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Entamoeba spp. diagnosis in patients with inflammatory diarrhea by staining, copro-antigen ELISA and multiplex PCR methods

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ABSTRACT

Objective: To evaluate *Entamoeba* spp. diagnosis in patients with inflammatory diarrhea by staining, copro-antigen ELISA and multiplex PCR methods.
Methods: In this descriptive cross-sectional survey, 200 stool samples were randomly collected during 2015–2016. The stool samples were evaluated microscopically for the presence of the parasite using direct and formalin-ether concentration and trichrome staining methods. Then, the stool samples were examined by copro-antigen ELISA (Biomerica Company) and multiplex PCR methods.
Results: Of 200 samples, 17, 29 and 23 cases were positive for *Entamoeba* species by the staining, copro-antigen ELISA and multiplex PCR methods, respectively. Of 23 positive samples in multiplex PCR test, 13 and 10 samples were positive for *Entamoeba dispar* (*E. dispar*) and *Entamoeba histolytica* (*E. histolytica*), respectively.
Conclusions: Our finding indicated a relatively high prevalence of *Entamoeba* species in patients with inflammatory diarrhea in Ahvaz city. Due to the complications of *E. histolytica/dispar* infection, the health authorities of the city must pay more attention to control and prevent the transmission of *E. histolytica/dispar* to individuals.

1. Introduction

Intestinal parasitic infections (IPIs) have the high prevalence at around the world, especially in developing countries[1]. Approximately, one-third of the people of the world (more than two billion people) are in the influence of IPIs. About 450 million people suffer from IPIs that at least 50% of these individuals are children. Lack of health care, the tropical wet climate, lack of access to safe drinking water, poverty, and illiteracy are some factors associated with IPIs[2,3]. In recent years, the prevalence of IPIs has been reported in different areas of Iran[4-7]. *Entamoeba histolytica/dispar* (*E. histolytica/dispar*) is known as the second factor that causing death among individuals with parasitic diseases. The parasites are one of the main causes of threatening individual health, especially in travelers[8]. Studies have been reported that *E. dispar* and *E. histolytica* are morphologically similar but genetically and biochemically are different that only *E. histolytica* is causing of the disease[9]. Also, 90% of *E. histolytica* infections and all *Entamoeba moshkovskii* and *E. dispar* infections are asymptomatic that *E. histolytica* infection can lead to amebiasis that is defined as extra intestinal or invasive intestinal, asymptomatic disease. The infections are one of the most predominant parasitic infections worldwide that can be leading to 40 000–100 000 deaths per year and infecting approximately fifty million individuals, especially in developing countries[9].

In a systematic review and meta-analysis study, in Iran during 1988–2009, Karambaigi *et al.* showed that the prevalence of *E. histolytica*/dispar was observed 1.3% (2.5% and 0.8% at rural and urban areas, respectively)[10]. *E. histolytica/dispar* infections are one of the serious concerns of public health, especially in the tropical and subtropical areas. On the other hand, in summer season, Ahvaz city has a temperature of about 50 ℃. Thus, Ahvaz has the tropical and subtropical climates. Hence, due to the climatic and ecological conditions in the city, evaluation of the prevalence rate of *E. histolytica/dispar* infections was essential. Therefore, the aim of this study was to evaluate *Entamoeba* spp. diagnosis in patients with inflammatory diarrhea by staining, copro-antigen ELISA and

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multiplex PCR methods.

2. Materials and methods

2.1. Study area

Ahvaz is a city in the center of Khuzestan Province in southwest of Iran. The city is 375 square km and its population has been reported as 1 425 891 until 2006. The city has a desert climate with temperatures above 50 ℃ that is one of the warmest cities in the world. The average annual rainfall is about 230 mm. According to report by the World Health Organization, Ahvaz has the most polluted climate in the world[11].

2.2. Sample collection

In this descriptive cross-sectional survey, the study population was individuals referred to the teaching hospitals and health centers of Ahvaz city under Ahvaz Jundishapur University of Medical Sciences during 2015–2016. At first, the purpose and nature of the study were told to the referred individuals. Then, 200 stool samples (83 from females and 117 from males) were randomly collected among them. The data such as sex, age, and disease symptoms were collected using the questionnaire[6,11].

2.3. Stool examination and trichrome staining

The stool specimens (1–3 g) were collected in labeled plastic vials without any preservatives and were tested immediately (less than 60 min after collecting). Then, we macroscopically evaluated the consistency of stool samples, the presence of mucus, blood and the worm parasites. Finally, the stool samples were microscopically evaluated for the presence of the parasite trophozoites, cysts, and eggs using the direct and formalin-ether concentration methods. Briefly, 0.5–1.5 g stool samples were mixed with 4 mL formol saline. After filtering, diethyl ether was added and mixed once again. After centrifuging, the sediment was stained with trichrome and was examined by light microscope (40× magnification)[11-13].

2.4. Copro-antigen ELISA

The stool samples were examined by copro-antigen ELISA (*E. histolytica/dispar* ELISA kit from Biomerica company) method[14,15].

