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A Nested-PCR Assay for Detection of *Cryptosporidium parvum* Oocysts in Water Samples

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Abstract

Cryptosporidiosis is a gastroenteric disease caused by the protozoan parasite *Cryptosporidium parvum.* Water–borne transmission of this organism has become more prevalent in recent years. Current method for detection of *C. parvum* oocysts in water is immunofluoresence assay (IFA). The method is time consuming, laborious and particularly not–specific. It cannot determine the infectivity of detected oocysts. We have evaluated a nested- PCR assay for sensitive detection of *C. parvum* oocysts in water samples. Water sample concentrates were spiked with *Cryptosporidium* oocysts and after DNA extraction and purification by QIAamp DNA mini kit, detection was achieved by nested PCR amplification of a 200 bp region of hsp70 gene specific for *C. Parvum.* The method could detect as few as one oocyst in seeded tap water samples. On the basis of these results, PCR could be a useful tool in the monitoring of water samples for the detection of *Cryptosporidium* oocysts.

Keywords: Cryptosporidium parvum*, Nested-PCR, Iran*

Introduction

Cryptosporidium parvum, an intestinal protozoan parasite is an important cause of waterborne gastrointestinal disease worldwide (1). Since low numbers of *C. parvum* oocysts are often found in the environment (2, 3) and the number of oocysts required to cause infection is relatively low (4), a rapid and sensitive pathogen detection method is essential for the water quality industry. Current method which is widely used for detection of *Cryptosporidium* in water samples is immunofluoresence assay (IFA). The method is time-consuming, labour intensive, and is subject to false positive and negative results. Furthermore the method could not distinguish between species of *Cryptosporidium* oocysts and this is important because not all species of *Cryptosporidium* are infectious (5). PCR-based method has the potential to address many limitations of the current method. The advantages of PCR include specificity, greater sensitivity and more rapidity (6, 7). The aim of this study was to evaluate a nested-PCR assay for rapid and sensitive detec-

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tion of *Cryptosporidium* oocysts in water samples. To determine the sensitivity, nested–PCR was first performed on nucleic acids extracted from *Cryptosporidium* oocysts serially diluted in distilled water and then it was performed on concentrate of tap water samples spiked with serially diluted *Cryptosporidium* oocysts.

Materials and Methods

C.parvum *oocysts* *C. parvum* oocyts were obtained in purified and enumerated form from National Institute of Infectious Disease (NIID, Tokyo-Japan). Lower densities were obtained by serial dilution in distilled water.

Water samples 50 liters of tap water samples was filtered through a 142 mm diameter membrane filter with a pore size of 1.2 µm. The solids captured on the filter were removed by eluting in phosphate buffer saline (PBS) contain %0/02 SDS and Tween 80. The eluate was collected in 50 ml conical centrifuge tube and then centrifuged at 3000 g for 10 min. Packed pellets were spiked with serially diluted *C. parvum* oocysts in triplicate for each dilution.

DNA extraction Purified samples: DNA was extracted from purified oocysts by eight cycles of freezing in liquid nitrogen for 1 min, followed by thawing at 98ºC for 1 min.

Spiked water samples: DNA was extracted and purified from spiked concentrate of water samples by QIAamp DNA mini kit (QIAGEN K.K., Japan) according to the manufacturer's protocol, with a little exception. The exception includes eight cycles of freezing and thawing after incubation of samples at 56ºC.

Nested–PCR amplification The nested PCR primers which were designed for this study, with the aid of computer software, amplify a region within the hsp70 gene (accession no. U11761). External primers CPHSP2F (5́-AAATGGTGAGCAAT CCTCTG) and CPHSP2R (5́–CTTGCTGCTCTTACCAGTAC) which amplify a 361 bp fragment of hsp70 encoding gene was described previously (8). Internal (nested) primers NesCPHF (5́- TGGTGGTGTTATGACCAAGC) and NesCPHR (5́-TGGTACACCTCTTGGTGCT G) which amplified a 199 bp product within the first amplicon were designed by using DNASIS software (Hitachi software, Japan). The external PCR mixture incorporated a 300nM concentration of each primer (CPHSP2F and CPHSP2R), 1x PCR buffer, 100 µM dNTP, 1.25 unit of Taq polymerase, 1 µl of bovine serum albumin (10%) and double distilled water. Twenty micro liters of *C. parvum* template DNA was added to give a total volume of 50 µl. The nested PCR master mix was the same as the first PCR mastermix with the excerption of internal primers (NesCPHR and NesCPHF) and concentration of dNTP (20 µM). Bovine serum albumin also was not included in the reaction mixture. One micro liter of the first PCR product was added as the DNA template. The amplification reactions for external primers were initiated by denaturation at 94º C for 5 min, and then subjected to 35 cycles of denaturation at 94º C for 1 min, annealing at 55º C for 2 min, and extension at 72ºC for 1 min with an addition 10 min extension at 72ºC. Nested PCR parameters included an initial denaturation at 94ºC for 5min followed by 30 cycles of 94º C for 30s, 58º C for 45s, and 72º C for 30s. Final extension was carried out at 72ºC for 10 min. PCR was performed in a Takara PCR thermal cycler.

