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Original Article

Differential detection of Entamoeba histolytica from Entamoeba dispar by parasitological and nested multiplex polymerase chain reaction methods

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Abstract

Introduction: Amebiasis is an intestinal illness caused by a one-celled parasite (amoeba) called Entamoeba (E) histolytica. E histolytica and E dispar are morphologically undistinguishable but have genetic and functional differences. E. histolytica is invasive and cause amoebiasis, but E dispar cause an asymptomatic colonization which does not need to be medically treated. We have performed a nested multiplex Polymerase Chain Reaction (PCR) targeting small subunit rRNA (Ribosomal ribonucleic acid) gene for differential detection of E histolytica and E dispar directly from stool samples.

Methods: All the fecal samples collected without preservation and were screened for amebic cells by parasitological methods. Fecal samples that containing amebic cells were stored at -20ºC until DNA extraction. DNA extraction was down by using a DNA extraction kit. The genus specific primers were designed using nucleotide sequences of 18S-rRNA gene of Entamoeba.

Results: Thirty one (4.28%) stool samples out of 724 samples were positive for E histolytica/ E dispar. The nested multiplex PCR illustrated that the size of diagnostic fragments of PCR products was obviously different for two Entamoeba species, the specific product size for E histolytica and E dispar was 439 and 174 bp. The nested multiplex PCR was positive in 25 out of 31 stool specimens that 17 (54.8%) samples were positive for E dispar and 8 (25.8%) samples were positive for E histolytica.

Conclusion: Nested multiplex PCR was useful for the specific detection of E histolytica and E dispar in stool samples. In current study we detected that E dispar was more prevalent in our study area.

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Introduction

Entamoeba histolytica causes intestinal and extra intestinal amoebiasis written by Krishna K et al. The prevalence of amebiasis is differs with the population of individuals affected and varies between areas with different socioeconomic conditions.[1,2] E. histolytica infection is endemic in many parts of the world where sanitation hygiene is deprived.[3] Both sexes, in different ages, were exposed to chance of infection because all of them were living under the equal situations of infection in an area.[4,5] Entamoeba histolytica is a parasite of the human intestine; usually infect

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the hosts by ingesting contaminated water or food. According to many studies, amoebiasis and giardiasis are common causes of intestinal protozoal infection in the most parts of the world.[6] In endemic areas, contact can be enormously high; the global prevalence of infection was estimated to be about 10% of the world's population.[7] Of these, about 90% were estimated to be asymptomatic carriers while 10% developed invasive amoebiasis and effectuate 40000 to 100000 deaths per year.[7,8] Invasive amoebiasis occurs when trophozoites attack the intestine wall, and can cause diarrhoea, dysentery and in some cases dissemination to organs where abscesses result.[9] This disease is the second leading cause of death from parasitic disease worldwide.[10] E histolytica and E dispar are morphologically identical but have genetic and functional differences; in 1997; the World Health Organization (WHO) with the committee of the conspicuous world parasitologists in Mexico mentioned the E. histolytica as pathogenic specie and E dispar as nonpathogenic specie.[11,12] Identification and isolation of E histolytica from E dispar by microscopic method and staining is impossible. In order to avoid unnecessary treatment of those with non-pathogenic Entamoeba species, it is essential to discriminate these species from the pathogenic specie.[13]

Thus, the use of molecular methods is necessary for the detection of E histolytica from E dispar.[7] PCR based on amplification of the small subunit ribosomal ribonucleic acid (rRNA) gene (SSU-rDNA) was reported to be 100 times more sensitive than ELISA (Enzyme Linked Immunosorbent Assay) and other immunological methods. SSU-rDNA is widely used as target for detection and differentiation of Entamoeba species.[14] The present study was designed to detect Entamoeba species by parasitological and molecular method, in medical centers of Miandoab city, Azerbaijan Province, Iran's north-west and avoid to excessive and unnecessary use of anti-protozoal drugs for E dispar treatment.

Methods

During January 2011 to June 2012, 724 stool samples were collected from Fatima Hospital of Miandoab city, west Azerbaijan province, and northern west Iran. All the fecal samples were examined by microscopic examinations. Samples collected without preservation and used wet-mount, formalin-ethyl acetate concentration and trichrome staining technique for identification of cysts and trophozoites.[15] Positive samples were stored at -20°C.

