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ORIGINAL ARTICLE

Comparison and evaluation of four methods for extracting DNA from *Giardia duodenalis* cysts for PCR targeting the *tpi* gene

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Abstract *Giardia duodenalis* is an intestinal flagellated protozoan and the common cause of gastrointestinal diseases in human. This parasite can be seen in two different forms in its life cycle including as cyst and trophozoite. Due to presence of resistant cyst wall, DNA extraction inhibitors along with artifact in stool specimens, this study was performed aiming to evaluate four methods for DNA extraction from *G. duodenalis* cysts. Seventy *G. duodenalis* positive stool specimens that were confirmed by light microscope were included in this study. All stool samples were concentrated using four layered discontinuous sucrose flotation technique (0.5, 0.75, 1, and 1.5 M) and single-layered sucrose solution (0.85 M). The isolated cysts were then subjected to DNA extraction by four methods. To remove the artifacts, the extracted DNA were evaluated by PCR. The results of the present study showed the high level of optical density (OD) in the method I ($P < 0.01$) with the following steps; *Giardia* cysts plus crushed cover glass were vortexed. Then, the samples were boiled and then followed by freeze–thaw cycles, yet this method yielded the lowest concentration. Furthermore, the highest concentration were observed in the method II ($P < 0.01$) with the following steps; *Giardia* cysts plus crushed cover glass and TAE buffer were mixed and then shaken, followed by boiling. Based on the results of the present study, using crushed cover glass, boiling and freeze–thaw cycles can be effective in destruction of *G. duodenalis* cyst wall and have enough efficiency for extracting DNA from *G. duodenalis* cysts.

Keywords *Giardia duodenalis* · DNA extraction · tpi gene · Cyst

Introduction

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) is an intestinal flagellated protozoan and a common cause of gastrointestinal disease in a wide range of vertebrates, including humans (Feng and Xiao 2011; Inpankaew et al. 2014). The parasite can be seen in two different forms in its life cycle including cyst and trophozoite. The cysts are resistant to the adverse environmental factors and trophozoites, the vegetative form, are present in intestinal lumen (Babaei et al. 2011). About 200 million people with symptomatic giardiasis live in Asia, Africa and Latin America. Additionally, almost 280 million new cases of the disease were reported without any clinical sign of giardiasis (Lane and Lloyd 2002; WHO 1996). High prevalence of giardiasis can be seen in tropical and developing countries such as Isfahan, Iran (Pestehchian et al. 2012), where infections are associated with poor personal hygiene, sanitary conditions, poor water quality control and overcrowding. Clinical manifestations of giardiasis vary from asymptomatic infection (60–70 % of the cases) to acute or chronic diarrhea with malabsorption (WHO 1996). Several parasitological techniques such as microscopic stool examination with direct wet mount, concentration and

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staining methods are used routinely for detection of *Giardia* trophozoites and cysts, also immunofluorescence assay and enzyme immunoassay for detection of soluble antigens in fecal samples have been applied (Guy et al. 2004; Rayani et al. 2014), yet these methods have not enough sensitivity for detection of infections with low cyst excretion and for epidemiological investigations (Guy et al. 2004; Johnston et al. 2003; Nantavisai et al. 2007).

In the recent decades, especially since the introduction of DNA amplification techniques, genetic characterization has been extensively used for evaluation of genetic variability of *G. duodenalis* in order to develop methods for tracing source of infection and clinical diagnosis (Caccio et al. 2008). Identification of *G. duodenalis* genotypes in clinical specimens as well as differentiation of *Giardia* at the assemblage and genotype levels have relied on the analysis of the SSU-RNA, β-giardin *(bg)*, glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef-1*), triose phosphate isomerase (*tpi*) genes. Molecular approaches to further discrimination of *G. duodenalis* isolates are reliant on the efficient extraction of DNA from *Giardia* cysts (Abbaszadegan et al. 2007; Machiels et al. 2000).

Recently, a numerous direct DNA extraction methods have been introduced to extract DNA from *G. duodenalis* cyst, but due to the presence of resistant wall surrounding the cysts, and also presence of many microorganism and artifact in the stool specimens such as lipids, hemoglobin and mucosa, these methods are not very much efficient for extraction and detection of DNA from *Giardia* cysts (Amar et al. 2004; Nantavisai et al. 2007).

Therefore, the present study aimed to confirm previously methods and evaluate efficiency of four routinely used methods used for extraction of DNA from *Giardia duodenalis* cysts for PCR amplification of *tpi* gene.

Materials and methods

Fecal specimens

Seventy *G. duodenalis* fecal samples were obtained from infected humans referred to Khorramabad, West of Iran, from November 2013 to September 2014. For further examination, the samples were transferred to the Intestinal Parasites Researcher Laboratory, Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences.

