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## Molecular identification of *Entamoeba histolytica* from stool samples of Ilam, Iran

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### ABSTRACT

**Introduction:** Amoebiasis is a multifactorial, life-threatening public health issue and the third parasitic disease cause of mortality in worldwide, particularly in low- and mid-income countries. The aim of this study was to attempt to explore genetic encoding differences of CP8 (conserved gene) of *Entamoeba histolytica/Entamoeba dispar* in its various infectious properties isolated from Ilam located at a southwest part of Iran.

**Materials and methods:** A total of 2023 stool samples were collected between 2016 and 2018 from the hospital in Ilam, of which only 30 isolates were identified as *E. histolytica/E. dispar*. These isolates were collected from the intensive care unit, infectious disease, and surgery settings. The isolates were identified and the polymerase chain reaction (PCR) was performed to detect the CP8 gene. In all stages, *Entamoeba histolytica* HM1: IMSS was used as a positive control.

**Results:** In genotype confirmation, only two isolates had the CP8 gene found in the PCR technique. The sequencing results confirmed the mentioned gene with 99%–100% specificity.

**Conclusion:** It is concluded that PCR is highly sensitive to detect *E. histolytica* and indicating this important role as screening tools in direct DNA extraction from stool samples and valuable technique in early detection of symptomatic and asymptomatic *E. histolytica* patients.

### 1. Introduction

Diarrhea is a multifactorial, life-threatening public health issue and the second cause of mortality in young children, particularly in lower-income and middle-income countries [1]. Several factors such as infectious agents and nutritional deficiencies contribute to the incidence of diarrhea [2,3]. *Entamoeba histolytica* is an intestinal protozoan parasite which is mainly transmitted by infect food [4] and water and is considered as one of the major causes of diarrhea [5]. It has been documented that *E. histolytica* third parasitic disease cause of mortality in worldwide, particularly are the most common in tropical and subtropical areas and industrialized countries, Approximately 50 million people around the world suffer from *E. histolytica* infection (Amoebiasis) with over 40,000 to 100,000 million rates of mortality [6,7]. In a systematic review and meta-analysis study by Ali Haghghi et al in 2018 regarding Amoebiasis in Iran, it was shown that *Entamoeba dispar* is the most commonly detected and non-pathogenic agent. Also, these findings are indicative of a low burden of amoebiasis in Iran [8].

Amoebiasis also known amoebic dysentery colonization of the large bowel, featuring severe abdominal pain chronic intestinal amoebiasis, amoebic hepatitis and amoebic liver abscess (ALA), bloody diarrhea, pyrexia, colitis and peritonitis, [9] Hence, devising an easily, rapid and non-invasive diagnostic test for accurate detection of *E. histolytica* is an important step in prevention strategies [9,10]. However, Due to the low sensitivity of the microscopic method in the detection of *E. histolytica* is estimated about 60%, Therefore, we aimed to perform this investigation in order to discern the molecular prevalence of CP8 of *E. histolytica* isolated from diarrheal patients in central laboratory and Imam-Khomeini hospitals in Ilam, Western Iran.

### 2. Materials and methods

#### 2.1. Parasite isolation and identification

A total of 2023 stool samples were collected between 2016 and 2018, of which only 30 isolates were identified as *E. histolytica/ E.*

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*dispar*. A single fresh stool specimen was collected from each patient without fixative. The presence of parasites was determined by microscopic examination of fresh stools using direct slide smear, formalin-ether concentrated specimens and trichrome stain. In all stages, *Entamoeba histolytica* HM1: IMSS was used as a positive control.

### 2.2. *Entamoeba histolytica* cultures

Samples that were identified as *Entamoeba* in terms of the microscopic specification were cultured in a coagulated horse serum medium (Horse serum, ringer, egg + starch) for final confirmation. Using this method, it was found that 2 of the samples were *Entamoeba histolytica* and the rest of the samples were *Entamoeba dispar*. (Coagulated horse serum was used for cultures of *E. histolytica*/*E. dispar* trophozoites).

