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Original Article

Application of Multiplex PCR for Detection and Differentiation of *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii*

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Abstract

Background: *Entamoeba moshkovskii* and *E. dispar* are impossible to differentiate microscopically from the pathogenic species *E. histolytica*. Multiplex polymerase chain reaction (Multiplex PCR) is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment.

Methods: For detection and differentiation of the three-microscopy indistinguishable *Entamoeba* species in human, multiplex PCR assay using different DNA extraction methods was studied. A conserved forward primer was derived from the middle of the small-subunit rRNA gene, and reverse primers were designed from signature sequences specific to each of these three *Entamoeba* species.

Results: A 166-bp PCR product with *E. histolytica* DNA, a 580-bp product with *E. moshkovskii* DNA and a 752-bp product with *E. dispar* DNA were generated in a single-round and multiplex PCR reaction.

Conclusion: We recommend this PCR assay as an accurate, rapid, and effective diagnostic method for the detection and discrimination of these three *Entamoeba* species in both routine diagnosis of amoebiasis and epidemiological surveys.

Introduction

Entamoeba moshkovskii, *Entamoeba dispar* and *Entamoeba histolytica* are morphologically identical but biochemically and genetically are different and microscopic examination is unable to detect and differentiate these three *Enatamoeba* spp. Although *E. histolytica* is known to be pathogen, the other two species are non-pathogen or the ability of them to cause disease is unclear (1, 2). Before redescription of *E. histolytica* and *E. dispar* in 1997 (3, 4), several epidemiological studies in Iran have shown *Entamoeba* spp. infection rate of about 2.2 to 30 percent (5-7). In the past decade, these three *Entamoeba* have been differentiated and reported by molecular methods in some areas of Iran (2, 8-17).

Developing of a new method for differentiation of those microscopy identical amoebas is highlighted. Multiplex PCR is a molecular biology technique for amplification of multiple targets in a single PCR experiment. The multiplex PCR was used extensively for pathogen identification, Single Nucleotide Polymorphism (SNP) genotyping, mutation analysis, gene deletion analysis, template quantitation, linkage analysis, RNA detection, forensic studies and diet analysis (18, 19). In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture (18).

While the single template PCR reaction uses a single template along with several pairs of forward and reverse primers the multiple template PCR reaction uses multiple templates and several primer sets in the same reaction tube (18, 19). Extraction of DNA is often an early and important step in many diagnostic processes used to detect bacteria, viruses and parasites in the environment as well as diagnosing disease and genetic disorders (20).

In the presence study, we investigated the presence of *E. histolytica, E. dispar* and *E. moshkovskii* by single-round and multiplex PCR using six different DNA extraction methods.

Materials and Methods

Entamoeba samples

Twenty-seven DNA templates from 20 *Entamoeba histolytica* and 7 *Entamoeba dispar* samples were analyzed. All the 20 *E. histolytica* DNAs were extracted previously in Japan from Japanese patients (21). The DNA of 7 *E. dispar* was also extracted previously from Iranian isolates (22). DNA of *E. histolytica* HM-1: IMSS, *E. dispar* SAW760, and *E. moshkovskii* Laredo cells as positive controls that were maintained alive in liquid nitrogen tank in Department of Medical Parasitology and Mycology, Shahid Beheshti University of Medical Sciences, were recovered in TYI-S-33 medium and their DNAs were extracted to study the single and multiplex PCR assays.

DNA preparation

The growing trophozoites were harvested by centrifugation at 800 ×g for 5 min. About 200 µl of cultured trophozoites was washed with phosphate-buffered saline (pH=7.2). The genomic DNA of cultured trophozoites was extracted and compared using five DNA extraction kits: DNG™ plus and DNP™ kit(CinnaGen Inc., Tehran, Iran), Chelex (Bio-Rad), QIAamp DNA mini kit and QIAamp DNA stool mini kit (QIAGEN, Germany) according to the manufacturer's directions and also the traditional phenol-chloroform method (23). The procedure of DNG™ -plus and DNP™ requires about 60 minutes and does not require phenol extraction or proteinase digestion for DNG™-plus. In QIAamp DNA stool mini kit and QIAamp DNA mini kit for tissue extraction, purification requires no phenol-chloroform extraction or alcohol precipitation, and DNA is eluted in low-salt buffer and is free of protein, nucleases, and other impurities or inhibitors. Chelex is a chelating material from Bio-Rad. It is a fast, cheap, and effective method of DNA extraction. The Chelex protects the sample from DNAases that might remain active after the boiling and could subsequently destroy the DNA. The concentration and purity of the extracted DNA was assessed by Spectrophotometer WPA (Biowave II, Eng) reading of the absorbance at the 260/280 nm. The DNA concentration and the corresponding A260 values, for the six DNA isolation methods of three positive controls are shown in Table 1.

