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Original Article

Evaluation of a Single PCR Assays on Cp5 Gene for Differentiation of *Entamoeba histolytica* and *E. dispar*

*S Rostamighalehjaghi[1], R Jamali[1], S Rezaie[2], Z Babaei[3], H Hooshyar[4], *M Rezaeian[2]*

[1]Dept. of Parasitology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Division of Molecular Biology, Department of Medical Mycology & Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[3]Dept. of Parasitology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
[4]Dept. of Parasitology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

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Abstract

Background: We examined a molecular method with a single-PCR for amplification of a part of CP5 gene enabling us to differentiate the pathogenic species, *Entamoeba histolytica*, from the non-pathogenic species, *E. dispar*.

Methods: We developed a single PCR method for this purpose. After investigation of GenBank, primer pairs were designed from highly conserved regions of cysteine proteinase (CP5) gene. The primers were utilized in PCR using isolated genomic DNA template of *E. histolytica* and the PCR products were then sequenced. The same primer and method for PCR was used for isolated genomic DNA template of *E. dispar*.

Results: A fragment of about 950 bp was isolated in PCR by using DNA from *E. histolytica*, however, no banding pattern was produced by using the same primers for *E. dispar*. We characterized CP5 gene at molecular level in *E. histolytica* isolates from 22 positive; including 20 non-dysentery samples isolated from both cities as well as two dysentery samples isolated only from Tabriz. Nucleotide sequence comparison in gene data banks (NCBI, NIH) revealed significant homology with CP5 gene in *E. histolytica* isolates

Conclusion: We developed a PCR method, which could detect simply and rapidly *E. histolytica* by amplifying a specific PCR fragment.

Keywords: *Amebiasis,* Entamoeba dispar, Entamoeba histolytica*, Nucleic acid sequencing*

Introduction

Amebiasis caused by *Entamoeba histolytica* is still mentioned as one of the major health problems in tropical and subtropical areas (1). It is the cause of various infectious diseases ranging from dysentery to abscess of liver or other organs. It is estimated that amebiasis is responsible for up to 110,000 deaths per year (2-4). This infection is usually predominant in low socioeconomic status and poor hygienic situations that favor the indirect fecal-oral transmission of the infection (5). Previously two morphologically identical species of *Entamoeba* had been found, and was shown that only one of them is able to cause infection in kittens or human volunteers (6). However, *E. histolytica* has recently been re-described as two distinct species; the pathogenic species *E. histolytica* and the nonpathogenic species *E. dispar*. As these two species are morphologically similar, development of new methods for their rapid differentiation is currently under investigation (6, 7).

Cysteine proteinases are among the most important enzymes in many microorganisms and are known to play essential roles in pathogenesis of such organisms (8, 9). From known Cysteine proteinases, CP1 and CP5 exist in *E. histolytica* and not in *E. dispar* (10, 11).

In the present study, we have examined a molecular method with a single-PCR for amplification of a part of CP5 gene enabling us to differentiate the pathogenic species, *E. histolytica*, from the non-pathogenic species, *E. dispar*.

***Corresponding author:** Tel: +98 21 88951392, Fax: +98 21 88951392, E-mail: Rezaiian@sina.tums.ac.ir

Materials and Methods

Collection of samples

2332 stool sample were collected randomly from patients referring to local hospital or laboratory of public health center located in Tabriz and Bandar abbas in 2005. The examinations were done in Parasitology laboratory in Emmam Khomaini Hospital, Shahid Rajaei Hospital in Tabriz and Karadj respectively and also protozoalogy lab in Tehran University of Medical Sciences.

Microscopic examination were done by direct examination and formalin ether concentration method for detection of *E. histolytica/E.dispar* trophozoite or cyst respectively and 148 positive specimens were cultured immediately or stored in refrigerator without any preservative before culturing .

Culture and preservation

Coagulated horse serum media (Hrs+s) was used to transform cysts to trophozoites, then Robinson's culture media were used for mass culture and the adaptation of trophozoites.

Preparation molecular study

After 3-4 subcultures, the upper layer of 43 Robinson's media was removed and the deposit was kept in centrifuge tube, then 10 ml of PBS solution with pH:7.2 added to tube and mixed adequately twice with speed of 1600g for 5 min using centrifuge. The upper layer was removed and deposit was mixed with 10ml of PBS suspension and centrifuged again. The sediment was then suspended in 1 ml PBS finally divided equally in 1.5 ml ependorf tube and kept at -80° C until DNA extraction.

Method of DNA Extraction from trophozoites

For DNA extraction from trophozoites a slightly modified procedure that has been previously described (12), using Phenol-Chloroform-Isoamylacohol (PCI), was utilized. Briefly, the harvested amoeba cells were suspended in DNA extraction buffer containing: 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 3% SDS, and 50 µl of proteinase-K (20 mg/ml). The suspension was then incubated at 65° C for 1 h and the cellular debris was removed by centrifugation at 2500 g for 15 min. After addition of 25 µl RNase-H (10 mg/ml), the suspension was incubated at 37° C for 30 min, extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of isopropanol, followed by centrifugation at 15000 x g for 30 min. The DNA pellet was rinsed with 70% ethanol and resuspended in distilled water.

