Detection and enumeration of *Cryptosporidium* oocysts in environmental water samples by Real-time PCR assay

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Abstract

Introduction: The protozoan parasite, *Cryptosporidium* Spp., widely spreads in both raw and drinking waters. It is the causative agents of waterborne diarrhea and gastroenteritis in the world. In the present study, a molecular assay was used for the detection and quantification of *Cryptosporidium* oocysts in environmental water samples.
Materials and methods: Thirty surface water samples were collected from Rasht City rivers and lagoons during 2009-2010. The samples were analysed for *Cryptosporidium* oocysts using Real Time PCR method. Samples were filtrated through a 1.2µm pore size cellulose nitrate membrane filter and then purified and quantified by Real-time PCR technique.
Results: *Cryptosporidium* oocysts were found in 19 of 30 the samples. Oocyst concentration was ranging from 0.007 to 27 oocysts per liter of the examined waters.
Conclusion: The present study showed that the investigated water supplies were contaminated by *Cryptosporidium* oocyst. This study indicated that in this level of oocysts there is a potential risk of waterborne cryptosporidiosis due to direct or indirect consumption of these waters by humans and animals. Real-time PCR is a technique that provides high sensitivity for detection quantitative purposes.

Keywords: *Cryptosporidium* spp., Water, Iran, Real-time PCR

Introduction

Cryptosporidium is one of the important agents of diarrhea and gastrointestinal disorder among children and patients with AIDS and it has been frequently responsible for waterborne outbreaks resulting from contaminated drinking water and recreational waters (1, 2). Ninety percent of reported outbreaks of these pathogenic protozoans occur through water, while 10% are related to food (3).

It seems that effective levels to outbreaks with these protozoa ordinarily occur when oocyst concentrations raised to 5 *Giardai* cyst in 100 liters of water sampled (4, 5) and 10 to 30 oocysts for *Cryptosporidium* (6). Detection of low numbers of organisms in water samples is difficult, it needs very sensitive techniques. In the past decades, conventional methods such as fluorescence labeled antibodies by immunofluorescence assays (IFA) were

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used to detect the *C. parvum* oocysts in water, but these methods have some limitations as difficulty in applying them in turbid samples and also in their need for advanced equipment for microscopically oocyst seeing. Conventional PCR techniques is the end point, therefore, it cannot be made a quantitative assessment of the copy number of parasites. But instead, quantitative Real-time PCR is capable of quantitative evaluation from early exponential phase of amplification and PCR products show that this rate is proportional to the initial DNA concentration (7-11).

IFA method is a prevalent method for the detection of *Cryptosporidium* in water samples. But this technique has some limitation and it is unable to species determination, so recently researchers paid more attention to molecular methods. Number of oocysts and species determination are two important factors for designation of health risk level. The small number of oocysts can cause disease in humans and the water sources are introduced as an important way of transmitting for this protozoan parasite. So, only a few studies have described a TaqMan quantitative PCR specific to *Cryptosporidium* (7-11). So, in present study we decided to use Real-time PCR technique for the determination and quantification of these microorganisms in water.

Materials and methods

Samples collection and filtration: Totally 30 environmental water samples were collected from surface water in Guilan province, north of Iran. Dependent to water turbidity, 2-35 liters of water were filtered. Water passes through a 142 mm diameter membrane filter with a pore size of 1.2 µm by means of vacuum device. For the recovery of particles, the filter was rinsed by 50 ml of 0.1% PBS-Tween 80 and particulates concentrated by centrifugation in at 3000 g for 10 min. For the purification of oocysts, the pellet was subjected to sucrose-flotation according to our previously paper (12).

Genomic DNA extraction: Total DNA from samples were extracted using QIAamp DNA minikit Qiagen(GmbH, Hilden, Germany) as recommended by Jiang et al. (2005) (13) with some modification including suspension then it was subjected to 15 freeze–thaw cycles (1 min in liquid nitrogen and 1 min at 65 °C per cycle).

Quantitative Real-time PCR: The Primer Design™ genesig Kit for *Cryptosporidium* (Crypto) Genomes was used for the quantification of *Cryptosporidum* genomes. A pair of oligonucleotide primers used for real-time PCR in this kit were designed to detect the 18S rRNA gene of *Cryptosporidium* species, with sequences matching with *C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis* and *C. suis* completely and with a single base pair (bp) mismatch at forward primer position 5 of 22 for *C. felis* and position 18 of 22 for *C. muris* (PrimerDesign Ltd). PCR mixtures were prepared according to the manufacturer's instructions. 2x Precision TM Master Mix 10 µl, Crypto Primer/Probe mix 1 µl, internal extraction control primer/probe mix 1µl, RNase/DNase free water 3µl and 5µl of diluted DNA template (suggested concentration 5ng/µl). PCR amplification program was performed for 50 cycles' denaturation (10 seconds at 95°C) and data collection (60 seconds at 60°C).