Table 1: DNA concentration of the three *Entamoeba* spp. strains, and their corresponding A260 values, for the six DNA extraction methods

***Entamoeba* samples**	***E. histolytica* (HM1:IMSS)**		***E. dispar* (SAW 760)**		***E. moshkovskii* (Laredo strain)**	
Methods	ng/µl	A260/280	ng/µl	A260/280	ng/µl	A260/280
Phenol chloroform	11.5	1.91	11.27	1.54	3.5	3.50
DNG plus kit	10.5	1.16	8.00	2.00	1.5	1.50
DNP kit	26.0	1.26	33.00	1.26	9.0	1.38
QIAamp DNA mini kit	17.0	1.25	79.00	1.43	69.5	1.99
QIAamp DNA stool mini kit	8.5	1.20	53.00	1.02	12.0	1.20
Chelex kit	11.0	1.58	56.00	1.47	11.0	1.57

Polymerase chain reaction

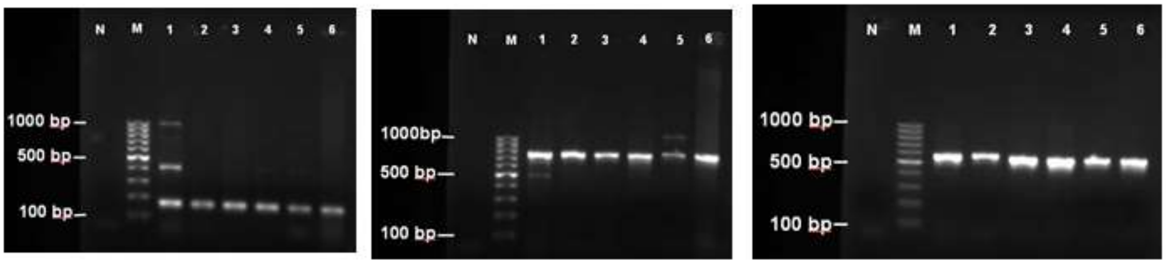
Single-round PCR amplification as well as multiplex PCR were used in the study. The sequence of a forward primer used (EntaF, 5'-ATGCACGAGAGCGAAAGCAT-3') was conserved in all three *Entamoeba* spp., whereas the specific reverse primers, EhR (5'-GATCTA-GAAACAATGCTTCTCT-3' X64142), EdR (5'-CACCACTTACTATCCCT-ACC-3' Z49256), and EmR (5'-TGACCGGAGCCAG-AGACAT-3' AF149906), were specific for *E. histolytca* , *E. dispar, E. moshkovskii,* respectively (24).

PCR was performed using Amplicon (Taq DNA Polymerase Master Mix Red, Denmark) as a ready-made solution. The reaction mixture contained 5 µl of distilled water, 7.5 µl of amplicon, 20 pmol of forward and reverse primers, and about 5-10 ng of extracted DNA template to achieve a final volume of 15 µl. Amplification of each species-specific DNA fragment started with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. Amplified products were visualized after electrophoresis on 1.5% agarose gels by ETBr staining.

Results

Species-specific PCR products

The forward primer in combination with the appropriate reverse primer amplified a 166-bp PCR product with *E. histolytica*, a 752-bp PCR with *E. dispar*, and a 580-bp with *E. moshkovskii* DNAs. By using the separate *Entamoeba* spp. DNA template, with species-specific *E. histolytica* primers (EntaF/EhR) amplified DNA from the HM-1:IMSS strain with all of the six DNA extracted methods observed , but no band were seen when *E. dispar* SAW760 or *E.moshkovskii* Laredo DNA were used. The *E. dispar* species-specific primers (EntaF/EdR) and the *E. moshkovskii* primers (EntaF/EmR) also showed the expected specificities in single-round PCR. Similar results were also observed when the forward and reverses' primers for *E. histolytica*, *E. dispar*, and *E. moshkovskii* were mixed in a single DNA template reaction. Amplified PCR bands of the six DNA extracted methods using the three *Entamoeba* isolates visualized on a 1.5% agarose gel are presented in Fig. 1.