DNA purification was done with genomic DNA extraction kit (QIAGEN, and Hilden, Germany). DNA samples were kept at -20°C until usage. The primer sequences designed for E dispar and E histolytica were subjected to a basic local alignment search tool (BLAST) in the genome database of all organisms (www.ncbi.nlm.nih.gov/blast) and were confirmed to be specific for this study.

Final concentration of the mixture contained 200 µM each of dNTP, 10 pmol of each primer, 1.0 U Taq DNA polymerase (fermantase), 1.5 mM $Mgcl_2$ and approximately 2.5 µl of template DNA was added in genus specific and species specific PCR. The PCR tubes were finally placed in an automated PCR machine (Biometra I Uno Thermoblock). Thirty cycles of polymerase chain reaction (PCR) were performed as follows: initial denaturation step at 96°C for 2 min, denaturation at 96°C for 1 min, Annealing at 56°C for 1 min, extension 72°C for 1.5 min and final polymerization step at 72°C for seven min. In the species specific nested multiplex PCR (which had multiple primer sets in the same tube), only the annealing temperature was changed to 48°C, leaving the other parameters of the amplification cycles unchanged. Amplified products (10 µm) were run to electrophoresis in 1% agarose gels, and the existence of specific bands was visualized with UV light after ethidium bromide staining.

Two standard strains used in this study were E histolytica HM-1: IMSS and E dispar SAW760. These were used as a positive control in the present study.

Results

A total of 31 samples (4.28%) out of 724 stool samples were positive for E histolytica and E dispar by using parasitological methods. The nested multiplex PCR was carried out on 61 stool specimens including 31 stool specimens positive for E histolytica and E dispar and 30 negative controls. All the primer pairs selected for this study were quite specific, and did not amplify DNA derived from the negative control. The E1 and E2 primers were designed to amplify SSU-rDNA in genus of Entamoeba, when an optimum annealing temperature of 56ºC was employed, and to yield amplicon of about 1.070 bp. This E1/E2 primer pair was positive in 28 out of 31 samples. Three samples could not be detected by nested multiplex PCR which may be due to loss of DNA during the extraction procedure. The nested multiplex PCR demonstrated that the size of diagnostic fragments of PCR products was obviously different for all the two Entamoeba species, the species-specific product size for E histolytica was 439 bp and E dispar was 174 bp. The nested multiplex PCR was positive in 25 out of 31 stool specimens; that 17 samples were positive for E dispar and 8 samples were positive for E histolytica. Six samples could not be detected by nested multiplex PCR and with consideration that in genus nested multiplex PCR 28 samples was positive, 3 samples may be of other Entamoeba species for example E Coli or E Moshkovskii (Figure 1).

In this study, mixed infection with E histolytica and E dispar was not identified and despite some reports that E dispar in patients with gastrointestinal, symptoms were seen. In this study, there were no gastrointestinal symptoms in patients with E dispar, while all the 8 cases of infection with E histolytica had gastrointestinal symptoms from mild diarrhea to severe.

Discussion

E histolytica is the pathogenic and the cause of amoebic colitis and liver abscess; while the E dispar is a non-pathogenic species and has never been related to the disease.[11] Differential diagnosis between the two species is important both for treatment decision and public health awareness.[16,17] WHO have recommended that E histolytica should be specifically identified and treatment is crucial; while E dispar is not recommended to treat.[12] E dispar as a separate species which cannot be recognized by parasitological methods from E histolytica, has prompted the WHO to recommended the development of improved methods for the specific detection of E histolytica.[12] Accordingly, we have assessed the application of nested multiplex PCR technology to recognize and differentiate of E histolytica and E dispar directly from stool samples.

