so dependent on the aim and design of the study, a combination of detection methods should be applied on water samples to detect whether that water is, or is not, contaminated. To our knowledge, this is the first study performed in Iran for the purpose of detection and enumeration of Giardia and Cryptosporidium in an Iranian water supply. The study provides new information on the contamination of rivers by the pathogenic protozoa Cryptosporidium and Giardia in Rasht City. Furthermore, the study reveals that Goharrood and Zarjoob Rivers, which provide water for farming, are heavily contaminated by Cryptosporidium and Giardia; Anzali Lagoon will also be affected. Among the samples collected from the drinking water sources (Sangar Dam, Sefid Roud Dam and Bijar Dam) both parasites were detected by at least one of the IFA, PCR or LAMP methods. Molecular identification of Cryptosporidium from humans and animals in Iran has been reported before, but the most common detection methods
were staining methods, such as the Ziehl Neelsen procedure. The prevalence and epidemiological features of giardiasis were variable in different parts of Iran, from 5 – 23%.

The presence of *Giardia* and *Cryptosporidium* in aquatic ecosystems in Iran makes it imperative to develop prevention strategies for water and food safety. National statistics on outbreaks linked to contaminated water are not available for Iran. It is now clear, through the analysis of outbreaks, that the pathogens can pass into recreational and drinking water.  

In Iran there are no requirements for testing surface water for the presence of these parasites, and the detection of *Giardia* and *Cryptosporidium* and the prevalence of these organisms in water have not been studied before. The present work may contribute to this gap and will function as a platform for future research. Additional and more systematic studies and further monitoring of these pathogens in Iran are required.

**Authors’ contributions:** M-RM and BK conceived, designed, analyzed, interpreted, and critically revised the manuscript. M-RM, AM, AS and AM undertook the literature searches and analyzed the data. M-RM and PK wrote the manuscript; PK provided suggestions and additional information and critically revised the manuscript. All authors have approved the final version of the manuscript for submission. M-RM, BK and PK are guarantors of the paper.

**Acknowledgments:** We would like to express our appreciation for Dr. Ali Haghighi, Dr. Keyhan Ashravi, Mrs. Johantab, Mrs. Raeesayan, Mrs. Parvar, Isaia Sotiriadou for his kind cooperation and assistance. This work was supported by the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences and Tehran Province Water & Wastewater Co. (TPWW).

**Funding:** This work was supported and funded by the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences and Tehran Province Water & Wastewater Co. (TPWW), and by the host Universities of the authors in Iran and in Germany.

**Competing interests:** None declared.

**Ethical approval:** Not required

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