### 2.3. DNA extraction and PCR

DNA was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer’s protocol. Method and quantified with a NanoDrop device at 260–280 nm. In the next step, the polymerase chain reaction (PCR) technique was done to amplify the *CP8* gene using the specific primers (Table 1), as follows: 1- primary denaturation step at 95 °C for 5 min, 2–35 cycles of secondary denaturation at 95 °C for 1 min, annealing at 56.7 °C for 1 a min and primary extension at 72 °C for 30 s, and 3- final extension at 72 °C for 5 min. Then, the PCR products were electrophoresed on a 2% (w/v) agarose gel and the amplified 86 bp band was observed in a gel-doc system under ultraviolet illumination.

### 2.4. Statistical analysis

Obtained information was analyzed by ANOVA and chi-squared statistical tests using SPSS® software. A significant level of 0.05 ≥ was considered for *P* value.

## 3. Results

### 3.1. Results of morphological identification of the isolated intestinal protozoa

The results of this study showed that among the 2023 patients, the age of patients ranged < 1 to 89years is 975 (48.2%) males and 1045 (51.7%) female patients. A total of 620 (89%) patients positive for intestinal protozoa, the highest prevalent species was related to the *Blastocystis hominis* (67.1%), The frequency of the *Giardia lamblia*, *E. coli*, *E. histolytica*/*E. dispar*, *Endolimax nana*, and *Iodamoeba bütschlii* was (10.%), (8.1%), (4.8%), (4.2%) and (5.8%) isolates, respectively. The prevalence of intestinal parasites is shown in Fig. 1.

### 3.2. Molecular characterization of the isolated *E. histolytica*

Among 30 collected isolates from diarrheal patients, only two were recognized to be positive regarding the *CP8* gene. The results of *CP8* gene found in the PCR are shown in Fig. 2.

## 4. Discussion

Parasitic diarrhea is usually caused by various parasites that are

**Table 1**

The primers used for the detection of *CP8* gene and product length of this gene.

Primer name	Sequence (5’ to 3’)	Product length
CP8-E1, Forward	5'-ACGAAGTATCAGCAGTTGGGT-3'	86 base pair
CP8-E2, Reverse	5'-CTCTCCCGCATGTGTTCCTCC-3'	

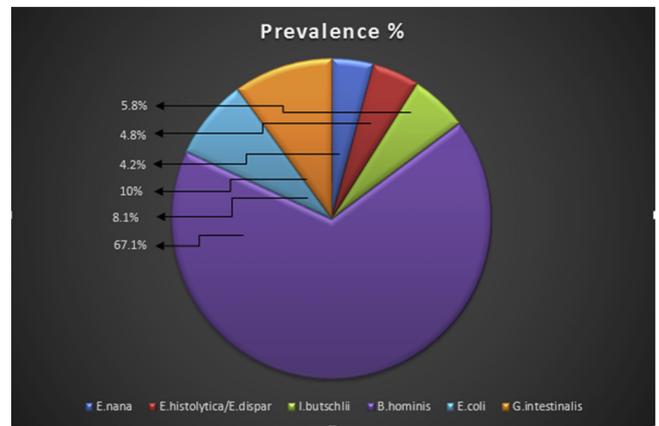


Fig. 1. Microscopy examination result for prevalence of intestinal parasites.

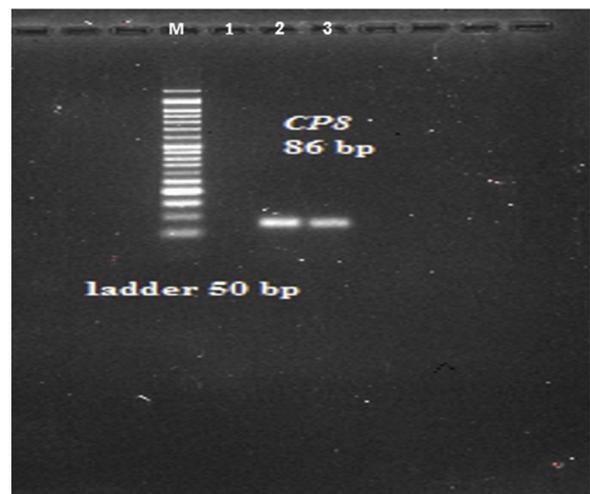


Fig. 2. PCR result for *Entamoeba histolytica*. M: marker (50 bp); 1- negative control; 2- positive control (*Entamoeba histolytica* HM1: IMSS); 3- *E. histolytica* isolate with *CP8* gene.