DNA Extraction from the cyst

DNA extraction from the cyst was carried out by using, the QIAamp stool Mini Kit.

Primer designing

Oligonucleotide primers were designed based on GenBank investigation from CP5 gene sequences (accession numbers: X91654, M64721, M94163, and M64712). One pair of primers was designed for amplification of approximately 950 base pair of the CP5 gene and synthesized as follows: 5' GTT CACTGTCTCGTTATTAG 3' as forward and 5' CATCAGCAACCCCAACTG 3' as reverse.

DNA amplification by PCR

In the first step ,a part of collagen binding protein (cbp-30) gene was amplified by PCR for confirming the existence of *E. histolytica/dispar* DNA and also for substantiation of absence of other amoeba in the 43 positive isolates that has been previously described (13). On the other step, all 43 positive and confirmed *E. histolytica/ dispar* samples were used for analysis of the CP5 gene using a single PCR with the specific designed primers. Two standard strains used in this study were *E. Histolytica* HM-1, *E. dispar* AS16IR.These were used as a positive control in the present study.

First the primers were tested by two standard strains DNA, then amplification of the CP5 gene was performed on DNA from the 43 clinical samples diagnosed *E.histolytica/dispar*. DNA was amplified using Primus, MWG-BIOTECH, Thermal Cycler under the following conditions: 5 min at 95° C as a initial hot start step, followed by 35 cycles, of 1 min at 95° C, 2 min at 46° C, 2 min,

3 sec at 72° C, and a final extension step 10 min at 72° C. Distilled water used as a negative control and The PCR reaction mixture was consisted of 3 µl of template DNA, 10 µl of 10X PCR buffer (Roche), 1 µl of deoxynucleotide triphosphate (dNTPs) mix, 3 µl of $MgCl2$, 1 µl of Taq polymerase (CinnaGen Tehran, Iran), and 1 µmol l of each primer and 30 µl of dd H2O. Finally, the PCR products were electrophoresized on ethidium bromide-staining 1% (W/V) agarose gel. In the case of detection a banding pattern with approximate size of 950 bp, it was calculated as *E. histolytica*. Positive and negative control reactions were included with each batch of samples analyzed by single PCR. The twenty of PCR products were sequenced on both strands and analyzed using the align two sequences (bl2 seq) program.

Sequencing of the PCR fragments

Sequencing of the 20 PCR products were performed with the Dye Terminator Cycle Sequencing Kit (MWG, Germany). We used the isolated double stranded DNA as template and the synthetic internal primers designed according to the investigated DNA sequence fragments from other eukaryotic cells. Sequencing of fragment was repeated three times for both strands. The nucleotide sequence of DNA was compared with the sequences available in gene data banks from National Center for Biotechnology Information (NCBI, NIH).

Results

A total of 2332 samples were investigated in this study, among which, 148 (6.35%) were infected with *E.histolytica/E.dispar* by microscopic examination. Forty three isolates were successfully cultured. In order to confirm the *E. histolytica/ dispar* infection, these samples were used for molecular analysis on (cbp-30) gene firstly, then the PCR amplification of the 950 bp fragment of the CP5 gene was performed on DNA from the confirmed isolates. A fragment of about 950 bp was isolated in PCR by using DNA from *E. histolytica*, however, no banding pattern was produced by using the same primers for *E. dispar*.

We characterized CP5 gene at molecular level in *E. histolytica* isolates from 22 positive; including 20 non-dysentery samples isolated from both cities as well as two dysentery samples isolated only from Tabriz (Fig.1, 2).

Nucleotide sequence comparison in gene data banks (NCBI, NIH) revealed significant homology with CP5 gene in *E. histolytica* isolates (Data not shown). The nucleotide sequences of the CP5 gene of two isolates of *E. histolytica* from Bandar Abbas and Tabriz were submitted to the GenBank (NCBI) and released for public access under the numbers: DQ899178, DQ899179.

