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### Original Article

## Development of Sensitive Detection of *Cryptosporidium* and *Giardia* from Surface Water in Iran

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### ABSTRACT

**Background:** The protozoan parasites *Cryptosporidium* spp. and *Giardia* are known to occur widely in both raw and drinking waters. They are two of the causative agents of waterborne outbreaks of gastroenteritis throughout the world. In the present study, a PCR assay and FA were developed for detection of *Cryptosporidium* oocysts and *Giardia* cyst in environmental samples.

**Methods:** We have detected *Cryptosporidium* spp. oocysts and *Giardia* cysts in seeded and unseeded environmental water samples by PCR method. Water samples were spiked with oocysts (50, 100,300,500) and filtrated with a 1.2- $\mu$ m pore size cellulose nitrate and follow by DNA extraction and purification by QIAamp DNA mini kit. Nested-PCR assay amplified an 850 bp fragment of 18s rRNA gene specific for *Cryptosporidium* and 435 bp fragment of glutamate dehydrogenase (GDH) target gene for *Giardia*. Also many river water from north of Iran, be checked by these methods.

**Results:** *Cryptosporidium* and *Giardia* DNAs were detected in seeded water sample and *Giardia* was detected in all 5 water samples from river in north of Iran by nested- PCR and FA. Also in one river water sample, *Cryptosporidium* was detected.

**Conclusion:** This protocol is effective for detection of these waterborne parasites in treated and untreated water samples. This study can also serve as a platform for further investigations and research water source in Iran.

**Keywords:** *Cryptosporidium* spp., *Giardia duodenalis*, Nested PCR, Iran

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## Introduction

The presence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in water source are growing problem throughout the world (1).

Although *Giardia duodenalis* causes an intestinal illness called *Giardiasis* (1) but *Cryptosporidium*, infect the gastrointestinal or extra intestinal of mammals, including humans (2) zoonotic transmission plays an important role in epidemiology of *Cryptosporidium*(3) and

*Cryptosporidium* causing chronic diarrhea in people with weakened immune systems and is sometimes fatal (4). Pollution of the rivers and lakes, by oocyst leading to waterborne disease, thus, ultimately the natural transmission cycle is completed (2).

Infective oocysts are environmentally resistant, are small enough to penetrate the physical barriers of water treatment, and are resistant to many disinfectants used in the water industry (5).

Cases of *Cryptosporidiosis* outbreak (4) and *Giardiasis* (6) due to drinking water contamination with these parasites have been reported.

In order to control outbreaks and prevent transmission of water, rapid and effective monitoring methods for presence of *Cryptosporidium* and *Giardia* are needed at drinking water facilities. Development of such methods would improve decisions concerning treatment, contamination, and public health risks.

The widely used method for detection of oocysts in environmental samples is the immunofluorescence assay (FA). However, FA is time-consuming and expensive and requires skilled and experienced technician (7). Recently PCR-based methods have been used increasingly for detection and analysis of *Cryptosporidium* oocysts and *Giardia* cysts in water by many researchers (8, 9). In

addition to high sensitivity and specificity of PCR for the detection of protozoan parasites in water samples, the PCR method is relatively quick and easy if you had enough experience.

In the present study, a PCR assay and FA were developed for detection of *Cryptosporidium* oocysts and *Giardia* cyst in environmental samples.

## Materials and Methods

*Cryptosporidium* spp. oocysts and *Giardia* cysts were obtained in feces from naturally infected calves and infected human respectively. Environmental water samples were seeded with purified oocysts ( $\leq 50, 100, 300, 500$ ) oocysts. All samples were filtrated by membrane filters, concentrate by sucrose flotation and evaluated by the PCR assay and IF.

In addition, five lit of five surface water samples were collected in the period between 2009 and 2010 from North of Iran that was evaluated by the PCR assay.