The fractional cycle number at which real-time fluorescence signal mirrors progression of the amplification reaction over the background noise level is used as an indicator of successful target amplification. Commonly, this is called the threshold cycle (Ct) (14). All the reactions were performed in triplicate. Data were collected from the green (FAM) channel and post-run analysis performed using the Rotor gene 6000 software version 1.7 (Corbett Research)

Conversion of 18S rRNA gens number of *Cryptosporidium* spp. to number of

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***Cryptosporidium* oocysts:** It has been reported that each *Cryptosporidium* genome in one oocyst has 20 copies of 18S rRNA genes (15). Therefore, it is possible to numerate *Cryptosporidium* oocysts by knowing the copied numbers of 18S rRNA gene in each sample. Then the copy number was converted to the number of *Cryptosporidium*. In the present study, accordingly the Real Time PCR data as shown in Table1 the number of oocyst were calculated based on the following formula (Log Copy number= (CT single copy - CTS)/Slop).

Results

Altogether, 30 surface water samples from Rasht city (Guilan Province North of Iran) rivers were collected and Cryptosporidium oocysts were successfully quantified. 19 out of 30 samples were positive by Real Time PCR. Oocyst concentration was ranging from 0.007 to 27 in different samples. The lowest number of Oocyst belongs to Zarjoob and highest number to Goharrood River (Table 1).

Table 1. Number of oocyst detected in water samples by Real Time PCR.

River name	Mean C_T*	C_T Single copy	Copy number	Oocysts per litter
Zar-Joob	31.855	39.72	5.893	1
Zar- Joob	19.525	39.72	27.372	2
Zar- Joob	36.255	39.72	0.806	0.2
Zar- Joob	19.025	39.72	28.745	2.9
Zar- Joob	15.91	40.33	51.891	4.5
Gohar-Rood	34.91	40.33	2.556	0.08
Gohar-Rood	30.145	40.33	9.027	0.28
Zar- Joob	10.375	40.33	78.080	4.87
Gohar-rood	29.895	38.45	6.294	0.6
Zar- Joob	33.6	38.45	2.023	0.25
Gohar-rood	34.54	38.4	1.281	0.1
Zar- Joob	33.095	38.45	2.466	0.31
Zar- Joob	18.04	38.45	35.824	3
Zar- Joob	30.77	38.45	5.072	1
Zar- Joob	30.37	38.45	5.615	1
Eynak lagon	19.97	38.89	30.250	10
Gohar-Rood	13.53	38.89	54.348	27
Zar- Joob	18.585	38.89	34.841	8.5
Eynak lagon	17.985	38.89	36.930	18
Zar- Joob	37.765	38.89	0.107	0.007
Zar- Joob	34.8	38.89	1.414	0.11
Gohar-Rood	33.035	38.89	2.897	0.16

*Concentration

Discussion

The oocysts, infectious form of protozoa, are very resistant to stresses such as desiccation and disinfectant materials (chlorine) they can remain infectious for a long time in the environment (16). The human ID50 of 30 oocysts reported for *Cryptosporidium parvum* (17). There have been several studies about Cryptosporidium in different parts of Iran. The prevalence in children has been reported to be 2.5 to 10.4% (18, 19) and in HIV patients in Iran was reported to be 1.5% to 9.4% (20-22). The prevalence of *Cryptosporidium* in cattle from different regions of Iran were 1.61% to 18.8% (23-25).

There are many studies that only qualified *Cryptosporidium* in Iranian surface water by PCR-RFLP method (26, 27). In our previously study *Cryptosporidium* was detected by IFA nested PCR and LAMP methods in water samples (28).

It seems that effective levels to outbreaks with *Cryptosporidium* ordinarily occur

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when oocyst concentrations raised to 10 to 30 oocyst in 100 liters of water sampled (6). So, detection and quantification of low numbers of organism is difficult and needs high sensitive techniques.

It should be noted that this is the first study performed in Iran for the detection and enumeration of *Cryptosporidium* in water supply by Real Time method. In the present study, 19 out of 30 samples were positive by Real Time PCR. This high prevalence was similar to other countries which are from 60 to 96% in the United States (29, 30) and from 20 to 64% in Canada (31).

In addition to the identification of oocysts in water, the concentration of oocysts in water samples is helpful to design a program for health management and protection of water resources. In this study, the concentration of oocysts in surface waters was determined to be 0.007 to 27 per liter (Table 1). The lowest concentration belongs to Zarjoob and the highest to Gohar Rood River, Rasht, Guilan, Iran.

Zarjoob River was more polluted than Gohar Rood River (Table1). It may due to more entrance of urban and industrial sewage of Rasht city to Zarjoob River.

Low numbers of oocysts have been reported in some other studies in other countries(29,32,33) There levels in surface waters are very low, ranging from 0.5 to 5,000 organisms in 100 liters of water (31, 34-37).

Although it may be a relatively low number of oocysts in water samples, but relatively infectious dose is low too and even as low as 1 oocysts can cause infection (17). So there is a potential risk of waterborne cryptosporidiosis due to direct or indirect consumption of these rivers specially Zarjoob river by humans and animals.

Conclusion

Although our detection and quantification of low numbers of organisms is difficult but in the present study, *Cryptosporidium* oocysts successfully enumerated by Real Time PCR. So we can use this technique for the quantification of *Cryptosporidium* in water samples especially in samples with low number of oocysts that requires very sensitive techniques.

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