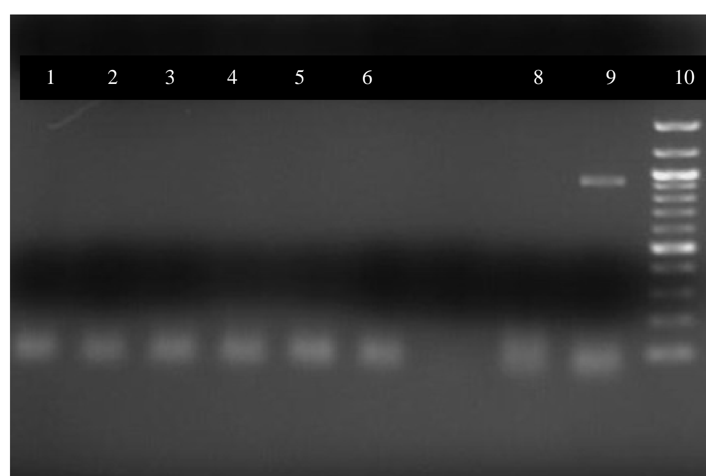

Fig.1: PCR products of the CP5 gene by using the designed primers. lane 1-5: isolated samples revealed as *E. dispar* , lane 6: standard species of *E. dispar*(AS16IR) ,lane 8 Negative control, lane9 : standard species of *E. histolytica*(HM1) and lane10: DNA ladder 100 bp

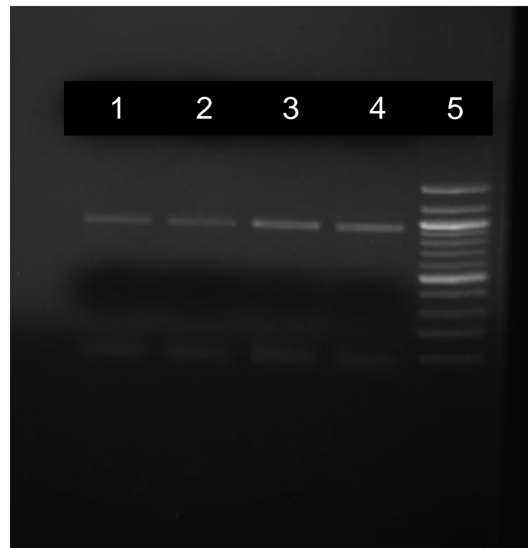

Fig.2: PCR products of the CP5 gene by using the designed primers, lane 1-3: Isolated samples revealed as *E. histolytica*, lane 4: standard species of *E. histolytica*(HM1) , lane 5 : DNA ladder 100 bp

Discussion

Entamoeba.histolytica is the second protozoan parasite reported as a cause of lethal infectious disease in humans (3) resulting in 500 million infections caused by *E. histolytica/E.dispar* worldwide and over 100000 death caused by *E. histolytica*. There is a clear evidence for necessity of additional research of this protozoa and its pathogenicity (2, 3). Cysteine peptidases of *E. histolytica* (EhCPs) are important in amoebic invasion process (14, 15). Up to now, about 50 CP genes have been characterized in genome of *E. histolytica* though some including ehcp1, ehcp2, ehcp-5 and EhCP-A7 are the major CPs. However, only gene products from five of these genes, EhCP1, EhCP2, EhCP3, EhCP5, and EhCP112, have been identified in cultured trophozoites (16).
Bruchhaus et al. have reported that the EhCP1, EhCP2, and EhCP5 enzymes contribute to approximately 90% of the total CP activity from the parasite. They have compared cysteine proteinase genes and their expression in *E. histolytica* and in non-pathogenic twin *E. dispar*. Recently a study has shown that two of CPS genes including ehcp1 and ehcp5 are unique to *E. histolytica*, as the former(ehcp1) is absent and the latter (ehcp5) is nonfunctional in *E. dispar*, a morphologically identical but noninvasive *Entamoeba* species (10,16,17).
Identification and differentiation of *E. histolytica* from *E. dispar* has been the most important advancement in intestinal protozoalogy with clinical and epidemiological application (18). Assays such as Isoenzyme (19) detection of specific antigens by monoclonal antibodies (20) and PCR based assays (2, 13, 21-24) have shown to be successful strategies for identifying and differentiating these amoebas (19). These methods have showed high specificity and sensitivity, although some cannot generally be used in developing countries due to their cost and complicated methodology. Recently nucleic acid-based approaches have successfully been developed for detection and discrimination of *E. histolytica* and *E. dispar* (12, 19). For example, researchers in Japan used two pairs of specific primers, one pair amplified a 100 bp fragment, and the other amplified a 101 bp fragment on gene encoding 30 kDa surface antigen for *E. histolytica* and *E. dispar* (22).
In spite of the fact revealed that ehcp5 expresses in *E. histolytica* trophozoites and also is nonfunctional in *E. dispar*, we use the CP5 gene for discrimination of them and report a PCR-based molecular method which can simply and rapidly

identify *E. histolytica* from *E. dispar*. The described method has shown that this gene was a suitable locus for detection *E. histolytica* in stool specimens because our results revealed that a fragment of about 950 bp only was isolated in PCR by using DNA from *E. histolytica* extracted from clinical samples as well as standard strain, HM1, however, no banding pattern was produced by using the same primers for *E. dispar*.

In conclusion, the new diagnostic method reported here can aid in easier and less costly identification of *E. histolytica* by routine laboratories compared to other methods and may help the health care system by avoiding use of unnecessary drugs in patients infected with *E. dispar.*

Ethical Considerations

Ethical issues Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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