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Original Article

A Sensitive and Specific PCR Based Method for Identification of *Cryptosporidium* Sp. Using New Primers from 18S Ribosomal RNA

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Abstract

Background: The main goal of the present study was to develop a new sensitive and specific PCR based method for Identification of *Cryptosporidium* sp. using novel primers from 18S ribosomal RNA. Cryptosporidiosis in high-risk host groups particularly in neonates and immuno-compromised individuals may result in death. To the best of our knowledge this is the first study regarding develop a new PCR based method to diagnose the cryptosporidiosis in Iran.

Methods: A total of 850 human fecal samples from patients clinically suspected to cryptosporidiosis and 100 healthy and diarrheic cattle stool specimens were collected. The simplified formol-ether concentration method was carried out for all samples. They were then examined microscopically by modified Ziehl-Neelsen staining method. Total DNA was extracted by QIA amp DNA stool mini kit. PCR and nested-PCR was carried out by using designed primers.

Results: Twenty nine cases of cryptosporidiosis infection in human and 30 samples from cattle microscopically were positive. The described primary and nested PCR method could detect all *Cryptosporidium* positive samples from human and cattle. Regards to suspected negative samples in primary PCR examination, the Nested PCR could approve two more positive results. Furthermore, Nested PCR analysis was able to detect one more case which was negative in both microscopically examination and primary PCR. Specificity of the test was 100%. Sensitivity of Nested PCR in comparison to our gold standard; microscopy after Ridley concentration modified ziehl-Neelsen, was 100 %.

Conclusion: Our developed PCR based method by using new primers devised from 18S ribosomal RNA revealed the ability for identification of the *Cryptosporidium* species such as *C. parvum* and *C. huminis* with high specificity and sensitivity.

Keywords: *Cryptosporidium*, PCR, Identification

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Introduction

Cryptosporidium sp. are monoxenous protozoan parasites causing water-food borne gastrointestinal infections in both human and animal (1-3). They are common seen in childhood, pregnancy and immune-compromised people such as AIDS patients (4). Children are the most infected group in developing countries (5-7). *Cryptosporidium* oocysts were detected in 13% of parsitologic stool investigations in developing countries (5-7). Species which involve human are *C. muris*, *C. parvum*, *C. hominis*, *C. felis* and *C. canis*. Oocysts contaminating food or water and direct contact with infected animals or humans cause acute gastroenteritis and diarrhea in healthy people but in immunocompromised patients, individuals with AIDS and malnourished children, *Cryptosporidium* parasites cause a chronic and life-threatening disease (1, 8, 9).

Common methods for detection of *Cryptosporidium* are parasite visualization using acid-fast staining as well as fluorescent staining after concentration. Because they are obligate intracellular parasites, cultivating of the organism is not routine in the laboratory (10, 11).

To obtain high sensitivity in the diagnosis with microscope, a modified Ziehl- Neelsen staining and a minimum amount of 500,000 oocysts in each gram of examined stool required (11). Besides, identification of oocysts in direct microscopic detection is depended to the time as well as experience of stool examiner (12,13). In addition, the lack of morphological characters to discriminate *Cryptosporidium* species causes low sensitivity and specificity for detection of this parasite (3,4). Furthermore, Immunofluorescent-antibody assays (IFA) methods which used to detect *Cryptosporidium* oocysts in environmental samples are not useful for species identification (14, 15-17).

The PCR techniques have proved both specific and sensitive methods for detection of protozoan infections and *Cryptosporidium* specimen types (18, 19). In the present study, based on a primer designed from 18S ribosomal RNA, we tried to set up a sensitive Nested-PCR for detection of *Cryptosporidium* species from human, and cattle feces. This method may help us for detection of these parasites in a more rapid and sensitive way.

Materials and Methods

Fecal specimens

A total of 850 fecal samples were obtained from patients clinically suspected to cryptosporidiosis. One hundred stool specimens from diarrheic and/or healthy cattle were also collected from rural area in south of Iran. The samples were referred to Parasitology Laboratory, School of Public Health, Tehran University of Medical Sciences the simplified Formol-Ether concentration method was carried out for all samples (20, 21). They were then examined and evaluated microscopically by modified Ziehl-Neelsen staining method. Briefly, thin smears of fecal suspension were prepared on glass slides. The slides were flooded with carbol fuchsin for 1 hour following fixing by absolute alcohol (20). Then they were washed and decolorized in 3% acid-alcohol for about 30 seconds. The slides were then washed and stained with 1% methylene blue for 4 minutes. After washing and air drying, the slides were investigated microscopically by 40× as well as 100× objectives (20). Positive samples were separated for performing PCR analysis.

DNA extraction

Total High Molecular weight DNA was extracted by QIA amp DNA stool mini kit (Qiagen, Hilden, Germany). In according to manufacturer's instructions, we performed a pre treatment as follows; 180 to 200 mg portion of each stool sample was transferred into an Eppendorf tube and dissolved in 700 µL of ASL buffer of DNA extraction kit. The samples were then exposed to a five cycles of freeze and thaw within liquid nitrogen and boiling water in order to disruption of oocyst cells. Afterwards, 700 µL of ASL buffer was added into the sample tube and then the procedure was followed based on instruction of DNA extraction kit.