Negative control in which oocysts or DNA replaced with sterile distilled water was included in the spiking step and in the PCR amplification.

Detection of PCR products PCR products were analyzed by electrophoresis on a 1.6% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized with an Image Master VDS system (Pharmacia, Biotech).

Results

To evaluate the sensitivity of designed nested–PCR primer set described in this report, serial dilutions of purified *C. Parvum* oocysts were made, and nested-PCR was performed with de-

signed primers. Fig. 1 shows that using CPHSP primers and one step PCR, oocyst dilutions were detected at a level of approximately 10^2 oocysts. But with performing nested PCR by NesCPH primers it could be detected as few as 1 oocyst (Fig. 2). Comparison between fig 1 and 2 shows that performing nested-PCR could increase detection sensitivity from 10^2 cells to 1 cell.

Limit of detection of the oocysts in tap water

To asses the sensitivity of nested-PCR for detection of *C. Parvum* in water samples, dilutions of enumerated *C. Parvum* oocysts were seeded into packed pellets of tap water, and nested PCR was performed following extraction and purification of DNA by QIAamp DNA mini kit. The nested-PCR results from tap water sample concentrates seeded with oocysts are shown in Fig. 3. Even for the lowest inoclum level, the nested-PCR amplification product is clearly visible on agarose gel stained with ethidium bromide. The results also demonstrate that QIAamp DNA mini kit is an efficient procedure for removing of PCR inhibitors existing in water samples.

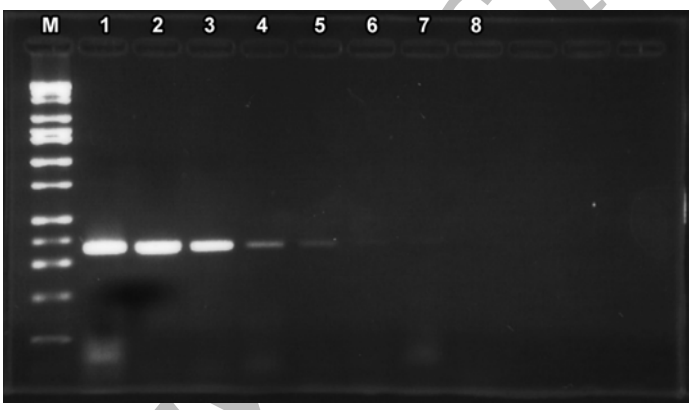

Fig. 1: Sensitivity of the PCR assay for detection of *C. parvum* oocysts as determined by 1.6% agarose gel electrophoresis. M: 100 bp ladder, Lane 1: positive control, Lane 2: 10^4 oocysts, Lane 3: 10^3 oocysts, Lane 4: 10^2 oocysts, Lane 5: 10 oocysts, Lane 6: 5 oocysts, Lane 7: 1 oocyst, Lane 8: negative control.