For unseeded environmental sample, oocysts were counted by using sucrose-flotation and follow by FA. Five liters of seeded water samples (50, 100, 300, 500 oocyst) and 5 liters of 5 environmental water samples from river water in Guilan- north of Iran were filtered through a 142 mm diameter membrane filter with a pore size of 1.2  $\mu\text{m}$ .

For recovery of particles, the filter was rinsed by 50 ml of 0.1% PBS-Tween 80. This process was repeated two times and particulates concentrated by centrifugation in at 3000 g for 10 min. The reaction was subjected to sucrose-flotation and different DNA extraction methods for nested PCR technique. For unseeded environmental samples, plus PCR, we also did FA for detection and counting of oocyst. In most studies, oo-

cyst is spike in distilled water, but in this study, we used environmental water to set up these protocols in a natural condition. In addition, freezing thawing, followed by phenol-chloroform extraction and the QIAamp DNA mini kit (Qiagen) were tested for their ability to extract relatively DNA from seeded and unseeded environmental water samples.

#### ***Detection and enumeration of cysts and oocysts by FA***

The prepared suspensions of *Cryptosporidium* spp. oocysts and *Giardia* cysts were mounted onto slides, fixed with methanol and staining with fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* spp. and anti-*Giardia* sp. monoclonal antibodies (MAb) (Cellabs Pty Ltd.). Each slide was scanned completely for detection and enumeration of cysts and oocysts based on criteria including as the right size, shape, by using an immunofluorescent microscope.

#### ***DNA extraction with the QIAamp DNA mini kit***

DNA was extracted according to the manufacturer's instructions with the addition of 10 time freeze-thaw has been described previously (9). Briefly, by adding 180 µl of the ATL buffer from the QIAamp DNA minikit, the suspension was transferred into a 1.5-ml Eppendorf tube and subjected 10 time, freeze-thaw cycles (10-min 56 °C and liquid nitrogen). After that, DNA was extracted with the QIAamp DNA minikit and the manufacturer-recommended procedures.

#### ***DNA extraction with phenol-chloroform***

Briefly, 500 µl of Tris-EDTA buffer was added to 0.5 ml of water pellets, and flowed by ten cycles of freeze-thaw (-196°C and 56°C). The samples were processed further by the conventional phenol-chloroform DNA extraction method (10).

#### ***Optimizing of PCR assay***

PCR optimized by using extracted DNA of different dilution of oocyst ( $\leq 50, 100, 300, 500$ ). PCR reactions were set up with BSA and without BSA.

The GDH target gene for *Giardia* and the ssUr DNA for *Cryptosporidium* were amplified by PCR. *Cryptosporidium* oocysts in water samples were identified by a previously described nested-PCR technique and 850 bp encoding 18s rRNA was amplified (11). Each sample was amplified using different volumes of DNA preparation (0.5, 1 and 2 µl). Each PCR reaction contained 30µl. of 200 nmol of each primer, 0.2 mM of dNTP, 1.5mM of MgCl<sub>2</sub>, 2.5U DNA polymerase, 3µL of 10x PCR buffer (CinnaGen), and 1 µL of bovine serum albumin (BSA, 10 mg/mL). The following thermal protocol was used: nest I PCR consisted of a predenaturation at 94 °C for 3 min; 35 cycles of 94 °C for 45 second, 60 °C for 50 second and 72 °C for 1 min and final extension at 72 °C for 7 min. The nest II (second) PCR parameters were as nest I (first) PCR, exception for annealing step which was done at 58 °C for 50 seconds. Positive and negative controls were run with every PCR set. A semi-nested PCR was performed using the primers and PCR conditions published by Read et al to amplify a 432-bp fragment of *Giardia* glutamate dehydrogenase gene (GDH) (12).

The PCR was performed in standard mixtures of 30µl containing 200 nmol of each primer, 0.2 mM of dNTP, 1.5mM of MgCl<sub>2</sub>, 2.5U Taq DNA polymerase, 3µL of 10x PCR buffer (CinnaGen, Tehran, Iran), and 1 µL of bovine serum albumin (BSA, 10 mg/mL), (CinnaGen). The templates were subjected initial denaturation at 94 °C for 2 min, 35 cycle of 94 °C for 2 min, 55 °C for 10 second, 72 °C for 30 second and final extension at 72 °C for 5min.

