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ORIGINAL PAPER

Molecular Identification and Prevalence of *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii* in Erbil City, Northern Iraq

SHLER AKRAM FAQE MAHMOOD[1, 2*] and HAWRI MUSTAFA BAKR[2]

[1] Microbiology Department, College of Medicine, Sulaimani University, Kurdistan Region, Iraq
[2] Basic Sciences Department, College of Medicine, Hawler Medical University, Kurdistan Region, Iraq

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Abstract

The present study was conducted to evaluate the infection rates of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* among asymptomatic individuals in Erbil City, northern Iraq. The research intent was to discover whether pathogenic or nonpathogenic species cause a high rate of symptomless *Entamoeba* infections. Stool samples were microscopically examined, and the 18S-rRNA gene was targeted utilizing the nested PCR technique in the positive specimens. Initial results based on morphological features showed that the *Entamoeba* prevalence rate was 7.4%. Significantly higher rates of infections were seen in females than in males and in low-income people than in moderate-income people. The incidence rates among the asymptomatic individuals, as determined by molecular analysis, were as follows: *E. histolytica* – 6%, *E. dispar* – 4.3%, and *E. moshkovskii* – 0.3%. Of all the *Entamoeba* positive samples, a single infection with *E. histolytica* was identified in 41.4% samples; the single infection with *E. dispar* in 18.6% samples, 35.7% samples had mixed infections with two *Entamoeba* species, and 4.3% had mixed infections with three species. The current study concluded that 7.4% of healthy people, who live in the endemic area under investigation, carry *Entamoeba* species asymptomatically. Additionally, the majority of asymptomatic *Entamoeba* infections were caused by the pathogenic *E. histolytica* (81.4%) compared to *E. dispar* (58.6%), and *E. moshkovskii* with the lowest rate of infection. Single and co-infections with *E. histolytica* and *E. dispar* were noted. *E. moshkovskii*, which was identified for the first time in the region, was only seen in mixed infections.

Key words: *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii,* epidemiology, asymptomatic infections

Introduction

Parasitic infections are endemic to most tropical and subtropical regions of developing countries (WHO 1997). *Entamoeba histolytica*, a protozoan parasite that inhabits the human gastrointestinal tract, causes asymptomatic infections in about 90% of infected people playing a significant role in spreading the parasite. Prolonged asymptomatic infection can lead to invasive amoebiasis, whose symptoms may include bloody diarrhea, abdominal pain, flatulence, nausea, and vomiting. In some cases, the amebae may spread from the gastrointestinal tract to the liver and cause the formation of ulcerations and abscesses, resulting in amoebic liver abscesses (Haque et al. 2003).

Entamoeba dispar and *Entamoeba moshkovskii* are nonpathogenic intestinal protozoa that are morphologically identical to *E. histolytica* but are genetically and biochemically different (Clark and Diamond 1991; Diamond and Clark 1993). Previous studies showed that the infection rate of *E. dispar* in developed countries is much higher than *E. histolytica* (Pillai et al. 1999; Fotedar et al. 2007b). High levels of *E. moshkovskii* infection were reported on the Indian subcontinent. However, fewer studies have been conducted into the prevalence of this species. Human isolates have been reported in South Africa, North America, Italy, and Bangladesh (Ali 2003; Singh et al. 2009).

Amoebiasis develops in 50 million individuals globally, with an annual mortality rate of 40,000 to 100,000 (WHO 1997). This high infection rate is likely inflated as a result of false positives caused by the morphologically indistinguishable, nonpathogenic *E. dispar*/*moshkovskii*, and/or polymorphic nuclear leukocytes