Primer designing and Nested PCR amplification

A part of 18S ribosomal RNA gene from *Cryptosporidium* genus which described previously (NCBI, Accession Nr. GQ259149.1) was selected to design specific primers for detecting of all *Cryptosporidium* species. The mentioned gene fragment was amplified with the primer pairs including: Cry18S-S2, 5' GGTGACTCATAATAACTTTACGG 3' as forward and Cry18S-As2, 5' ACGCTATTGGAGCTGGAATTAC 3' as reverse primers. Nested PCR was performed by the following primers; Cry18S-S1, 5' TAAACGGTAGGGTATTGGCCT 3' as forward and Cry18S-As1, 5' CAGACTTGCCCTCCAATTGATA 3' as reverse. PCR was carried out by using a peqSTAR thermocycler (Peqlab, Germany) under the following conditions for both primary and Nested analysis: 94 °C for 5 min, 35 cycles of 1 min at 94 °C, 1.30 min at 60 °C, 2 min at 72 °C, followed by 35 cycles comprising 1 min at 94 °C, 1.30 min at 60 °C, 2 min at 72 °C and a final extension step of 10 min at 72 °C. 20 ρM of each primer was added in a volume of 50 µl containing: 20 mM $(NH4)_2$ SO_4, 75 mM Tris-HCl (pH. 8.8), 1 mM $MgCl_2$, 0.2 mM dNTP mix, 1.2 Units of thermo stable DNA polymerase (Roche), and 1 µl of template (genomic DNA). The amplification products were subjected to electrophoresis on a 1 % agarose gel.

Result

From 850 human samples and 100 cattle stool samples which have been investigated microscopically, 29 cases of cryptosporidiosis infection in human and 30 positive samples from cattle have been revealed. These positive samples were nominated for further PCR analysis. In addition, A total of 100 microscopically negative human samples which, based on clinical manifestations, were suspected to cryptosporidiosis were selected for further PCR analysis.

Isolation of DNA from positive feces samples mentioned above has been done by DNA stool mini kit (Qiagen, Hilden, Germany).

Synthesized Designed Primers from *Cryptosporidium* 18S ribosomal RNA utilized in a double-tube nested PCR. Both outer and inner primers optimized at a same protocol. Using the outer primers, a fragment with approximate size of 347 bp was amplified (Fig. 1), while by using inner primers a gene fragment with 240 bp has been produced (Fig. 2). The described primary and nested PCR method could detect all *Cryptosporidium* positive samples from human and cattle (Fig. 1&2).

Regarding to suspected negative samples in primary PCR examination, the Nested PCR could approve two more positive results (Fig. 1&2). Furthermore, Nested PCR analysis was able to detect one more case which was negative in both microscopically examination and primary PCR (Fig. 3).

The specificity of the designed primers was approved by performing the same PCR

analysis using isolated DNAs from *Entamoeba histolytica*, *Giardia duodenalis* and *Blastocystis hominis* and showed no banding pattern.

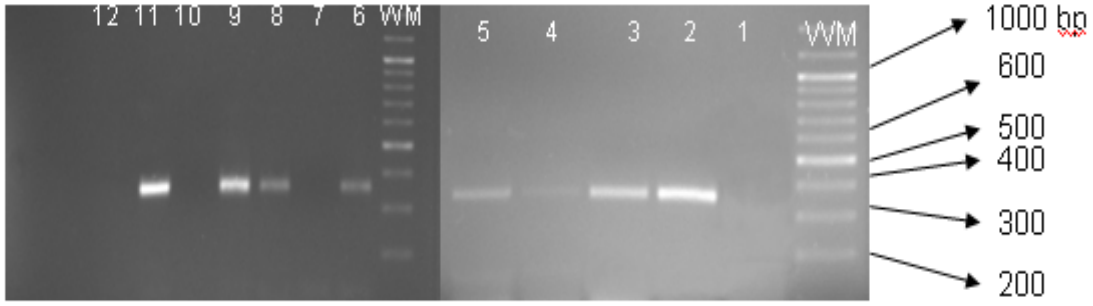

Fig. 1: PCR products of human samples (1-5) and cattle samples (6-9) on 1% agarose gel. WM: 100 bp molecular weight marker; lane 1: sample 229; lane 2: sample 145; lane 3: sample 142; lane 4: sample 233; lane 5: sample 234; lane 6: sample 192; lane 7: sample 183; lane 8: sample 163; lane 9: sample 194; lane 10: human negative fecal sample; lane 11: positive control; lane 12: negative control (Distilled water instead of DNA template)