(A) (B) (C)

Fig. 1: Single PCR assay with EntaF primer, combined with EhR, EdR, and EmR primers in a single PCR reaction by using 6 DNA extracted method from (A) *E. histolytica* (HM1:IMSS), (B) *E. dispar* (SAW760) , (C) *E. moshkovskii* Laredo strain
Lane M, 100 bp weight marker; lane N, negative control; DNA extracted by: 1. Phenol-chloroform, 2. DNG™-plus kit, 3. DNP™ kit, 4. QIAamp DNA mini kit, 5. QIAamp DNA stool mini kit, 6. Chelex kit

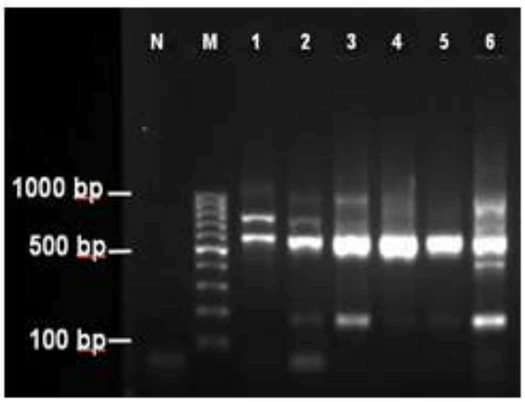


Fig. 2: Multiplex PCR fragments amplified using EntaF primer combined with EhR, EdR, and EmR reveres primers in a single reaction mixed with DNAs of *E. histolytica* (HM1:IMSS), *E.dispar* (SAW 760) and *E. moshkovskii* Laredo DNAs extracted from cultured trophozoites

Lane M, molecular weight marker (100-bp); lane N, negative control ; lanes 1-6, DNA extracted by six DNA extracted methods: 1. Phenol chloroform, 2. DNG™-plus kit, 3. DNP™ kit, 4. QIAamp DNA mini kit, 5. QIAamp stool mini kit, 6. Chelex kit

When DNAs of *E. histolytica* (HM1:IMSS), *E. dispar* (SAW760), and *E. moshkovskii* Laredo strain were mixed together in a multiplex PCR assay using the entire forward and three reverses' primers, the same fragments of PCR results were observed.

However, the PCR bands intensity of some of the DNA extracted methods for *E. histolytica* and *E. dispar* were weak (Fig. 2).

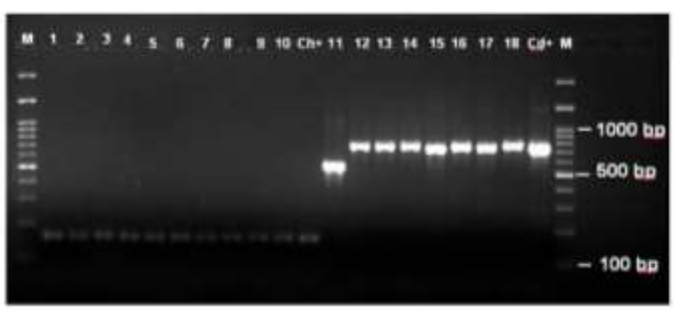
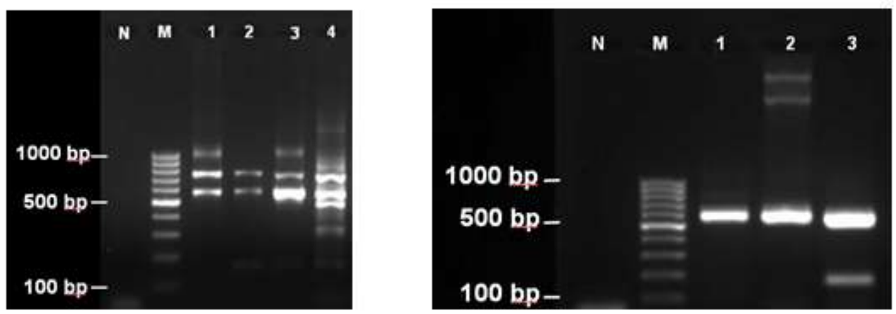


Fig. 3: The single PCR assay of *E. histolytica* DNA with EntaF and EhR primers (lanes 1-10), *E. moshkovskii* DNA with EntaF and EmR primers (lane 11), and *E. dispar* DNA with EntaF and EdR primers (lane 13-18), in a single reaction by using DNA samples from E. *histolytica* and *E.dispar*, isolates./ Lane M, Molecular weight marker (100-bp); lane Ch+, positive control of *E. histolytica;* and lane Cd+, positive control of *E. dispar*, DNA were extracted by QIAamp DNA stool mini kit methods of DNA extraction

Evaluation of the PCR assay with DNA samples

Overall, seventeen (62.96%) samples from 27 isolates including ten *E. histolytica* and seven *E. dispar* as well as extracted DNAs from *E. histolytica* HM-1:IMSS, *E. dispar* SAW 760, and *E. moshkovskii* Laredo cells as positive control using single-round PCR reaction were identified (Fig. 3). A mixture DNA of some of those positive *E. histolytica*, *E. dispar* isolates, and *E. moshkovskii* Laredo strain, which were tested using multiplex PCR assay, are also shown in Figs. 4. Multiplex PCR fragments of the *E. histolytica* (HM-1:IMSS), *E. dispar* (SAW760 and *E. moshkovskii* Laredo strain amplified in a single reaction using forward primer combined with the three reverse primers are shown in Fig. 5.



