*Epidemiology and Infection*

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# Differential detection of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* in faecal samples using nested multiplex PCR in west of Iran

Fares Bahrami[1,2], Ali Haghighi[2], Ghasem Zamini[3] and Mohammadbagher Khademerfan[3,4]

[1]Zoonoses Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran; [2]Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [3]Department of Parasitology and Mycology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran and [4]Cellular and Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran

## Original Paper

**Cite this article:** Bahrami F, Haghighi A, Zamini G, Khademerfan M (2019). Differential detection of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* in faecal samples using nested multiplex PCR in west of Iran. *Epidemiology and Infection* **147**, e96, 1–7. https://doi.org/10.1017/S0950268819000141

Received: 12 February 2018
Revised: 20 January 2019
Accepted: 20 January 2019

**Key words:**
*Entamoeba* complex; differential detection; Iran; nested multiplex PCR

**Author for correspondence:**
Ghasem Zamini, E-mail: kurdistan_parasitologist@yahoo.com

## Abstract

This study aimed to determine the prevalence of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* (collectively referred to as *Entamoeba* complex), using microscopic and molecular methods in Kurdistan Province, northwest of Iran. The relationship between positive *Entamoeba* species and clinical symptoms was also investigated. Eight positive *Entamoeba* complex, as well as four *Entamoeba* complex-like isolates, were detected by microscopic stool examination. DNA was extracted from all positive and from 55 randomly selected negative stool samples. PCR was performed using species-specific 18S rRNA primers for the *Entamoeba* complex. All positive PCR samples were sequenced. In total, 14 (1.01%) out of 1383 isolates, i.e. 12 microscopy-positive and *Entamoeba* complex-like isolates and two out of 55 microscopy-negative isolates, were identified via PCR and sequencing. Overall, 0.58% (8/1383) of the isolates were *E. dispar*, 0.14% (2/1383) *E. histolytica*, 0.07% (1/1383) *E. moshkovskii* and 0.22% (3/1383) were mixed of *E. histolytica* and *E. dispar*. Based on our findings, the prevalence of *E. dispar* is greater than that of *E. histoltyica*. On the other hand, a case of *E. moshkovskii* was reported for the first time in this region. It seems that some gastrointestinal symptoms may be attributed to *Entamoeba* species.

## Introduction

Amoebiasis, an infection caused by *Entamoeba histolytica*, is a neglected re-emerging disease, causing serious morbidity and mortality in humans [1, 2]. This infection is manifested as either commensal or pathogenic forms of intestinal parasite [1]. Although *E. histolytica*, *E. dispar* and *E. moshkovskii* are morphologically identical, the pathogenicity of *E. dispar* and *E. moshkovskii* remains unclear [3, 4].

According to reports from different parts of the world, most cases of amoebiasis are asymptomatic [5]. However, there are controversies regarding the pathogenesis of this disease. Some researchers believe that the species and strain of the parasite are involved in the pathogenesis, while some suggest that the severity of infection and host conditions can intensify the clinical symptoms [6–8].

Traditionally, laboratory detection of *Entamoeba* species in human faeces was dependent on the microscopic examination of stool samples. However, this method cannot differentiate pathogenic *E. histolytica*, commensal *E. dispar* and ubiquitous *E. moshkovskii*. Also, researchers have recently identified a new species in humans, called *E. bangladeshi*, which is highly similar to other members of the *Entamoeba* complex [9]. Therefore, molecular methods are necessary for differentiating these amoebae [10, 11].

Molecular investigations revealed that the prevalence of *E. dispar* is 10 times higher than that of *E. histolytica* worldwide [5]. So far, most molecular studies on *Entamoeba* species have used single polymerase chain reaction (PCR) assays to detect *E. histolytica* and *E. dispar*, while detection of *E. moshkovskii* has been disregarded [12]. Therefore, nested multiplex PCR assay has been developed for the rapid detection and identification of these three *Entamoeba* species [13].

According to recent studies, gastrointestinal disorders (GIDs) are caused by *E. moshkovskii*, and humans may be proper hosts for this *Entamoeba* [4, 14]. In addition, previous studies have indicated an association between *E. dispar* and clinical symptoms [15, 16]. Therefore, we designed and implemented the present study to assess the prevalence of *E. histolytica*, *E. dispar*



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and *E. moshkovskii* in Sanandaj, capital of Kurdistan Province in west of Iran, using nested multiplex PCR and to investigate the relationship between these *Entamoba* species and clinical symptoms.