non-detectable [11]. One of the most important causes of this parasite diarrhea is *E. histolytica* which is responsible for nearly 10,000 deaths each year [12]. Since the species of *E. histolytica* and *E. dispar* stage are morphologically the same in the trophozoites stage and in the cyst, the microscopic method cannot distinguish between these two species [13]. For this reason, the use of molecular methods for the isolation of *E. histolytica* from *E. dispar* and the exact diagnosis of the disease is necessary to unnecessary prevent and consumption of antiprotozoal drugs and prevent drug resistance [14,15]. Microscopic tests and culture in media are used to detect *E. histolytica* and *E. dispar*, but these methods are not suitable for differentiating these two parasites, so using molecular methods such as PCR to differentiate them was used. One of these methods is the use of the *CP8* gene, which has *E. histolytica*, and the isolates of *E. dispar* lacking this gene. Therefore, using a simple method such as PCR, these two parasites can be easily differentiated from each other.

In recent years, PCR has been widely used to diagnose all types of infectious diseases, including parasitic diseases [10,13]. In this study, we also used this method to detect *E. histolytica*. In the present study, the results of a microscopic study were compared with PCR results using *CP8* gene. Of the 30 samples that were identified as *E. histolytica*/*E. dispar* in a microscopic study, only were two samples (3.35%) detected by PCR of *E. histolytica* and the rest were known as non-pathogenic *E. dispar*. In a study by Hoshyar et al. for the isolation of *E. histolytica* from *E. dispar* in the central regions of the country by PCR, the predominant species of *E. dispar* was reported [16] which is consistent

with the present study. Nazem alhosseini et al. conducted a molecular study of 22 samples of cysts and/or trophozoites excisers in Tehran, and all reported to be diagnosed with *E. dispar* and only one isolates had for *E. histolytica* found in the PCR test [15] which is very similar to our study, and most of the samples were related to *E. dispar*. In 2004–2005, Haghighe et al. examined 450 patients with symptomatic symptoms of dyspepsia referred to Taleghani hospital in Tehran microscopic studies and reported 5 cases of *E. histolytica* or *E. dispar*, Using PCR, four of them were detected by *E. dispar*, and one of the positive samples was not exposed to PCR [17]. In a similar study by Maria Teresa Nascimento Silva in Brazil in 2014. 456 specimens were evaluated using *E. histolytica* II® ELISA kit for the study of *E. histolytica*. However, the results showed that adhesin of *E. histolytica* was not detected and samples related to non-pathogenic *E. dispar* [18]. According to researchers, a small number of contaminated infections are caused by a high level of personal and general health in the study area, which is contrasted with the relatively large number of contamination cases in the present study, although the determination of the species of *E. dispar* as the predominant species is in line with the present study.

## 5. Conclusion

It is concluded that PCR is highly sensitive to detect *E. histolytica* and indicating this important role as screening tools in direct DNA extraction from stool samples and valuable technique in early detection of symptomatic and asymptomatic *E. histolytica* patients.

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## Authors' contributions

RN and AK were involved in designing and supervising in interpreting the data; AN and AG was involved in the collection of data; RN supervised all steps of the study. RN, AK, AM, JA and AN wrote the manuscript. ME critically revised the manuscript. All authors have read and approved the final manuscript.

## Compliance with ethical standards

The study was approved by the Ilam University of Medical Sciences Ethics Committee and registered with NO: EC/92/H/132.

## Conflict of interest

The authors declare that they have no competing interests.

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