Parasitological examination

Microscopic confirmation for the presence of *G. duodenalis* was performed by direct wet mount examination. Direct microscopy was done on all formed and liquid stool samples. All samples were confirmed positive for *Giardia* cysts (8–10 cysts in a 400× magnification field). After the microscopic analysis, all samples were kept at 4 °C without any preservative. At the final step, cysts of 70 *Giardia*-positive specimens were concentrated using four layered discontinuous sucrose flotation technique (0.5, 0.75, 1, and 1.5 M) and single-layered sucrose solution (0.85 M). Among samples, the fresh specimens with a high number of *G. duodenalis* cysts were kept frozen at −20 °C until DNA extraction (Pestechian et al. 2014).

DNA extraction

In the first step, in order to elevate the DNA concentration and removal the extraction inhibitors, all the concentrated samples were treated by the four methods (Table 1). Then, genomic DNA of all treated samples were extracted using genomic DNA extraction kit (GennAll, Seoul, Korea) according to the manufacturer's instruction. Finally, the OD and concentration of extracted DNA were measured at the wavelengths of 260 and 280 nm by Nano Drop device (WPA, England). Conventional methods were used for the calculation of mean and confidence interval.

Data of ODs and concentrations were analyzed by SPSS (version 16.2, SPSS Inc., Chicago, IL, USA) through Anova/Tukey test. P value < 0.01 was considered as significant.

Method I

Concentrated samples (200 µL) were mixed with 200 mg crushed cover glass (0.4–0.5 mm) and vortexed for 1 min. Then the samples were boiled at 100 °C (for 3 min) followed by freeze–thaw cycles using liquid nitrogen and a 100 °C heating block (for 6 steps-each step for 3 min).

Method II

Concentrated samples (200 µL) + 200 mg crushed cover glass (0.4–0.5 mm) and 200 µL TAE buffer (EDTA 0.001 M, Tris–Acetate 0.04 M) were mixed and were shaken in 2000 rpm, followed by boiling at 100 °C (for 3 min).

Method III

Concentrated samples (200 µL) were mixed with 50 µL 1/10 diluted 2ME (20 µL 2ME + 180 µL Distilled water) and incubated at room temperature for 10 min, followed by freeze–thaw cycles using liquid nitrogen and a 100 °C heating block (for 6 steps-each step for 3 min).

Table 1 Four treatment methods for DNA extraction from *Giardia* cysts

Method I	Samples (200 µL) + 200 mg crushed cover glass, vortex for 1 min, boiling (100 °C/3 min), freeze–thaw (for 6 steps)-kit
Method II	Samples (200 µL) + 200 mg crushed cover glass and 200 µL TAE buffer, shacked in 2000 rpm,, boiling (100 °C/3 min)-kit
Method III	Samples (200 µL) + 50 µL 1/10 diluted 2ME, incubation (room temp./10 min), freeze–thaw (for 6 steps)-kit
Method IV	Samples (200 µL) + 200 mg glass bead, vortex for 10 min, freeze–thaw (for 6 steps)-kit

MI Method I, *MII* Method II, *MIII* Method III, *MIV* Method IV

Method IV

Concentrated samples (200 µL) were mixed with 200 mg glass beads (0.4–0.5 mm) and vortexed for 10 min. Then, freeze–thaw cycles were performed using liquid nitrogen and a 100 °C heating block (for 6 steps-each step for 3 min).

Polymerase chain reaction (PCR)

A genus-specific PCR targeting the *tpi* gene was carried out using pair of following primers [forward(CTTCATCGG YGGTAACTT) and reveres (TTCTGYGCTGCTATY YTC)] as described by Ping Zhang et al. (2012). The PCR reaction performed in 25 µL of final volume containing 12 µL master mix, 5 µL distilled water, 2 µL each primer and 4 µL template DNA. The amplification condition were as follows: 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, 55 °C for 27 s and 72 °C for 20 s. Amplification was performed by Rotor-Gene 6000 device (Hilden, Germany). Analysis of PCR products was done by electrophoresis using 2 % agarose gel containing ethidium bromide.

Results

In this study, four treatment and extracting methods were done on 70 *Giardia*-positive stool specimens. The results for all methods for DNA extraction of 70 *Giardia*-positive cysts are illustrated in Table 2. As the table shows, the mean level of OD in method I is significantly higher than the other methods ($P < 0.01$), and also the mean level of concentration in method II is significantly higher than the other methods ($P < 0.01$). Furthermore the method I has lowest mean of concentration.