## Methods

### Study setting and sampling

This cross-sectional study was conducted from June 2015 to November 2016 on 1383 individuals, attended to 14 medical laboratories in Kurdistan Province, Iran. After collecting faecal samples from the medical laboratories and completing the questionnaires, the samples were directly transferred to the Research Laboratory of the Department of Parasitology and Mycology (School of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran) for daily microscopic examinations.

### Questionnaire

A structured questionnaire was used to collect information on the following causes of referral: (i) routine evaluation (i.e. check-up and receiving a health certificate); (ii) diagnosing agents of GIDs (i.e. diarrhoea, abdominal pain, weight loss, loss of appetite, vomiting, nausea, etc.); and (iii) detecting diseases other than GIDs (e.g. cancer, diabetes, autoimmune diseases, immunodeficiency disorders, etc.).

### Microscopic examination

All faecal samples were examined to detect *Entamoeba* cysts or trophozoites, using direct wet mount examination and formalin–ether sedimentation technique under microscopic observation (Zeiss, Germany, 40× magnification). Following that, trichrome staining was performed for determining and confirming *Entamoeba* samples under high-power microscopic observation (Zeiss, 100× magnification).

All microscopy-positive isolates and those resembling the *Entamoeba* complex, in addition to 55 microscopy-positive isolates for *Entamoeba coli*, *Endolimax nana* and/or negative stool samples, were kept in 70% alcohol at 4 °C for DNA extraction and molecular analysis.

Molecular investigations were conducted in the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences (Tehran, Iran). However, due to funding limitations, we were unable to perform PCR assays for all the samples.

### Genomic DNA extraction

Almost 300 µl of faecal specimens were washed three times with triple-distilled water through centrifugation to remove any traces of alcohol. Then, genomic DNA was extracted directly from the samples, using FavorPrep® Stool DNA Isolation Mini Kit (YTA, FavorGen, Cat. No YT9032, Taiwan) with slight modifications. After adding a glass milk matrix and 1 ml of lysis buffer, the samples were frozen in liquid nitrogen and thawed at 90 °C in a water bath. The genomic DNA was then eluted in 50 µl of elution buffer and stored at −20 °C until PCR amplification.

### DNA amplification by PCR

A nested multiplex PCR assay using species-specific primers was performed to amplify the region of 18S rRNA gene for the *Entamoeba* complex. The sensitivity and specificity of this method for the detection of the *Entamoeba* complex have been examined in the literature [13]. The first pair of primers, E-1 (5′-TAAGATGCACGAGAGCGAAA-3′) and E-2 (5′-GTA CAAAGGGCAGGGACGTA-3′), was used to amplify about 900 bp of 18S rRNA gene. For the second round of nested multiplex PCR, the reaction conditions were optimised for amplifying species-specific product sizes (439, 553 and 174 bp for *E. histolytica*, *E. moshkovskii* and *E. dispar*, respectively).

The PCR assay was performed in a multiplex reaction mixture under similar conditions by combining three pairs of primers: EH-1 (5′-AAGCATTGTTTCTAGATCTGAG-3′) and EH-2 (5′-AAGAGGTCTAACCGAAATTAG-3′); Mos-1 (5′-GAAACC AAGAGTTTCACAAC-3′) and Mos-2 (5′CAATATAAGGC TTGGATGAT-3′); and ED-1 (5′-TCTAATTTCGATTAGAAC TCT-3′) and ED-2 (5′-TCCCTACCTATTAGACATAGC-3′). The primer sequences were examined for specificity by conducting Basic Local Alignment Search Tool (BLAST) searches in the National Center for Biotechnology Information (NCBI). The primers were synthesised using the Macrogen® system (South Korea). For confirmation of the multiplex PCR, single-round PCR was also carried out using the described primers. The PCR assay was repeated four times in the samples (twice by multiplex-nested PCR and twice by single-nested PCR) under similar conditions.

The first PCR reaction was performed in a final volume of 25 µl, containing 12.5 µl of 2X PCR kit master mix (Ampliqon ApS, Literbuen 11, DK-2740 Skovlunde, Denmark), 15 $\rho$M of each primer and 10 ng of extracted DNA. The second PCR reaction was performed in a final volume of 30 µl, containing 15 µl of 2X PCR master mix, 15 $\rho$M of each primer and 10 ng of the first PCR product. The reaction conditions for the second PCR were optimised to combine the primers of *E. histolytica* (EH-1 and EH-2) with *E. dispar* (ED-1 and ED-2) and *E. moshkovskii* (Mos-1 and Mos-2) primers in a single reaction mixture under the same conditions.