Second PCR was done as denaturation at 94 °C for 20 s, annealing at 53 °C for 20 s,

extension at 72 °C for 40 s, and final extension at 72 °C for 5min. PCR products were electrophoresed on a 1.5% agarose gel contained ethidium bromide and visualized with the UV Transilluminator device. These optimized protocols were used for evaluation of environmental water samples, too.

## Results

Clearly, the first PCR reaction did not amplify the oocysts DNA in seeded water, but all environmental water samples that are spiked with oocysts ( $\leq 50, 100, 300, 500$ ) were positive by nested PCR (Fig. 1). Nested PCR increase the sensitivity detection of samples with low parasite loading. Nested PCR also was positive with different volumes of DNA preparations (0.5, 1 and 2  $\mu$ l).

In the present study, 2 $\mu$ l of DNA (extracted by QIAamp mini kit and phenol-chloroform from oocysts -negative river water samples) was spiked into the PCR mixture of a pure *Cryptosporidium* spp. DNA for analysis of PCR inhibitors. Results indicated that method QIAamp mini kit removed more PCR inhibitors than the phenol-chloroform extraction method.

conjugated anti-*Cryptosporidium* spp. monoclonal antibodies (MAb). (A and B: x400 and C: x200 magnifications)

In this study, the inclusion of 1  $\mu$ l of BSA (10mg/ml) to the PCR mixture reduced the impact of PCR inhibitors in DNA extracted and increases the sensitivity of PCR. In compare of QIAamp DNA mini kit DNA extraction, freezing-thawing followed by phenol-chloroform extraction, didn't gave consistently high yields of DNA for water samples (Fig. 2).

All oocysts-negative river water samples, seeded by oocysts, concentrated by membrane filter and follow by DNA extraction, were positive by PCR method.

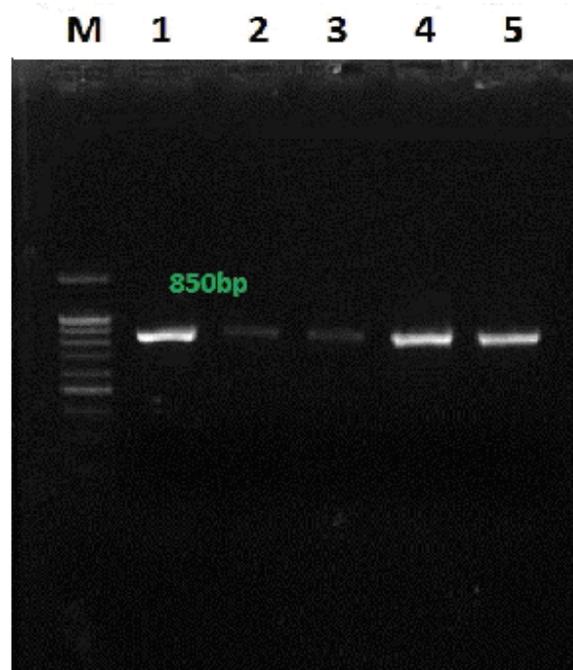
Five unseeded environmental water samples with various turbidity (Table 1) from north of Iran have been tested by PCR and FA assay, in one sample a few *Cryptosporidium*-like oocyst (2-3 oocyst) were detected by FA (Fig. 3 ) but it was negative by PCR (Table 1).

Application of the PCR test has shown the presence of *Giardia* cysts in 5-liter volumes of all of environmental water samples. In the same samples, all samples were positive for *Giardia* by FA (Table 1).

In river water, a single PCR did not always detect the desired target but nested PCR increased the chance of obtaining a positive result.

