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Sensitive Detection of *Giardia* Cysts by Polymerase Chain Reaction (PCR)

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Abstract

Giardia is one of the most common human parasites and causes a lengthly course of nonbacterial diarrhea. Disease outbreaks due to *Giardia* infection are often attributed to contaminated water supplies. A major problem associated with detection for this organism is the lack of sensitive and reliable methods. PCR has the potential to address many of the limitations.We have performed a PCR-based method for sensitive detection of *Giardia* cysts. Because the sensitivity of PCR is a function of the efficiency of DNA extraction from cysts, we have also compared some different methods for DNA extraction from the cysts. *Giardia* cysts were collected from infected human, partially purified and serially diluted samples were prepared. DNA was extracted by 3 different methods and we found that simple repeated freezing and thawing was the best method for extraction of DNA from the cysts. A 163 bp conserved fragment related to the giardial heat shock protein (HSP70) gene was used as the target for PCR amplification. We were able to detect as few as 5 cysts in the samples. The results suggest the potential utilities of PCR for sensitive detection of *Giardia* in water sources.

KeyWords: *Giardia* cyst, HSP70, Iran.

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Introduction

Giardia is an enteric protozoan parasite that can cause a severe gastroenteritis and a lengthly diarrhea in infected humans. *Giardia* has been associated with numerous outbreaks of water-born diseases (1, 2, 3, 4, 5). Currently, giardiasis represents a major public health concern of water utilities in developing and even developed countries (6). *Giardia* cysts are resistant to conventional chlorination, can persist and remain infective for extended periods in the water column or sediments, are produced in large numbers in fecal matters, difficult to detect in water, and cross-infect different animal species. Further, traditional indicators of microbial quality of water (e.g. total and fecal coli forms) do not correlate well with the presence of protozoan parasites. For these reasons detecting and monitoring of *Giardia* in water is very important in public health. A major problem with evaluating the health significance of the waterborne route of transmission of protozoan parasites is the detection of *Giardia* in water. These organisms are difficult to culture from environmental samples and current methods of detection rely on direct examination of water concentrates. Detection methods need to be sensitive due to the low infective doses of these organisms in humans. Since an infection in humans can be initiated by as few as 1 to 10 viable cysts (7, 8, 9), the detection method must be very sensitive. In fact, one of the major problems related to *Giardia* is its detection in water supplies. Current methods for the detection of *Giardia* cysts in water rely primarily on microscopic observation of water concentrates, using phase-contrast microscopy or Immunofluresent Assay (IFA) (10, 11). These methods are laborious, time-consuming and costly (12). Other limitations include non specific antibody binding, interfering by sample debris and lack of sensitivity (12, 13). Therefore, a more rapid and sensitive method for the detection of *Giardia* cysts in water samples is needed. A recently developed technique that offers the greatest potential for the detection of a wide range of microorganisms, including *Giardia,* in water, is Polymerase Chain Reactions (PCR). PCR has the potential to address many of the limitation of the current methods. PCR methods for the detection of *Giardia* in clinical and environmental water samples have been described (5, 12, 14, 15) and several target gene, including small subunit rRNA gene (16), giardian gene (15, 17), and heat-shock protein (HSP) gene (12) have been used for this purpose. In the present study, we have performed a PCR-based method, using a primer pair complementary to HSP70 gene, as the target gene, to establish a rapid, sensitive and specific detection of *Giardia lamblia* in partially purified samples as a tool for subsequent detection of this parasite in water samples. Advantages of the primers have been showed previously (9). This is

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among the first molecular biological experiments in Iran for detection of parasites, and hopefully will progress in future for monitoring environmental samples.

Materials and Methods

***Giardia* cysts.** Several fecal samples were collected from patients suspected to giardiasis. The samples were tested by normal light microscopy. *Giardia* cysts were isolated and partially purified by sucrose flotation (18). The semi purified and concentrated cysts were stored in strrile distilled water without adding any preservatives, up to two weeks in 4^oC.

Preparation of serially diluted cyst samples. Cyst densities were determined microscopically by hemocytometer, and lower densities (10^4, 10^3, 10^2, 10^1, 5, 12, 0) of cysts were obtained by serial dilution of stock samples in water.