For the first PCR assay, amplification was carried out in a thermocycler (Techne Ltd., Cambridge, UK) at 95 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s; and a final extension at 72 °C for 5 min. In addition, nested amplification included 35 cycles at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 30 s under identical conditions for the initial denaturation and final extension.

Both positive and negative controls were included in each round of PCR to validate the results. Then, 3 µl of PCR products was electrophoresed on agarose gel 1.5%, stained with ethidium bromide and visualised under UV light. The positive control DNA was collected from axenically grown *E. histolytica* HM-1: IMSS, *E. dispar* SAW760 and *E. moshkovskii* Laredo strains. All positive control DNAs were provided by Dr Seiki Kobayashi (Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Tokyo, Japan) for A. Haghighi.

### Sequencing of PCR products

The PCR-amplified products were subjected to direct sequencing, using a BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA) and a genetic analyser (3130 × 1; ABI Prism). The sequence chromatograms were observed using

https://doi.org/10.1017/S0950268819000141 Published online by Cambridge University Press

**Table 1.** Distribution of *Entamoeba* complex according to the multiplex PCR

| | Multiplex PCR | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Positive | | *Entamoeba* complex-like | | Negative | | Total | |
| *Entamoeba* complex[a] | No. | % | No. | % | No. | % | No. | % |
| *E. histolytica* | 1 | 7.14 | 1 | 7.14 | 0 | 0 | 2 | 14.28 |
| *E. dispar* | 4 | 28.57 | 3 | 21.43 | 1 | 7.14 | 8 | 57.14 |
| *E. moshkovskii* | 1 | 7.14 | 0 | 0 | 0 | 0 | 1 | 7.14 |
| Mixed *E. histolytica*/*E. dispar* | 2 | 14.28 | 0 | 0 | 1 | 7.14 | 3 | 21.43 |
| Total | 8 | 57.14 | 4 | 28.57 | 2 | 14.28 | 14 | 100 |

[a]Only cysts form were seen under microscopy.

Chromas Version 1.0 (Technelysium Pty Ltd, Unit 406, 8 Cordelia St, South Brisbane QLD 4101, Australia). The nucleotide sequences were manually edited, and the sequence representatives for each identified species were submitted to the GenBank/EMBL/DDBJ database under accession numbers KY884295 and KY823418 to KY823428.

### Statistical analysis

Data were entered in Microsoft Excel and analysed in STATA version 12.0 (StataCorp LP). The proportion percentage was measured to describe the characteristics of the participants, including the frequency of *Entamoeba* complex infection according to variables including age, sex, etc. The $\chi^2$ test or Fisher's exact test was used to analyse the association between the *Entamoeba* complex and different subgroups. The odds ratios (OR) and 95% confidence intervals (CI) were also determined, based on the binary logistic regression analysis to identify the potential contribution of each variable to the acquisition of *Entamoeba* complex infection. *P*-value <0.05 was considered statistically significant.

## Results

### Microscopic analysis

Using microscopic methods, the *Entamoeba* complex cysts were detected in 0.58% (8/1383) of the isolates. Four (0.29%) isolates were also considered similar to the *Entamoeba* complex cysts (e.g. *E. hartmanni*).

### PCR assay

Based on the nested multiplex PCR, all 12 microscopy-positive and *Entamoeba complex*-like isolates were considered positive for the *Entamoeba* complex. Additionally, among 55 microscopy-negative *Entamoeba* complex isolates, which were positive for other amoebae (e.g. *E. coli* and/or *E. nana*), two were detected as *E. dispar* and mixed of *E. histolytica and E. dispar* (Table 1).

### Prevalence and differential detection

Out of 1383 studied samples, 14 (1.01%) *Entamoeba*-positive isolates were identified. Two (14.28%) out of 14 samples were *E. histolytica*, eight (57.14%) were *E. dispar*, one (7.14%) was *E. moshkovskii* and three (21.43%) mixed *E. histolytica* and *E. dispar* (Table 1).

### Relationship between clinical symptoms and Entamoba species

Table 2 presents the relationship between the clinical symptoms and *Entamoba* species. All infected patients with *E. histolytica*, *E. moshkovskii*, or had both *E. histolytica* and *E. dispar* showed GIDs, including abdominal pain, diarrhoea and chronic dysentery. It should be noted that one *E. histolytica*-positive patient and one mixed infected patient were immunocompromised. However, only three (21.43%) patients, infected with *E. dispar*, had abdominal symptoms and chronic diarrhoea.

