Acta Tropica 162 (2016) 233–238

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

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

Nozhat Zebardast[a,*], Farshid Yeganeh[b], Mohammad Javad Gharavi[c], Alireza Abadi[d], Seyyed Javad Seyyed Tabaei[e], Ali Haghighi[e,*]

[a] Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[b] Department of Immunology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Parasitology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
[d] Department of Community and Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Deptartment of Parasitology and Mycology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 13 June 2016
Accepted 11 July 2016
Available online 14 July 2016

Keywords:
Simultaneous detection and differentiation
Entamoeba spp.
Giardia lamblia
Cryptosporidium spp.
Multiplex PCR
QPCR-MCA

ABSTRACT

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.

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

* Corresponding authors.
E-mail addresses: Nszebardast@gmail.com (N. Zebardast), Ahaghighi11@yahoo.com, haghighi@sbmu.ac.ir (A. Haghighi).

http://dx.doi.org/10.1016/j.actatropica.2016.07.004
0001-706X/

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-Mojarad 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 TYI-S-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 Kuzehkanan, 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, peroxyredoxin 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.

Table 1
Summary of oligo nucleotide primers used in multiplex PCR for detection and differentiation of *E. histolytica/E. dispar/E. moshkovskii* (5).

Protozoa parasites	Gene	Primer name and sequence	Amplicon size	Melting temperature	Accession number
E. histolytica/E. dispar/E. moshkovskii	18SrRNA	EntaF: 5′-ATGCACGAGAGCGAAAGCAT-3′		57 °C	
E. histolytica		EhR: 5′-GATCTAGAAACAATGCTTCTCT-3′	166 bp	46 °C	X64142
E. dispar		EdR: 5′-CACCACTTACTATCCCTACC-3′	752 bp	45 °C	Z49256
E. moshkovskii		EntaF: 5′-TGACCGGAGCCAGAGACAT-3′	580 bp	55 °C	Af149906

Table 2
Summary of oligo nucleotide primers used in multiplex PCR for detection and differentiation of *E. histolytica*, *G. lamblia*, *Cryptosporidium* (10).

Protozoa parasites	Gene	Primer name and sequence	Amplicon size	Melting temperature		Accession number
E. histolytica	CP8	EHCP-S1: 5′-ATTTGTTAAGTATTGTAAATGGG −3′ EHCP8-AS1: 5′-ATTGTAACCTTTCATTGTAACAT-3′	605 bp	47 °C	46.9 °C	AY156066
G. lamblia	CP6	GLCP6-S1: 5′-AATCTGTTGACTTAAGGGAGTA −3′ GLCP6-AS1: 5′-ATTGATCATTATAGGGATTGT-3′	463 bp	46.7 °C	46.3 °C	XM_001706220
Cryptosporidium SPP.	18S rRNA	Cry18S-S1: 5′-TAAACGGTAGGGTATTGGCCT-3′ Cry18S-AS1: 5′-CAGACTTGCCCTCCAATTGATA-3′	240 bp	54.2 °C	55.5 °C	GQ259149