DNA extraction. DNA was extracted from cysts by 3 separate methods for each sample:

A) Repeated freezing and thawing. This method was performed by 6 times freezing and thawing in liquid nitrogen for 60 seconds and in a 65^oC water bath for 60 seconds, respectively.

B) Freezing thawing and DNA extraction was performed based on the above-mentioned method and then followed by phenol-chloroform extraction of DNA. DNA presented in supernatant was precipitated by 0.1 volume 3M sodium acetate (pH 5.2), and 2.5 volumes 95% ethanol. The precipitant was washed with 70% ethanol and the purified DNA was resuspended in 30 µl of distilled water.

C) Chemical extraction of DNA: 150 µl of STE solution (100 mM NaCl, 1 M Tris-HCl, 0.5 M EDTA) , 15 µl of 10% SDS solution and 5 µl of proteinase K solution (20 mg/ml) were added to each 20 µl cyst sample. The suspension was mixed and incubated at 55^oC for 30 minutes. It was then added to 200 µl phenol-chloroform mixture and phenol-chloroform extraction was performed.

DNA amplification by PCR. The PCR was performed with 20 µl of template DNA, in a total reaction volume of 50 µl, consisting of 1xPCR amplification buffer, 0.2 mM each dATP, dGTP, dCTP and dTTP, 0.5µM each forward (5'-GTATCTGTGACCCGTCCGAG-3') and reverse (5'-AGGGCTCCGGCATAACTTTCC-3') primers and 2 unit of *Taq* DNA polymerase. Thirty five cycles of amplification were performed in a thermal cycler (model Eppendorf-Germany). After initial denaturation of DNA at 94^oC for 3 min, each cycle consisted of a denaturation step at 94^oC for 45s, an annealing step at 55^oC for 30s, an extension step at 72^oC for 45s and a final extension step at 72^oC for 10 min, following the last cycle. Appropriate positive (a given sample containing *Giardia* DNA) and negative controls (distilled water) were included in each test running.

Restriction enzyme analysis. We had to be sure that the PCR product belongs to giardial HSP70 gene. To meet this purpose, HSP70 sequences of *Giardia lamblia* were derived from GenBank database and on the basis of that sequence, the restriction sites of various restriction enzymes were analyzed by DNasis software (Hitachi software 1998 JAPAN) and the best enzyme was selected as *Hae*III. For restriction DNA digestion 21.5 µl of PCR products were treated directly by 10U (1 µl) of the restriction enzyme and 2.5 µl related buffer in a total volume of 25 µl followed by 60 minutes incubation at 37^oC.

Detection of PCR products. PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml), and visualized under a UV transilluminator. The restriction enzyme cleavage products were analyzed by electrophoresis on an acryl amide gel and stained with silver nitrate.

Results

PCR amplification of Giardia DNA. DNA extracted from *Giardia* cysts was amplified by the specific primers for HSP70 gene. After agorose gel electrophoresis, a 163 bp PCR product was seen (Fig. 1). To verify the band, the PCR products were digested with the restriction enzyme *Hae*III. After electrophoresis 2 bands with expected sizes of 111 bp and 52 bp (according to *Giardia lamblia* HSP70 gene sequence derived from GenBank, accession no. X16738) were observed (Fig. 2), confirming that the PCR products belonged to *Giardia.*

Sensitivity of the detection of Giardia cysts by PCR. To establish a PCR based sensitive method for detection of *Giardia*, special effort was put into optimization of the DNA extraction techniques. As a guideline for the DNA extraction, three methods were used. Table 1 shows the sensitivity of the PCR amplification of DNA after making 10–fold dilution series of *Giardia* cysts in water and DNA extraction with all the 3 methods. The best result was achieved by freezing and thawing, without any other treatment. We were able to detect as few as 5 *Giardia* cysts by this method (Fig. 1). Other DNA extraction methods were associated with decrease in sensitivity (Table 1). We were not able to get any PCR amplification with samples containing less than 1000 cysts, when DNA was extracted with or without freezing and thawing.