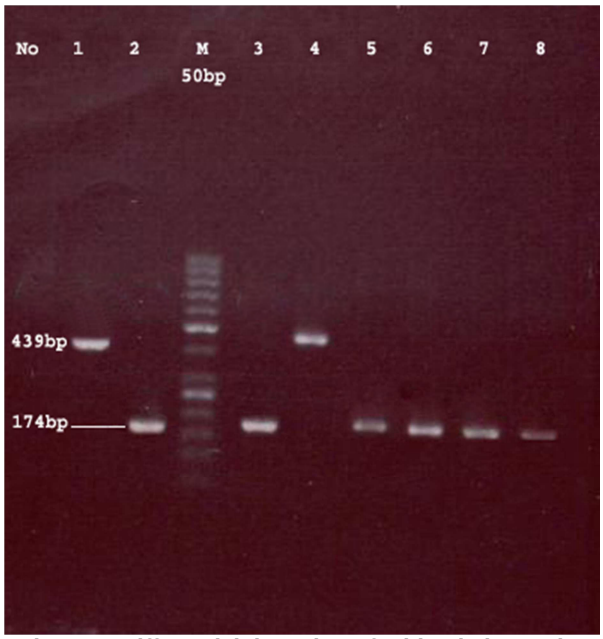

Figure 1. Differential detection of E histolytica and E dispar by nested multiplex PCR (polymerase chain reaction) on stool samples

The E histolytica and E dispar bands are 439 bp and 174 bp. Lane-No = Negative control, Lane-1 = genomic DNA of E histolytica HM1 = IMSS strain, Lane-2 = genomic DNA of E dispar SAW760, Lane-M = 50 bp DNA ladder, Lane-3, 5, 7, 8 = E dispar and Lane-4 = E histolytica.

Laboratory diagnoses of amoebiasis mostly depend upon the detection of parasite in the microscopic examination of fresh samples. However, there are some drawbacks in the microscopic diagnosis. Diagnosis of the pathogenic strain is currently done with molecular (PCR, DNA probs, and

riboprinting), immunoassay and isoenzyme analysis methods.[18]

In recent years, PCR and other molecular methods has been increasingly used for diagnosis of numerous infectious diseases. PCR analyses are based on the extra chromosomal circular rRNA gene, which approximately 200 copies are present in each Entamoeba cell.[19]

Before this time, DNA extraction was impossible from fecal samples without cultivation but at first, the researchers Apiradee and colleagues extracted the DNA of E histolytica and E dispar without cultivation and directly from fecal samples.[14]

PCR in comparison to isoenzyme classified all samples correctly into E histolytica and E dispar. Additionally, positive PCR for E. histolytica was strongly associated with serology analysis. Previous findings indicating that serology assays cannot differentiate between E dispar, E histolytica infections in some situations; because some asymptomatic cases usually induce a significant antiamebic response.[20-23] However, in comparison to microscopy or culture technique, PCR identified a considerably larger number of additional positive samples, suggesting that PCR is more sensitive. Sensitivity of microscopy for the detection of Entamoeba by examination of a single fecal sample is considered to be about 70%.[24]

However, PCR extension was continuously clean, but false-positive can occur in samples that contain DNA from several sources. However, with adequate primers, false-positive results can be predictable to occur at a very low rate.[25]

This study aimed to develop a reference method and to evaluate the particular proportions of E dispar or E histolytica infections in referred samples. In Iran, prior studies have also reported high prevalence of Entamoeba infection with prevalence rates ranging from 9.4% to 21.0%.[26-28] The results of our study show that E dispar (54.8%) was found to be the most common species detected in our study, as like as other studies in Iran and other parts of the world.[29] But two articles in Malaysia showed that E histolytica was the most prevalent species detected.[30] The results of current work indicate that the PCR is suitable for detection and differentiation of E histolytica and E dispar directly from human feces.

Because of the high sensitivity and specificity of the modified PCR assay, the failure of microscopy to distinguish between the two ameba species, and the time consuming of the culture and subsequently differentiate Entamoeba species by isoenzyme analysis, it is apparent that this protocol or similar technique are substantially more appropriate than microscopy or culture to correctly diagnose intestinal E histolytica or E dispar infections.

Conclusion

Molecular techniques are indeed promising tools for epidemiological studies, particularly in discriminating the pathogenic from the non-pathogenic species of the Entamoeba species.

Conflict of Interests

Authors have no conflict of interest.

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