Simultaneous detection and differentiation of *Entamoeba histolytica*, *E. dispar*, *E. moshkovskii*, *Giardia lamblia* and *Cryptosporidium* spp. in human fecal samples using multiplex PCR and qPCR-MCA

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A B S T R A C T

*Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* spp. are common causes of diarrheal and intestinal diseases all over the world. Microscopic methods are useful in the diagnosis of intestinal parasites (IPs), but their sensitivity was assessed approximately 60 percent. Recently, molecular techniques have been used increasingly for the identification and characterization of the parasites. Among those, in this study we have used multiplex PCR and Real-time PCR with melting curve analysis (qPCR-MCA) for simultaneous detection and differentiation of *E. histolytica*, *E. dispar*, *E. moshkovskii*, *G. lamblia* and *Cryptosporidium* spp. in human fecal samples. Twenty DNA samples from 12 *E. histolytica* and 8 *E. dispar* samples and twenty stool samples confirmed positive for *G. lamblia* and *Cryptosporidium* spp. were analyzed. After DNA extraction from the samples, multiplex PCR was done for detection and differentiation of above mentioned parasites. qPCR-MCA was also performed for the detection and differentiation of 11 isolates of above mentioned parasite in a cycle with a time and temperature. Multiplex PCR was able to simultaneous detect and differentiate of above mentioned parasite in a single reaction. qPCR-MCA was able to differentiate genus and species those five protozoa using melting temperature simultaneously at the same time and temperature programs. In total, qPCR-MCA diagnosed 7/11 isolation of *E. histolytica*, 6/8 isolation of *E. dispar*, 1/1 *E. moshkovskii* Laredo, 10/11 *G. lamblia* and 6/11 *Cryptosporidium* spp. Application of multiplex PCR for detection of more than one species in a test in developing countries, at least in reference laboratories has accurate diagnosis and plays a critical role in differentiation of protozoan species. Multiplex PCR assay with a template and multi template had different results and it seems that using a set of primers with one template has higher diagnostic capability in compare with multi template. The results of this study showed that the use of the qPCR-MCA can be an effective method to simultaneous distinguish of the above mentioned parasites.

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1. Introduction

Nowadays diarrhea is one of the most common health problems worldwide and it has considerable outbreak in developing and developed countries. It is one of the leading causes of morbidity and mortality of millions of people. The World Health Organization (WHO) has introduced diarrhea as a second leading cause of death for children less than five years old (World Health Organization, 2013). The main cause of diarrhea in humans is infectious agents such as viruses, bacteria and parasites. In particular, in communities that have low health standards, the role of parasites such as *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum* in the creation of diarrheal disease is remarkable (Allen and Ridley, 1970; Kosek et al., 2003). Accurate and rapid diagnosis of infectious agents causing diarrhea, such as *E. histolytica*, *G. lamblia* and *Cryptosporidium* plays a prominent role in the strategy for the prevention and treatment of the associated infection. Microscopic
techniques are useful and their application is mandatory for diagnosis of the majority of the intestinal parasites. But the sensitivity of the microscopic method is estimated about 60 percent (Haque et al., 1998). Meanwhile, in spite of considerable diagnostic sensitivity and specificity of molecular detection methods, there are some problems in routine usage of these methods in medical laboratories, such as the need of specialized equipment, trained personnel and high cost. However, the simultaneous detection of more than a parasite by multiplex PCR is suggested very efficient (Hamzah et al., 2006; Khairnar and Parija, 2007). Adopting such an approach in developing countries, at least in reference laboratories, lead to cost reduction and ensure timely and accurate diagnosis of infectious agents associated with diarrhea. (Nazeer et al., 2013).

Concerning the importance of pathogen protozoan parasite E. histolytica and necessity to differentiate it from two similar protozoa E. dispar and E. moshkovskii, and also due to the prevalence of G. lamblia and Cryptosporidium in Iran, in this study, multiplex PCR and real-time PCR techniques were examined in diagnosis of the mentioned protozoa.