OD and concentration of each of 70 samples are available in Figs. 1 and 2, respectively. Products of amplification of gene fragment encoding *tpi* gene of *G. duodenalis* obtained from DNA extracted with all methods are shown in Fig. 3. In method I, DNA from cysts was well extracted and *tpi* gene amplified purely and in gel electrophoresis, PCR product produced a sharp band without any smear bands. However, in method II, DNA from *G. duodenalis* cyst and other microorganisms were extracted as well, but the resulted band was not sharp. In the other hand, in method III and IV, no DNA bands were observed in the gel electrophoresis.

Discussion

The current study aimed to evaluate and suggest an efficient method for extracting *G. duodenalis* DNA from cysts with the intension of improving tools for *Giardia* research and laboratory diagnosis in low resource settings in the *G. duodenalis* endemic countries. Several treatment methods were carried out and tested for the extraction of the parasite's DNA in this study. Different methods such as light

Table 2 Attributes of the four *Giardia* cysts DNA extraction methods

Method	MI		MII		MII		MIV	
	OD	Con.	OD	Con.	OD	Con.	OD	Con.
Mean	1.9*	0.4	1.4	49.4*	1.2	1.5	1.0	6.3
Minimum	1.6	0.2	1.2	20	0.8	0.5	0.8	1.9
Maximum	3	0.9	2	83	1.6	5	1.7	15
CI 95 %								
Lower	1.9	1.4	1.4	45.8	1.2	1.3	1	5.6
Upper	2.0	1.5	1.5	53	1.3	1.7	1.1	7.1
N	70		70		70		70	

OD Optical density, *Con.* Concentration (µg/mL), *CI* 95 % Confidence interval, *MI* method I, *MII* method II, *MIII* method III, *MIV* method IV, *N* number

* P (< 0.05) according to Anova/Tukey test

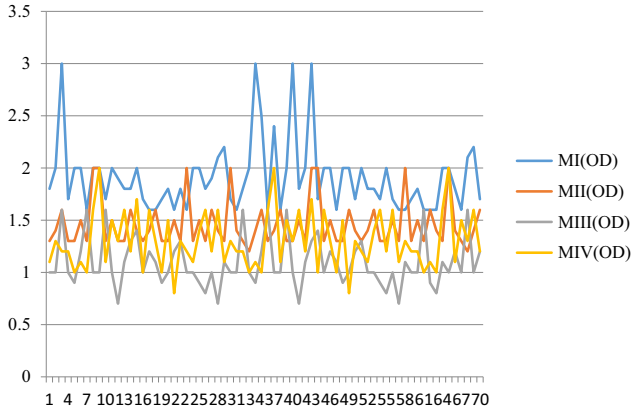

Fig. 1 Optical density of the each studied samples. Vertical axis: OD. Horizontal axis: Number of samples

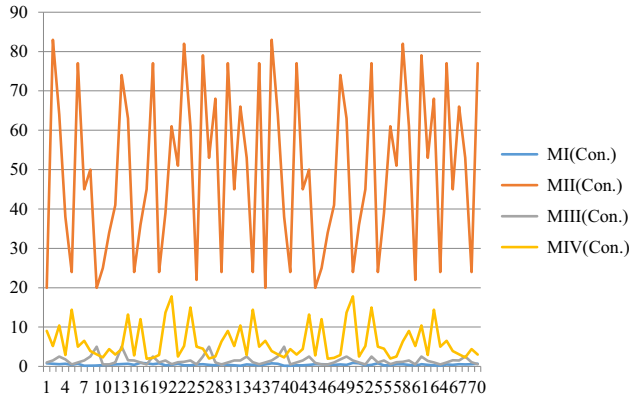

Fig. 2 Concentration of the each studied samples. *Vertical axis* concentration (μg/mL). *Horizontal axis* number of samples

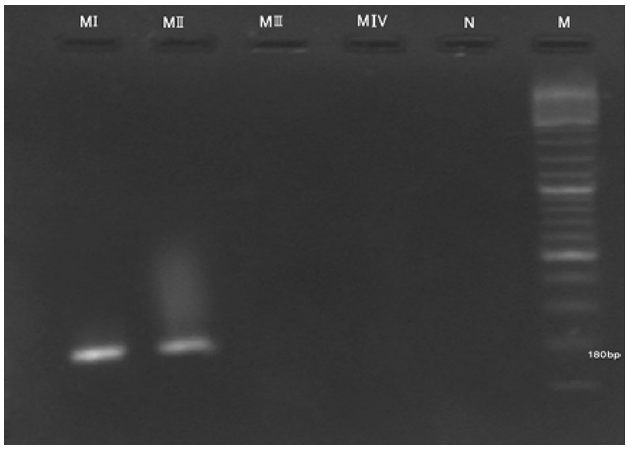

Fig. 3 Products of amplification of gene fragment encoding *tpi* gene of *G. duodenalis* on the gel electrophoresis. *M* Marker of molecular mass (100 bp). *N* Negative control. I: Method I, II: Method II, III: Method III, IV: Method IV. Products of amplification of gene fragment encoding *tpi* gene of *G. duodenalis* obtained from extracted DNA with all methods that were done by gel electrophoresis using 2 % agarose gel containing ethidium bromide