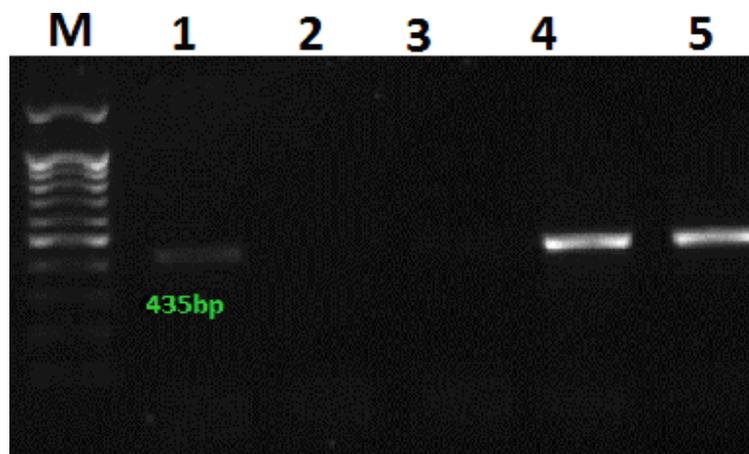
**Table 1:** Detection of *Giardia* and *Cryptosporidium* in surface water by PCR and FA

Sample	Turbidity (NTU)	<i>Giardia</i> spp.			<i>Cryptosporidium</i> spp.		
		PCR	Nested PCR	FA(No.)	PCR	Nested PCR	FA(No.)
1	11	-	+	+(300)	-	-	+(1-2)
2	75	-	+	+(50)	-	-	-(0)
3	21	-	+	+(320)	-	-	-(0)
4	16	-	+	+(1000)	-	-	-(0)
5	11	-	+	+(41)	-	-	-(0)

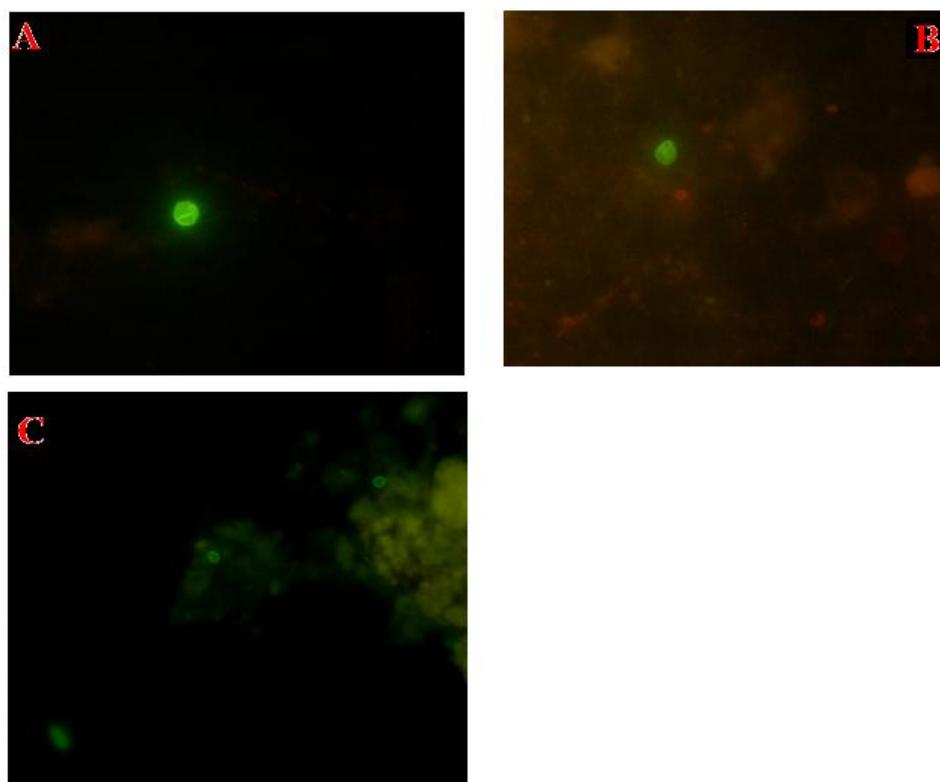


**Fig. 1:** Detection of *Cryptosporidium* by nested PCR of ssU rRNA gene using 1.5% agarose Gel electrophoresis

Lane 1: positive control; Lane 2:  $\leq 50$  oocyst; Lane 3: 100 oocysts; Lane 4: 300 oocysts; Lane 5: 500 oocysts