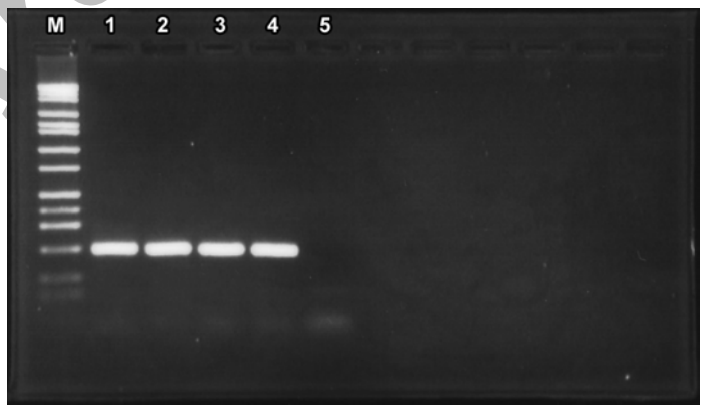

Fig. 2: Sensitivity of the nested- PCR assay for detection of *C. parvum* oocysts as determined by 1.6% agarose gel electrophoresis. M: 100 bp ladder, Lane 1: positive control, Lane 2: 10 oocysts, Lane 3: 5 oocysts, Lane 4: 1 oocyst, Lane 5: negative control.

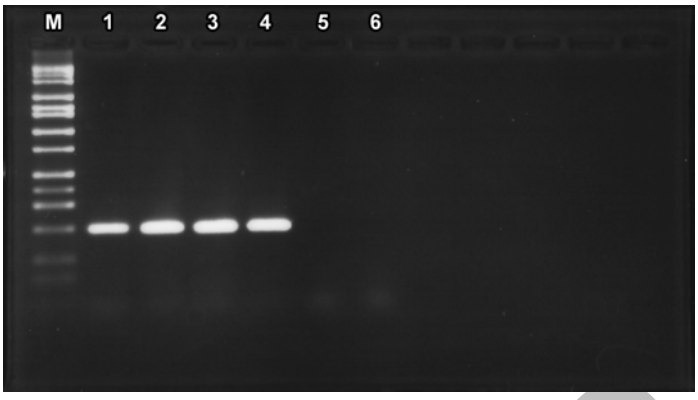

Fig. 3: Sensitivity of nested-PCR assay for detection of *C. parvum* oocysts spiked into packed pellet concentrates from 50 liters tap water samples after purification of DNA by QIAamp. M: 100 bp ladder, Lane 1: positive control, Lane 2: 10 oocysts, Lane 3: 5 oocysts, Lane 4: 1 oocyst, Lane 5: negative control without oocyst, Lane 6: negative control without DNA.

Discussion

The current method for detection of *Cryptosporidium* oocysts in water samples is immunofluoresence assay (IFA) which is time consuming; labour intensive, costly and the antibodies used cross-react with species other than *C. parvum* (5).

Therefore numerous attempts have been made to apply nucleic acid–based tests for sensitive and specific detection of *C. parvum* oocysts in water (8-11).

Amplification of a target gene by polymerase chain reaction (PCR) enabled detection of low numbers *Cryptosporidium* oocysts in purified samples (12, 13). The nested PCR procedure described in this paper further enhance the sensitivity of PCR, so that we could detect as few as 1 oocyst in purified samples. In contrast detection of *Cryptosporidium* oocysts in water samples is seldom possible at an equivalent sensitivity owing to presence of substances inhibitory to PCR that are difficult to remove by conventional DNA purification methods (14, 15). For example Johnson (12) could detect 1-10 oocysts in purified samples but the detection limit in water samples were 10-100 folds lower. In another study the PCR assay routinely detected 10 oocysts in 10 ml purified oocysts preparations, but sensitivity was found to be 10^3 -10^4 folds lower in environmental water samples (13). Therefore sensitivity of PCR for detection of microorganisms in water samples is a function of removing of inhibitors. Several procedures were used to counter the inhibition and enhance the PCR, including immunomagnetic separation (IMS) (9, 13, 16, 17, 18), spin column purification of extracted DNA (8) and hybridization to specific probes (19- 21).

In the study we used QIAamp DNA mini kit for purification of DNA. With the QIAamp system it was feasible to purify the DNA from PCR inhibitors that may be present in water samples. In our study nested-PCR assay could be readily applied to detection of *C. parvum* oocysts in spiked concentrate of tap water samples and we could achieve a detection limit as few as 1 oocyst. The results indicate that this PCR-based method is an attractive method with several advantages. The advantages include sensitivity, specificity, cost and speed (8, 22, 23). Moreover the data obtained established the basis for evaluating the method with environmental water samples. Environmental water samples may contain higher alge and organic and inorganic components, as well as various kinds of debris. Any one of these is a potential inhibitor of PCR. So the feasibility of using the method described here for detecting *C. parvum* in environmental water samples must be evaluated.

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