microscopy or immunofluorescence assay have been used to diagnose *G. duodenalis*. However, these techniques might not be highly sensitive and specific to detect cysts in stool samples. Recently, a few PCR-based techniques such as PCR–RFLP, quantitative PCR and HRM, have been developed for detection and genotype characterization of *G. duodenalis* (Sam brooks et al. 1989; Zhang et al. 2012). Some of these approaches were ineffective due to difficulty in releasing DNA from cysts, and also due to the presence of many compounds in stool samples such as proteases, DNase, polysaccharides and bile salts, which degrade DNA and inhibit enzymatic reactions (Abbaszadegan et al. 2007). The *tpi* gene locus for development of specific PCR assay was chosen in this study. *Giardia* cysts are oval in shape and their size range from 6 to 10 μm. The cyst's wall is different in thickness from 0.3 to 0.5 μm that is surrounded by an outer filamentous layer and also in the inner membranous layer they have two membranes that enclose the periplasmic space. The biochemical composition of the cyst wall is containing carbohydrate as N-acetyl galactosamine polymers (Gerwig et al. 2002). The results of the present study have shown the successful extraction of DNA from *G. duodenalis* cysts. To decrease the contamination of DNA extracted from *G. duodenalis* cysts by other microorganism, all samples were concentrated using four layered discontinuous sucrose flotation technique (0.5, 0.75, 1, and 1.5 M) and single-layered sucrose solution (0.85 M), Finally, 70 positive samples with *Giardia* cysts by flotation method 4 and single sucrose layer were all well condensed and effectiveness of DNA extraction with the use of each treatment was measured by PCR product of *tpi* gene of *G. duodenalis*. Comparison of efficiency of various treatment methods for DNA extraction methods have shown that the best results were obtained with method I. As the results show, using the mix of crushed cover glass, boiling (100 °C/3 min) and freeze–thaw cycles and glass beads (for 6 steps) have successfully extracted the DNA from *G. duodenalis* without any contamination with the other stool microorganisms. The result of the method I demonstrated that due to lowest volume and sharp structure of crushed cover glass compared to intact glass beads, they have effective role in the destruction of cyst wall. However, in the method II, even though the glass beads and freeze–thaw cycles were used, due to the usage of TAE buffer, the resulted DNA was contaminated by the other microorganisms, which produced a smear band in electrophoresis. In order to remove all peptide and disulfide bands that present in the cyst wall, we used diluted 2ME in the method III that this method is not succeeded for well extraction; however the incubation and freeze–thaw cycles also were used. Additionally, method III and method IV, without boiling, had not enough efficiency to extract the DNA from *G. duodenalis* cysts. In this study, all results of

OD and concentration of DNA in the studied methods were appropriate, but presence of artifact in treated specimens, inhibited the DNA amplification in method III and IV. Therefore, treatment and extraction methods should be able to remove the inhibitions of the DNA amplification. Our result demonstrate that using the crushed cover glass beads, freeze–thaw cycles and also boiling at 100 °C for 3 min can decrease inhibitors in the DNA amplification. Pestechian et al. (2014), reported the successful identification of genotypes of *G. duodenalis* in Isfahan, Iran by using the glass beads and freeze–thaw cycles with the QIA gene kit (QIA gene, Germany). Furthermore, Babaei et al. (2011) and Pestehchian et al. (2012), used the glass beads, freeze–thaw cycles and QIA gene kit (QIA gene, Germany) to remove the PCR inhibitor. In developing countries, usually for DNA extraction, Phenol–Chloroform Isoamyl Alchol (PCI) is used that this methods is not suitable for DNA extraction from blood smear or small helminthes, and also PCI is toxic as well (Blagg et al. 1955; Planelles et al. 1996). According to the results of the present and previous report, more studies on the standardization of the one effective way to break down the cyst wall of the parasite for DNA extraction and the removal of inhibitors seems necessary.

Conclusion

Based on the results of the present study, it can be suggested that using the crushed cover glass, freeze–thaw cycles and boiling can be effective in destruction of *G. duodenalis* cyst wall and this method can be used for standardization and improvement of DNA extraction and PCR product.

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