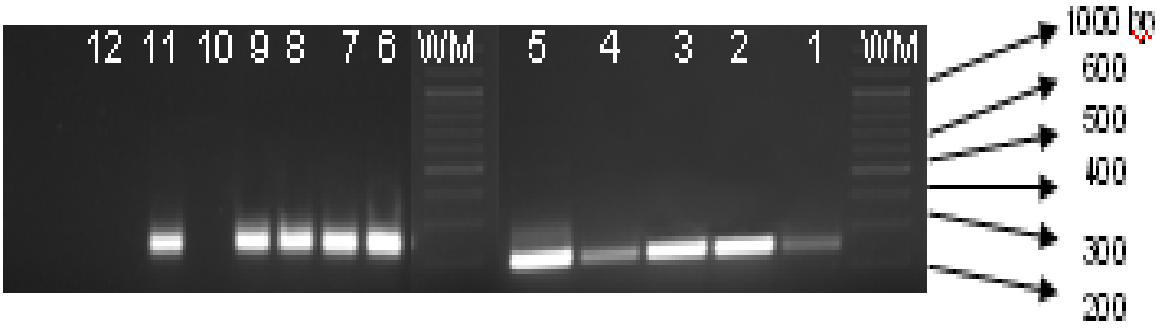

Fig. 2: nested-PCR products of human samples (1-5) and cattle samples (6-9) on 1% agarose gel. WM: 100 bp molecular weight marker; lane 1: sample 229; lane 2: sample 145; lane 3: sample 142; lane 4: sample 233; lane 5: sample 234; lane 6: sample 192; lane 7: sample 183; lane 8: sample 163; lane 9: sample 194; lane 10: human negative fecal sample; lane 11: positive control; lane 12: negative control (Distilled water instead of DNA template)

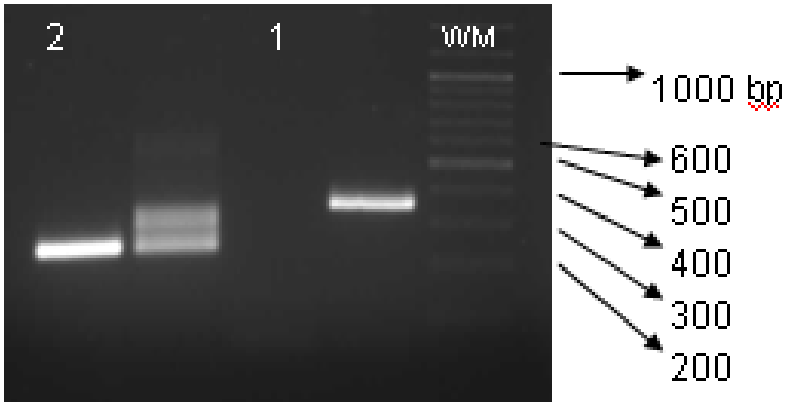

Fig. 3: PCR & nested-PCR products of a human sample that was negative in both microscopically and primary PCR analysis on 1% agarose gel. WM: 100 bp molecular weight marker; lane 1: sample k PCR pattern; lane 2: sample k nested-PCR pattern

Discussion

We described a sensitive and specific PCR based method for the rapid and direct detection of *Cryptosporidium* in stool specimens. Cryptosporidiosis, as mentioned before, is one of the most important emerging infectious diseases which are directly transmitted by the fecal–oral route and cause different kinds of diseases in mammals, birds, and fish (22). Although current laboratory methods for the detection of *Cryptosporidium* oocysts are primarily relied on examination by microscopy, however, morphological characters for identifying *Cryptosporidium* are few, and identification based on light microscopy alone is unreliable and relatively time consuming (23, 24). In addition, researches shown that methods based on immunological detections are not more sensitive than conventional microscopy (13, 25). In contrast, because of sensitivity and easily performance, PCR amplification seems to be an obvious choice for improved detection of *Cryptosporidium* from feces. To the best of our knowledge, PCR-based assays have not been previously developed locally in Iran for direct identification of *Cryptosporidium* genus. However, some investigations have been performed in order to differentiation the related species (26-28). In this study, we designed novel outer and inner primer pairs with a same annealing temperature in both analyses. Our investigation showed the ability of the first PCR with the outer 18S rRNA primers in detection of *Cryptosporidium* infected samples. In addition, in some cases with small amount of parasite, in which the direct microscopy as well as primary PCR were unable to detect the infection, the nested-PCR with inner 18S rRNA primers was able to recognize the infection. The study of Weber et al, revealed that the sensitivity of Immunofluorescence (IF) diagnostic methods for detecting *Cryptosporidium* oocysts in human stools was related to the amount of oocysts. The minimum of amounts of oocysts per gram were indicated as 10,000 in watery stool, 50,000 in formed stools (11). In addition, at least 500,000 oocysts per gram of formed stool were needed for a 100% detection rate

by modified cold Kinyoun acid-fast (AF) staining (11). Therefore, in cases of low amount of oocysts, we need more sensitive tests to identify infections. The method described in this study, was able to detect all *Cryptosporidium* infected samples by primary and nested PCR analysis. The specificity of our test was approved by the DNA extracted from other common intestinal parasites (*G. lamblia*, *E. histolytica* and *B. hominis*). In addition, using high temperature as annealing in PCR (60°C), nonspecific banding patterns was not observed in analysis. Sensitivity of the mentioned Nested PCR analysis in comparison with the standard microscopy was 100 %. Moreover, this method can be useful in epidemiological studies of cryptosporidiosis as well as environmental studies and water sources testing of this parasite.

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