### Sequencing analysis of PCR products

Twelve out of 14 positive samples, including one *E. moshkovskii*, five *E. histolytica* and six *E. dispar* samples, were sequenced with species-specific primers in forward directions, using an ABI 3730XL sequencer (Macrogen® Corp., Seoul, South Korea). The BLAST analysis showed that sequences of six *E. dispar* amplicons under accession numbers KY823418 to KY823423 were 100% identical to the available GenBank sequences for *E. dispar* with the accession number KP722600.1. On the other hand, five *E. histolytica* sequences, with accession numbers KY823424 to KY823427 and KY884295, showed high homology (99–100%) to the GenBank sequences of *E. histolytica* under accession number KP233840.1. The only detected isolate of *E. moshkovskii* amplicon, under accession number KY823428, showed 100% homology to the sequences of *E. moshkovskii* under GenBank accession number KP722605.1.

### Risk factors for Entamoeba complex infection

The results of single-variable logistic regression analysis for the evaluation of risk factors for *Entamoeba* complex infection and socio-demographic characteristics are presented in Table 3. According to Table 3, among the studied factors, none showed a significant relationship with *Entamoeba* complex infection.

## Discussion

Amoebiasis is one of the most common infections among humans worldwide [5]. The three studied species are

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**Table 2.** Frequency of *Entamoeba* complex isolated from symptomatic and asymptomatic attended individuals

| | With symptoms | | Without symptoms[b] | | Total | |
|---|---|---|---|---|---|---|
| *Entamoeba* complex[a] | No. | % | No. | % | No. | % |
| *E. histolytica*[c] | 2 | 14.28 | 0 | 0 | 2 | 14.28 |
| *E. dispar* | 3 | 21.43 | 5 | 35.72 | 8 | 57.14 |
| *E. moshkovskii* | 1 | 7.14 | 0 | 0 | 1 | 7.14 |
| Mixed *E. histolytica*/*E. dispar*[c] | 3 | 21.43 | 0 | 0 | 3 | 21.43 |
| Total | 9 | 64.28 | 5 | 35.72 | 14 | 100 |

[a]Only cysts form were seen under microscopy.
[b]All *Entamoeba* complex isolates except five of *E. dispar* were associated with clinical symptoms.
[c]Two patients were found immunocompromised, one with *E. histolytica* and another with mixed *E. histolytica*/*E. dispar*.

morphologically similar, despite genetic and pathogenic differences [13]. *E. histolytica* is generally considered a pathogenic species, while other *Entamoeba* species are classified as non-virulent [12, 14]; therefore, distinguishing of these species is of great significance.

Microscopic methods, as well as molecular approaches, were used in this study for the detection of *Entamoeba* species and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii*. In the medical laboratories of many countries, including our region, detection of *Entamoeba* is based on the microscopic identification of cysts or trophozoites. These methods are usually accompanied by misdiagnosis, and it is impossible to differentiate between the isolates of *Entamoeba* complex [17]. Therefore, molecular approaches have been developed to differentiate and detect *Entamoeba* species in faecal samples.

To the best of our knowledge, this study was the first to distinguish *Entamobea* species and to assess the prevalence of *E. histolytica*, *E. dispar* and *E. moshkovskii* in Kurdistan Province in west of Iran. Furthermore, we described the association of *Entamoeba* species with clinical symptoms among individuals, attended to 14 medical laboratories. The results of molecular studies showed that *E. dispar*, *E. histolytica* and *E. moshkovskii* infections are present in the study area. However, the prevalence of these amoebae and other parasites has dramatically decreased in recent years, similar to other regions of Iran.

According to the WHO/PAHO/UNESCO report and many conducted studies, the prevalence of *E. dispar* is greater than that of *E. histoltyica* and *E. moshkovskii* [5, 12]. Our findings also demonstrated that 78% (11/14) of the samples were attributed to *E. dispar* (eight samples with single infections and three samples containing both *E. dispar* and *E. histolytica*). The prevalence of *E. dispar* in the present study is close to most previous studies carried out in northern, central and southern Iran [18], Khoramabad [19], Gonabad [20], Zahedan [21], Ahvaz and Hamidieh [22] and Miandoab [23], as well as studies from Malaysia [12], Northern Ghana [24], South Africa [25], Australia [3], Northwest Ethiopia [26] and the Netherlands [27]. However, studies from Saqqez, Iran [28], south-west of Iran [29], United Arab Emirates [30] and Gaza Strip [31] reported different results in areas where *E. histolytica* was more prevalent.