**Fig. 2:** 1.5% agarose gel electrophoresis of PCR product. Lane 1: *Giardia* positive control by Phenol-chloroform extraction; Lanes 2 and 3: DNA extraction by Phenol-chloroform of surface water samples; Lanes 4 and 5: DNA extraction by QIAamp DNA mini kit of surface water samples



**Fig. 3:** Sample preparation for fluorescent microscope and *Cryptosporidium* like elements were detected. Binding of monoclonal antibodies to *Cryptosporidium* like cells (panels A, B and C were stained using a secondary antibody conjugated to fluorescein isothiocyanate (FITC)

## Discussion

PCR has some advantages over immunofluorescence microscopy in detection sensitivity (15-17) and ability to distinguish between species and genotypes (7, 9, 11, 13, 14). PCR technique needs DNA as low as a fraction of the DNA from one sporozoite (15).

Although most of these techniques perform successfully for fecal samples or purified oocysts, but it was reported the analysis of environmental samples are not as good as fecal samples (11, 16, 17).

It seems that water with higher turbidities will need extra purification prior to PCR. A

common problem encountered with environmental samples is inhibition of PCR by materials like humic acids that co extract with the DNA (10).

In three steps it is possible to somewhat overcome the effect of PCR inhibitors, including prior to DNA extraction, during or after DNA extraction, and when performing PCR (10, 18).

In this study, DNA purification after DNA extraction has been applied by using the QIAamp DNA mini kit to remove PCR inhibitors. Amplification for low oocysts

was achieved only with DNA extracted by QIAamp DNA mini kit in compare to phenol chloroform method.

Many studies have improved the amplification of PCR by inclusion of BSA (19, 20). In the present study, the effect of PCR inhibitors could be relieved somewhat by the addition of BSA too.

As said previously, through our optimized protocol, *Giardia* cysts were detected in all environmental water samples by FA and PCR assay protocol. All seeded environmental water samples were positive for *Cryptosporidium* by PCR method (Table 1), but about unseeded environmental water sample, in one sample, a few *Cryptosporidium*-like oocysts (2-3 oocysts) were detected by FA but it was negative by PCR.

It could be due to cross-reaction in IFA method with others organisms or to inhibition of PCR, enzymes by interfering substances, such as humic acids, present in the water samples (8).

“PCR detection sensitivities ranged from 1 to 10 oocysts or cysts for purified preparations and 5 to 50 oocysts or cysts for seeded environmental water samples” (21).

In this study, nested PCR detected less than 50 oocysts in seeded environmental water sample. Losses of oocysts have been reported during concentration by filtration (22). This suggests that sample preparation methods need to be developed in order for PCR to be efficient at too low dilutions in some environmental water samples types or drinking water. To our knowledge, it is the first study that evaluated sample processing and detection of *Cryptosporidium* and *Giardia* from environmental water in Iran by IFA and PCR methods.

In contrary of most previous studies, that the oocysts were inoculated into filtered water pellet suspensions (20, 21, 23,24), in the present study the oocysts were inoculated directly into the environmental water samples,

so mimicked a real analysis situation of the detection procedure.

We set up and perform satisfactory, IF and PCR methods for detection of these parasites in water samples. Therefore, this study could be a platform for future studies in water source of Iran. Therefore, detection and isolation of these organisms in water samples may be required in preventative public health programs. We must increase the public's awareness and knowledge and educate them about risk to human health and we must pay more attention to pollution of surface water to these parasites.

In conclusion, results of the present study suggest, the filtration by membrane filter and DNA extraction by QIAamp DNA mini kit was useful for detection of this water born parasites in water with high turbidity and can also use for some other protozoan in water samples.

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