2.5. Extraction of DNA

The DNA was extracted by the DNA extraction kit for stool (Bioneer) and the extracted DNA was stored at –20 ℃. This kit contained spin column that the parasite DNA was absorbed by the column and after twice washing with special buffers, the purified DNA was obtained.

2.6. Molecular analysis

The extracted DNA was examined by multiplex PCR. We used from the specific primers that including EntaF, 5'-ATGCACGAGAGCGAAAGCAT-3', EhR, 5'-GATCTAGAAACA ATGCTTCTCT-3', EdR, 5'-CACCACTTACTATCCCTACC-3' and EmR, 5'-TGACCGGAGCCAGAGACAT-3' for the diagnosis *Entamoeba* spp. [16]. These primers were designed based on small subunit ribosomal RNA (16S rRNA) gene that was used at the identification of all the different species of *Entamoeba*. The primers were purchased from Bioneer Company and were stored at –20 ℃. After molecular analysis, the amplified products of multiplex PCR were observed by the ethidium bromide staining after electrophoresis on agarose gels 1.5%[16].

2.7. Statistical analysis

SPSS statistical software of version 16 was used for the data analysis and *Chi*-square test for significance differences. The *P* value less than 0.05 was considered significant.

3. Results

Table 1 shows the symptoms of patients with inflammatory diarrhea in Ahvaz, southwest of Iran. Of 200 samples, 17, 29 and 23 cases were positive for *Entamoeba* species by the staining, copro-antigen ELISA and multiplex PCR methods, respectively. Of 23 positive samples in multiplex PCR test, 13 and 10 samples were positive for *E. dispar* and *E. histolytica*, respectively. On the other hand, among infected men and women significantly differences were seen and no significant differences were observed between vomiting, fever and abdominal pain with *Entamoeba* species infected patients ($P > 0.05$). Only, significant difference was observed between diarrhea and *Entamoeba* species infected patients ($P = 0.027$).

Table 1
The symptoms of patients with inflammatory diarrhea in Ahvaz, southwest of Iran.

Symptoms	Yes/No	Numbers	Percentage
Diarrhea	Yes	160	80
	No	40	20
Vomiting	Yes	72	36
	No	128	64
Fever	Yes	32	16
	No	168	84
Abdominal pain	Yes	104	52
	No	96	48

4. Discussion

IPIs are one of the serious concerns of the public health in several countries, in particular in the tropical and subtropical developing countries. The infections have been seen mainly in children. The prevalence of IPIs in each community is indicator of the health status of the area. Some environmental factors such as geographical location, climate, poverty, inadequate health conditions and economic situation, as well as personal factors such as nutrition, safety conditions, health status, cultural habits, literacy, and the high density of population facilitate the prevalence of IPIs[3,17]. Several evaluations were conducted in the different areas of Iran that showed the high prevalence of IPIs[4-7]. In the study, we demonstrated that of 200 samples, 17, 29 and 23 cases were positive for *Entamoeba* species by the staining, copro-antigen ELISA and multiplex PCR methods, respectively. These results were consistent with the conducted study by Koohsar *et al.* in Gorgan Province in 2013[18]. Also, in a systematic review and meta-analysis study, in Iran during 1988–2009, Karambaigi *et al*. showed that the prevalence

of *E. histolytica/dispar* was observed 1.3% (2.5% and 0.8% at rural and urban areas, respectively)[10].

Copro-antigen ELISA and the microscopic analysis of stool are appropriate screening methods for the identification of *Entamoeba* infections among patients with diarrhea. Due to the incapability of the initial screening methods for detection of *E. dispar* and *E. histolytica*, the confirmatory test of PCR is recommended. Serological methods are a appropriate indicator for *E. histolytica*; but occasionally, cross-reactions with *E. dispar* parasite are possible[19]. The cognition of *E. dispar* and *E. histolytica* has provided significant insights into the epidemic behavior of amoebiasis[20]. Hence, we indicated that of 23 positive samples in multiplex PCR test, 13 and 10 samples were positive for *E. dispar* and *E. histolytica*, respectively. Unlike our results, previous studies indicated that *E. dispar* was approximately ten times more common in stool samples than *E. histolytica* [19,21].

In the study, the prevalence of *E. histolytica/dispar* infections in males was higher than females. This difference can be explained by sample size, number of individuals referred to the hospital. Overall, the differences of present study with other studies may be attributed to number of study population, cultural habits of the region, methodology, type of sampling, occupations, sanitary status, geographical location and many other factors. Our findings showed that still the prevalence of *E. histolytica/dispar* infections is relatively high and it can be the serious risk threatening the public health, especially, in children. Therefore, the improvement of the life style of people is essential that its results can be leading to preventing the risk of *E. histolytica/dispar* infections. In conclusion, our finding indicated a relatively high prevalence of *Entamoeba* species in patients with inflammatory diarrhea of Ahvaz city. Due to the complications of *E. histolytica/dispar* infection, the health authorities of the city must pay more attention to control and prevent the transmission of *E. histolytica/dispar* to individuals.

Conflict of interest statement

We declare that we have no conflict of interest.

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