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Table 1: Comparison of different methods for DNA extraction of *Giardia* cysts based on the sensitivity of PCR assay

DNA extraction method	Sensitivity at a cyst concentration of:					
	10^4	10^3	10^2	10^1	5	1
Freezing & thawing	+	+	+	+	+	-
Phenol-chloroform extraction*	+	+	-	-	-	-
Chemical extraction	+	-	-	-	-	-

* Sensitivity can be increased up to five cysts by double PCR

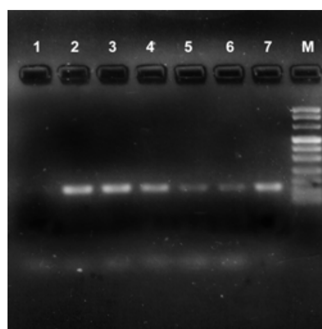

Fig .1: Agarose gel electrophoresis analysis of PCR amplified DNA of Giardia cysts after DNA extraction by freezing-thawing. Lane 1: negative control, Lane 2: 104 cysts, Lane 3: 103 cysts, Lane 4: 102 cysts, Lane 5: 10 cysts, Lane 6: 5 cysts, Lane 7: positive control, M: molecular weight marker VIII.

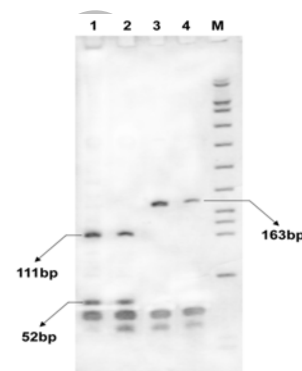

Fig. 2: Acryl amide gel electrophoresis of PCR products amplified by Giardial Hsp70 primers. Lane1s and 2: PCR product after digestion with the restriction enzyme HaeIII, Lane 3 and 4 before digestion, M: molecular weight marker VIII.

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Discussion

We applied a PCR-based method for detection of the pathogenic protozoan *Giardia*. Our method was able to detect 5 cysts or more, in partially purified samples. Several previous studies have described the development of specific PCR techniques for the detection of *Giardia* cysts and assessed their sensitivities. Amplification of a target gene by (PCR) could detect one *Giardia* cyst (15, 17), that was isolated from a pure culture with a micromanipulator. In another study (12) gene sequences coding for heat shock protein was Targeted (HSP) to specifically detect *Giardia* with a sensitivity of one cyst. Mayer (17) evaluated different methods such as PCR, nested PCR and fluorescent antibodies for detection of *Giardia* and *Cryptosporidium* in wastewater. They concluded that PCR may be a useful tool in the environmental analysis of water samples for protozoan contamination. Rochelle (19) evaluated eight primer pairs for the specific detection of *C. parvum* and *G. lamblia* in water. The detection sensitivity in their work was 1 to 10 cysts for purified preparations and 5 to 50 oocysts or cysts for seeded environmental water samples. In the present study we used *Giardia* gene for HSP70 as the target gene for amplification. This region is conserved among all *Giardia* species and thus, its amplification can be used as a diagnostic test for *Giardia lamblia*. By using primers, specific for this region, an amplicon with a size of 163 bp was observed. We also checked the amplicon by endonuclease digestion of the PCR products with *Hae*III restriction enzyme. To determine the sensitivity of the reaction, PCR was performed on nucleic acids extracted from partially purified *Giardia* cysts that were serially diluted in water. As it is shown in figure 1, our PCR set was able to amplify as few as 5 cysts of *Giardia lamblia*. This sensitivity is suitable and useful for monitoring water supplies with regard to the level of *Giardia* contamination. Although humic type materials are common problem encountered with PCR

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amplification of DNA extracted from environmental water samples, we could overcome this problem by procedures such as use of magnetic beads. We also evaluated different methods for extraction of DNA from *Giardia* cysts. In our experiment the simple repeated freezing and thawing of the cyst was most efficient as evidenced by the best PCR-amplification. Not only were the other methods, which employed chemical treatment, were time consuming, labor intensive and expensive, but, also showed poor DNA recovery. In conclusion, according to the results, it seems that PCR could be the method of choice with several advantages compared with other methods for detection of *Giardia* contamination in water reservoirs. The advantages include sensitivity, specificity, cost effectiveness and speed. We are planning to use this approach for detection of *Giardia* and other important protozoa such as *Cryptosporidium*, in water samples in Iran.

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