In 1997, WHO reported that most cases of *E. histolytica* may be in fact *E. dispar*, which is known to be non-pathogenic [5]. However, cases of *E. histolytica* infection have been reported in patients without symptoms. For instance, in studies from the Philippines [32] and Japan [33], most positive cases of *E. histolytica* were considered asymptomatic. The prevalence of *E. histolytica* (single and mixed infections) in our population was 0.36% (5/1383). GIDs, including abdominal pain and chronic diarrhoea, were reported in all cases infected with *E. histolytica* (single and mixed infections). This finding is consistent with several reports from Pakistan [34] and South Africa [25], which showed that *E. histolytica* commonly produces clinical symptoms in patients.

It is commonly believed that *E. dispar* is a non-virulent species [1]. In this regard, Espinosa *et al.* reported that *E. dispar* is non-virulent under *in vivo* conditions [35]. In addition, Oliveira *et al.* found that *E. dispar* was commensal and non-pathogenic to humans [36]. Dvorak *et al.* also suggested that *E. dispar* (SAW760 and SAW1734) strains are non-virulent [37]. These reports are in contrast with a study by Herbinger *et al.*, which showed that most *E. dispar* isolates were associated with GIDs in returning travellers [15]. According to some studies, the Brazilian strain of *E. dispar* is pathogenic and can produce amoebic liver abscess under *in vivo* conditions [38]. Based on our findings, eight out of 11 patients with *E. dispar* had single infection, while three cases showed GIDs including abdominal pain.

It was initially hypothesised that *E. moshkovskii* is a non-virulent and free-living *Entamoeba* species [39]. However, this is inconsistent with our findings, as gastroenteritis symptoms, such as abdominal pain and chronic diarrhoea, were observed in one patient infected with *E. moshkovskii*. Similarly, four studies from Australia, Tunisia, Malaysia and Bangladesh showed that humans can be true hosts for this species [3, 10, 11, 14]. Also, some studies from Australia, Malaysia and India linked *E. moshkovskii* infection to GIDs [4, 12, 40]. A study from Malaysia recommended that further investigations are necessary to determine the relationship between *E. moshkovskii* and GIDs and to identify the possible pathogenicity of this species [12].

In the present study, considering the low number of positive cases, besides practical and financial limitations, other probable factors, such as bacterial, fungal and viral infections, or other non-infectious diseases associated with gastroenteritis symptoms were not examined and cannot be ruled out. Therefore, we cannot confirm the association between clinical symptoms and *Entamoeba* complex infection, and future investigations are necessary in this area.

In conclusion, this study reported the presence of *E. histolytica*, *E. dispar* and *E. moshkovskii* in Kurdistan Province, especially among patients with GIDs, although these species were not commonly detected. Based on the findings, the prevalence of *E. dispar* is greater than *E. histoltyica* and *E. moshkovskii*. Only a few cases of *E. moshkovskii* have been reported in Iran, and a single isolate

https://doi.org/10.1017/S0950268819000141 Published online by Cambridge University Press

**Table 3.** Univariate analysis of risk factors associated with *Entamoeba* complex infection among individuals attended to the medical laboratories in Sanandaj County, Kurdistan, Northwest Iran($n = 1383$)

