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

Mohammad-Reza Mahmoudi¹, Mojgan Bandepour²,³, Bahram Kazemi²,³, Asad Mirzaei⁴*

1. Department of Parasitology, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
2. Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Department of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Parasitology, Faculty of Allied Medical Sciences, Ilam University of Medical Sciences, Ilam, Iran

*Corresponding author: Tel: +98 8432227122 Fax: +98 8432227122
Address: Department of Parasitology, Faculty of Allied Medical Sciences, Ilam University of Medical Sciences, Ilam, Iran
E-mail: amirzaeii@yahoo.com
Received: 2016/12/14 revised; 2017/01/13 accepted; 2017/01/28

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

*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
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 (Table 1). 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.

Acknowledgment

This paper was extracted from Mohammad-Reza Mahmoudi’s PhD thesis and supported by Vice Chancellor in Research Affair of Shahid Beheshti University of Medical Sciences (through grant number: 89-01-91-7138).

References