2. Material & methods

2.1. Parasite preparation and DNA extraction

Twenty DNA templates from twelve E. histolytica and eight E. dispar samples were analyzed. All the twelve E. histolytica DNAs were previously extracted from stool of infected Japanese patients (Haghighi et al., 2003). The DNA of eight E. dispar was also extracted previously from stool of infected Iranian patients (Nazemalhosseini-Monjariad et al., 2012). Extracted DNA from E. histolytica HM-1: IMSS, E. dispar SAW760, and E. moshkovskii Laredo were used as positive controls in multiplex PCR and real-time PCR. These species were maintained alive in liquid nitrogen tank in Department of Medical Parasitology and Mycology, Shahid Beheshti University of Medical Sciences, and were recovered in TYS-33 medium. Forty tested positive stool samples were used for G. lamblia (twenty) and Cryptosporidium spp. (twenty) in microscopic examination of direct smear, formalin-ether concentration and stained smears with modified acid-fast staining procedure, respectively. Also twenty five negative tested stool samples in microscopic examination (modified acid-fast staining) and conventional PCR were used as negative controls. To this end, three stool samples from each patient were examined three times within 10 days. Genomic DNA was extracted from samples using the QIAamp DNA stool mini kit (QIAGen, Germany). DNA extraction was done according to the manufacturer’s recommendation.

2.2. Multiplex PCR

Multiplex PCR was performed for detection and differentiation of E. histolytica/E. dispar/E. moshkovskii. Forward primer was derived from a conserved region of the small-subunit rRNA gene, and reverse primers were designed from signature sequences specific to each of these Entamoeba species (Table 1) (Hamzah et al., 2006). Also, we used the multiplex PCR for detection of E. histolytica, G. lamblia and Cryptosporidium spp. For this purpose, the cysteine protease 8, cysteine protease 6 and small ribosomal subunits genes were selected for E. histolytica, G. lamblia and Cryptosporidium spp., respectively (Table 2) (Bairami Kuzehkhanan, 2012). Multiplex PCR was performed using Amplicone (Multiplex Master Mix, Bioran) as a ready-made solution. The reaction components and amplification of each species–specific DNA fragment are presented in Table 3. Amplified products were visualized after electrophoresis on 2% agarose gels by ETBr staining.

2.3. Quantitative PCR assay (qPCR-MCA)

In this study, a quantitative PCR assay with melt curve analysis (qPCR-MCA) was used for detection of E. histolytica/E. dispar/E. moshkovskii, G. lamblia and Cryptosporidium spp. using Corbet Rotor Gene 6000 (Corbet life science, Australia). The genomic DNA was extracted from E. histolytica, E. dispar, E. moshkovskii Laredo, G. lamblia trophozoite grown cells in medium culture. In this assay, DNA of Cryptosporidium oocyst which was confirmed by sequencing was used as positive control. In the present study of analysis of the melting curve (Cm), we have used the increment of one degree from 70 to 95 and then the fluorescence recorded at each temperature. The cysteine protease 8, cysteine protease 6, peroxiredoxin and small ribosomal subunits genes were selected for E. histolytica, G. lamblia, E. moshkovskii and E. dispar, Cryptosporidium spp., respectively (Table 4). Summary of the reaction is listed in Table 5.
2.4. Ethics statement

This study involving human subjects is in accordance with the Helsinki declaration of 1975 as revised in 2000, and it has been approved by and carried out under the guidelines of the Ethical Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

3. Results

3.1. Multiplex PCR

The multiplex PCR was carried out with a combination of forward primer (EntaF) and reverse primers (EhR, EdR, EmR) in a reaction using DNA samples from *E. histolytica* (HM1:1MSS), *E. dispar* (SAW760), *E. moshkovskii* Laredo, as well as a mixture of DNA from the three amoeba based on amplification targeting 18S rRNA gene. Analysis of PCR products on agarose gel showed three

![Fig. 1. Multiplex PCR fragments amplified using EntaF primer combined with EhR, EdR, and EmR reverse primers in a single reaction by using extracted DNA samples from 1) *E. histolytica* (HM1:1MSS), 2) *E. dispar* (SAW760) and 3) *E. moshkovskii* Laredo strain, 4) *E. histolytica* (166 bp), *E. moshkovskii* (580 bp) and *E. dispar* (752 bp) (This lane is the result of PCR electrophoresis of above primers mixed three amoeba species DNAs in a reaction). Lane M, molecular weight marker (100-bp ladder); lane N, negative control.](image1)