(A) (B)

Fig. 4: Multiplex PCR with EntaF primer combined with EhR, EdR, and EmR primers in a single reaction mixture by using some of *E. histolytica*, *E.dispar*, *E. moshkovskii* Laredo strain DNAs
Lane M, molecular weight marker (100-bp); lane N, negative control ; A) lane 1, *E. moshkovskii* (580bp) and *E. dispar* (752 bp) and lanes 2-4, *E. histolytica* (166bp), *E. moshkovskii* (580bp) and *E. dispar* (752 bp) , B) lanes 1-2, *E. moshkovskii* (580) and lane 3, *E. histolytica* (166bp)

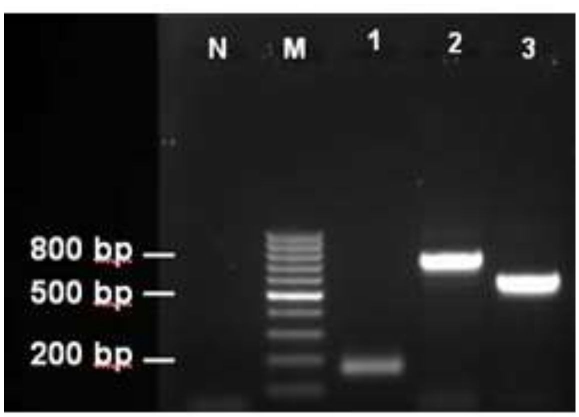


Fig. 5: Multiplex PCR fragments amplified using EntaF primer combined with EhR, EdR, and EmR reveres primers in a single reaction by using extracted DNA samples from 1) *E. histolytica* (HM1:IMSS), 2) *E. dispar* (SAW760) and 3) *E. moshkovskii* Laredo strain
Lane M, molecular weight marker (100-bp ladder); lane N, negative control

Discussion

A single-round PCR-based approach for differential diagnosis of three species of *Entamoebas*, *E. moshkovskii*, *E. histolytica*, and *E. dispar*, which share identical morphology as both cysts and trophozoites were described in this study. This simple diagnostic PCR technique does not require extra steps, as is the case with nested PCR (25), PCR restricted fragment length polymorphism (26), and PCR with reverse line blot hybridization (27).

Six simple methods including phenol chloroform, DNG™ -plus, DNP™, QIAamp DNA mini kit for tissue, QIAamp DNA stool mini kit and chelex kit for the extraction of DNA from *Entamoeba* Spp. were also compared and evaluated. In all 6 methods, DNA was extracted from cultured trophozoites of the three cryopreserved *Entamoebas*. In manual phenol chloroform DNA extraction method which is time consuming, two bands were seen above the expected band of *E. histolytica* cultured trophozoites (Fig. 1, A). All 6 methods had acceptable result considering of extracted DNA. All the commercial kits performed equally well in the PCR amplifications.

The study indicated that the multiplex-PCR consisted of multiple primer sets within single template had a better result compared with multiple primer plus multiple template PCR reaction.

Molecular tools are extensively used for epidemiological studies, particularly in differentiation of the pathogenic from the non-pathogenic species of the *Entamoeba* species. This study and a few reports by the other researchers showed single and multiplex PCR assay in a single sample is able to detect and differentiate *E. histolytica*, *E. dispar* and *E. moshkovskii* (28-30). Recently usefulness of nested multiplex PCR method for differentiation of *E. histolytica* from *E. dispar* on 31 stool samples was reported by Fallah et al. (31). Although a remarkable results were obtained for differentiation of the three microscopy identical *Entamoeba* species by a single-round and multiplex PCR in this study, but further studies on more positive stool *Entamoeba* samples as well as normal subjects and non-*Entamoeba* isolates in Iran are needed to evaluate sensitivity and specificity of those primers in a multiplex PCR.

Conclusion

We recommend the application of multiplex PCR assay as an alternative tool in routine diagnosis and epidemiological studies of amoebiasis. It is expected that this will provide better epidemiological data and a greater understanding of infections with these three amoebae in humans.

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