| Variable | Total | Positive *N* (%) | OR | 95% CI | | *P*-value |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| Sex | | | | | | |
| Male | 799 (57.8%) | 9 (1.13%) | Reference | – | – | 0.621 |
| Female | 584 (42.2%) | 5 (0.86%) | 0.758 | 0.253 | 2.274 | |
| Age group (years) | | | | | | |
| <6 | 271 (19.6%) | 0 | – | | | |
| 6–12 | 125 (9%) | 0 | – | | | |
| 13–18 | 66 (4.8%) | 1 (1.5%) | 1.069 | 0.118 | 9.725 | 0.953 |
| 18–30 | 252 (18.2%) | 4 (1.59%) | 1.121 | 0.277 | 4.529 | 0.873 |
| 30–50 | 387 (28%) | 5 (1.29%) | 0.912 | 0.243 | 3.423 | 0.892 |
| >50 | 282 (20.4%) | 4 (1.42%) | Reference | – | – | |
| Educational status | | | | | | |
| Preschool | 335 (24.2%) | 0 | – | – | – | |
| Illiterate | 277 (20%) | 3 (1.08%) | Reference | | | |
| Primary school | 357 (25.8%) | 6 (1.68%) | 1.561 | 0.387 | 6.298 | 0.528 |
| High school | 270 (19.5%) | 2 (0.74%) | 0.682 | 0.113 | 4.111 | 0.674 |
| Collage | 144 (10.4%) | 3 (2.08%) | 1.943 | 0.387 | 9.751 | 0.412 |
| Reasons for referral | | | | | | |
| Check-up | 508 (36.7%) | 5 (0.98%) | Reference | – | – | |
| GIDs | 629 (45.5%) | 7 (1.11%) | 1.132 | 0.357 | 3.589 | 0.833 |
| Non-GIDs | 246 (17.8%) | 2 (0.81%)[a] | 0.825 | 0.159 | 4.280 | 0.812 |
| Source of drinking water | | | | | | |
| Treated | 1319 (95.4%) | 12 (0.91%) | 0.280 | 0.061 | 1.278 | 0.100 |
| Untreated | 64 (4.6%) | 2 (3.12%) | Reference | – | – | |
| Contact with domestic animals | | | | | | |
| No | 1342 (97%) | 13 (0.97%) | 0.361 | 0.046 | 2.0843 | 0.333 |
| Yes | 41 (3%) | 1 (2.44%) | Reference | – | – | |
| Location | | | | | | |
| Urban | 1265 (91.5%) | 12 (0.95%) | 0.509 | 0.111 | 2.323 | 0.383 |
| Rural | 118 (8.5%) | 2 (1.7%) | Reference | – | – | |
| Job | | | | | | |
| Food staff | 204 (14.7%) | 3 (1.47%) | 0.567 | 0.057 | 5.598 | 0.624 |
| House wife | 286 (20.7%) | 3 (1.05%) | 0.403 | 0.041 | 3.971 | 0.422 |
| Self-employment | 222 (16%) | 3 (1.35%) | 0.521 | 0.053 | 5.136 | 0.570 |
| Student >6 years | 216 (15.6%) | 2 (0.93%) | 0.355 | 0.031 | 4.014 | 0.384 |
| Gov't employer | 99 (7.2%) | 2 (2.02%) | 0.784 | 0.069 | 8.895 | 0.844 |
| Farmer | 39 (2.8%) | 1 (2.56%) | Reference | | | |
| Child <6 years | 317 (23%) | 0 | – | – | – | |

(*Continued*)

https://doi.org/10.1017/S0950268819000141 Published online by Cambridge University Press

**Table 3.** (*Continued.*)

| Variable | Total | Positive *N* (%) | OR | 95% CI | | *P*-value |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| Seasons | | | | | | |
| Spring | 346 (25%) | 3 (0.87%) | 3.017 | 0.312 | 29.152 | 0.340 |
| Summer | 345 (25%) | 7 (2.03%) | 7.145 | 0.874 | 58.385 | 0.067 |
| Fall | 346 (25%) | 3 (0.87%) | 3.017 | 0.312 | 29.152 | 0.340 |
| Winter | 346 (25%) | 1 (0.23%) | Reference | – | – | |

*N*, number; OR, odds ratio; Reference, the subgroup is considered as baseline.
[a]Symptoms of gastrointestinal discomfort also occurred.

of this amoeba was detected for the first time in our study. Overall, we found that *E. dispar* and *E. moshkovskii* might be associated with GID symptoms.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0950268819000141.

**Author ORCIDs.** Ghasem Zamini, 0000-0002-7017-2647

**Acknowledgements.** We thank all technicians and personnel working in the medical laboratories, School of Medicine, Kurdistan University of Medical Sciences and also those who work at the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences who contributed to the study.

**Financial support.** This work was a part of the Ph.D. thesis of Fares Bahrami under supervision of Professor Ali Haghighi and Dr Ghasem Zamini, which was supported financially by Kurdistan University of Medical Sciences (Grant No. 335) and Shahid Beheshti University of Medical Sciences (Grant No. 6598).

**Conflict of interest.** None.

**Ethical approval.** The authors assert that all procedures contributing to this work comply with the ethical standards of the Declaration of Helsinki, revised in 2008. The trial was reviewed and approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

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https://doi.org/10.1017/S0950268819000141 Published online by Cambridge University Press