![Fig. 2. Multiplex PCR fragments amplified using EHCP-S1, GLCP-S1, Cry-18S-S1 primers combined with EHCP-AS1, GLCP-AS1, Cry-18S-AS1 reverse primers in a single reaction by using extracted DNA samples from 1) Cryptosporidium spp. (240 bp), *G. lamblia* trophozoite (463 bp), *E. histolytica* (HM1:1MSS) (605 bp) (This lane is the result of PCR electrophoresis of above primers mixed three protozoa DNAs in a reaction), 2) *E. histolytica*, 3) *G. lamblia*, 4) Cryptosporidium spp. Lane M, molecular weight marker (100-bp ladder); lane N, negative control.](image2)
different bands of 166 bp, 752 bp and 580 bp for *E. histolytica*, *E. dispar* and *E. moshkovskii*, respectively (Fig. 1). Likewise forward primers (EHCP8-S1, GLCP6-S1, Cry18S-S1) in combination with reverse primers (EHCP8-AS1, GLCP6-AS1, Cry18S-AS1) amplified a 605-bp PCR product for *E. histolytica*, a 463-bp for *G. lamblia*, and a 240-bp with *Cryptosporidium* spp. (Fig. 2). Seven trials of multiplex PCR with forward primers (EntaF, GLCP6-S1, Cry18S-S1) in combination with proper reverse primers (EhR, EdR, EmR, GLCP6-AS1, Cry18S-AS1) amplified 166-bp, 580-bp, 752-bp, 463-bp and 240-bp for *E. histolytica*, *E. moshkovskii*, *E. dispar*, *G. lamblia* and *Cryptosporidium* spp., respectively (Fig. 3). Meanwhile no band was detected in multiplex PCR reaction using negative fecal samples DNA.

### 3.2. Quantitative PCR assay (qPCR-MCA)

qPCR-MCA using forward primers (EHCP8-S1, EntaF, EhdmF, GLCP6-S1, Cry18S-S1), reverse primers (EHCP8-AS1, EdR, EhdmR, GLCP6-AS1, Cry18S-AS1) and SYBR Green was performed on DNAs of *E. histolytica* (HM1:1MSS), *E. dispar* (SAW760), *E. moshkovskii* Laredo, *G. lamblia* trophozoite from medium culture and *Cryptosporidium* DNA, respectively. In this study, the threshold cycles (Ct) for each of the five protozoan were 36.02, 28.62, 25.79, 21.56, 19.36 (Fig. 4) and melting curve analysis (MCA) showed the melting temperature (Tm) of 81.2, 84.7, 88.2, 62.8 (Fig. 5), respectively. In total, qPCR-MCA diagnosed 7/11 isolation of *E. histolytica*, 6/8 isolation of *E. dispar*, 1/1 *E. moshkovskii* Laredo, 10/11 *G. lamblia*, 6/11 *Cryptosporidium* spp. Given that the qPCR-MCA curves observed at temperatures below 78 °C with an irregular shape, nonspecific, so clearly distinguished from true positive cases.

### 4. Discussion

In order to increase the chances of detection and identification of parasites molecular methods has been used increasingly (Tavares et al., 2011). The exact identification of the parasite in a population causes the awareness of a real outbreak. In recent years, several molecular methods have been introduced and evaluated for identification of *E. histolytica*/*E. dispers*/ *E. moshkovskii* (Huston et al., 1999; Nunez et al., 2001; Tanyuksel and Petri, 2003; Ali et al., 2003; Parija and Kharinar, 2005; Roy et al., 2005; Fallah et al., 2014). The primary aim of this study was to demonstrate the application of the multiplex PCR assay for detection and identification of *E. histolytica*/ *E. dispers* and *E. moshkovskii*. We also developed a multiplex PCR assay for the simultaneous detection of *E. histolytica*, *G. lamblia* and *Cryptosporidium* in human fecal samples. Our method was also used
Fig. 4. Specific amplification chart of *E. histolytica* (HM1: MISS), *E. dispar* (SAW 760), *E. moshkovskii* Laredo, *G. lamblia* and *Cryptosporidium* spp.*.

*E. histolytica, E. dispar, E. moshkovskii and G. lamblia* DNAs were from trophozoites in medium culture.

Fig. 5. Melting curve analysis charts for *E. histolytica* (HM1: MISS), *E. dispar* (SAW 760), *E. moshkovskii* Laredo, *G. lamblia* and *Cryptosporidium* spp.*.

*E. histolytica, E. dispar, E. moshkovskii and G. lamblia* DNAs were from trophozoites in medium culture.

Table 5

| Body of qPCR-MCA and reaction components for *E. histolytica*/*E. dispar*/*E. moshkovskii*, *G. lamblia* and *Cryptosporidium* spp. |
|------------------|------------------|
| **qPCR-MCA** | **Reaction mixture** |
| | Master Mix | SYBR Green (Amplicon) |
| | | Forward & revers primers (10 pmol/μL) |
| | ddH2O | DNA (5–10 ng) |
| | | Total volume |
| | | Initial denaturation |
| | | Denaturation Temperature |
| | | Annealing Temperature |
| | | Extension Temperature |
| | | Temperature time |
| E. histolytica | 10 | 1 | 7 | 1 | 20 | 95 °C | 15 min | 95 °C | 15 s | 55 °C | 35 s | 72 °C | 45 s | 70 °C – 95 °C |
| E. dispar | | | | | | | |
| E. moshkovskii | | | | | | | |
| G. lamblia | | | | | | | |
| *Cryptosporidium* spp. | | | | | | | |

Our data confirm the effectiveness of using qPCR-MCA for detection and differentiation of the above mentioned parasites on positive microscopic samples and controls (Table 6). Several studies have used multiplex real-time PCR for diarrhea-causing protozoan parasites such as *E. histolytica*, *G. lamblia* and *Cryptosporidium* (Verweij et al., 2004; Nazeer et al., 2013). But qPCR-MCA requires only a single channel and does not use probes which are more cost-effective and easier to use compared to multiplex real-time PCR. Although this assay is able to differentiate genus and species using Tm but in order to get high resolution data, specific software needed for sequencing and melting curve analysis.

for synchronic detection of the five protozoa in one reaction in several experiences. Findings of this study and several reports by other researchers indicate that multiplex PCR is able to detect and differentiate *E. histolytica*/*E. dispar*/*E. moshkovskii* (Hamzah et al., 2006; Khairnar and Parjia, 2007; Ten Hove et al., 2007; Ngui et al., 2012; Fallah et al., 2014). This study showed that multiplex PCR using set of primers with a template for detection of the parasites have better results in comparison with several sets of primers with several templates (Table 6). It seems that the main reasons for this difference are the spatial limitations as well as materials division of multiplex PCR for several PCR.
Table 6
Summary of the data of multiplex PCR with a template and multi template, real-time PCR using SYBR Green.

<table>
<thead>
<tr>
<th>Gene (Primer)</th>
<th>Parasite</th>
<th>Multiplex PCR (single template)</th>
<th>Multiplex PCR (multi template)</th>
<th>Real-time PCR</th>
<th>SYBR Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene (Primer)</td>
<td></td>
<td>185 rRNA, CP6 (Entaf, Ehr, Edr, Emr, GLCP-51, GLCP-AS1, Cry-S1-Cry-AS1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. histolytica</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>E. dispar</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>E. moshkovskii</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>G. lamblia</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

In general, according to the survey results, multiplex PCR can be an effective way of detecting and differentiating E. histolytica, E. dispar and E. moshkovskii in human clinical specimens. Concerning the inability of microscopic methods to differentiate the three species of amoeba and time-consuming culture, followed by isoenzyme analysis to differentiate them, clearly, we conclude that (or we suggest that) multiplex PCR and similar methods are basically more suitable than microscopic and/or culture method in the detection and differentiation of E. histolytica, E. dispar and E. moshkovskii. Real-time PCR compared to microscopic method has advantages such as sensitivity, ease of use, ability to analyze large numbers of samples. The test has advantages compared to the multiplex PCR as; no need for analysis after amplification which reduce the risk of contamination, ability to better differentiate the protozoan parasite and easy interpretation compared to examine the stained gel in conventional PCR. This method can be a simple and valuable tool for rapid and reliable laboratory diagnosis of parasitic protozoa.

References