* Corresponding author: S.A.F. Mahmood, Microbiology Department, College of Medicine, Sulaimani University, Kurdistan Region, Iraq; **e-mail: shler.faqe@univsul.edu.iq**

and macrophages with similar morphology in the stool samples (Walsh 1986; Tanyuksel and Petri 2003). New methods have been developed that are better in distinguishing between the pathogenic *E. histolytica* and nonpathogenic amoebae in the stool sample. Emerging molecular-based techniques, such as polymerase chain reaction (PCR), have improved test specificity, or true positive rate of the target *E. histolytica* DNA (Tanyuksel and Petri 2003; Paul et al. 2007). Therefore, the most-recent epidemiological studies of *E. histolytica* use molecular methods to provide accurate data (Santos et al. 2010). To date, the PCR technique has never been used for assessing the prevalence rate of *E. histolytica* in Erbil City. Several studies have reported the infections with *E. histolytica* in almost all Iraqi cities, but only a few applied molecular methods; most relied on microscopic examination (Hamad and Ahmed 2011; Al-Sorchee et al. 2013; Saqur et al. 2017). To date, no research has been conducted on asymptomatic individuals in Iraq, the least-studied group globally. Moreover, it is mostly unknown whether the asymptomatic individuals have been infected with *E. histolytica* or the nonpathogenic *E. dispar* and/or *E. moshkovskii*. This study was conducted to fill this gap in research and strives to determine the prevalence rate of *Entamoeba* in Erbil City, first using microscopic examination and then molecular techniques, to confirm the presence of and differentiate between pathogenic and nonpathogenic amoebae.

Experimental

Materials and Methods

A total of 950 random stool samples (524 male and 426 female) were collected from asymptomatic healthy adults in a cross-sectional study. The Central Laboratory of Erbil Province provided specimens from asymptomatic individuals. Specimen donors filled out a structured questionnaire about personal status, residency, and source of water supply. The collected fresh stool samples were microscopically examined using the iodine and saline wet mount microscopy to detect *Entamoeba* trophozoites and/or cysts. About 0.2 g of each specimen was preserved at –80°C for molecular analysis.

DNA was extracted from specimens using the QiaAmp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer protocol. Finally, the purified DNA concentrate was eluted from the silica membrane spin column with a low salt buffer. DNA concentration was measured with a nanospectrophotometer; then, each sample was labeled and stored at –20°C. A nested PCR was performed. The first PCR targeted the *Entamoeba* genus by amplifying the 897 bp of the 18S rRNA gene, while the second PCR primers targeted *E. histolytica*, *E. dispar*, and *E. moshkovskii* by amplifying the 439 bp, 174 bp, and 553 bp respectively. This method was previously described by Khairnar and Parija (2007). The primers targeting the 18S-ribosomal RNA gene were confirmed for specificity by the Basal Local Alignment Search Tool (BLAST), the genome database of all organisms from the National Center for Biotechnology Information (NCBI). PCR amplification was performed using a thermal cycler (Techne Ltd., Cambridge, UK) with 20 µl reaction volumes that consisted of 10 µl Hot Start Master Mix (containing Taq DNA polymerase 1 unit/10 µl, 2 × reaction buffer, enzyme stabilizer, 4 mM $MgCl_2$, sediment, 0.5 mM each of dATP, dCTP, dGTP, dTTP, pH 9, and loading dye) (GeNet Bio, Daejeon, South Korea); 2 µl of both the forward and reverse primers (10 ρmole for each), 2 µl of DNA template, and 6 µl of water. The PCR cycling and running parameters were defined as one cycle of initial denaturation at 95°C for 10 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec with a final extension of 72°C for 5 min. The second PCR used the same cycling and running parameters except that the first step used 35 cycles, and the annealing temperature changed to 52°C. Negative and positive controls were used in both PCR rounds. Positive control DNA for *E. histolytica* HM-1:IMSS, *E. dispar* SAW760, and Laredo strains of *E. moshkovskii* were obtained from Kurdistan Biomedical Science University, Sanandaj, Iran. The PCR products were electrophoresed in 1%, 1.5%, and 2% agarose gels with a 1X Tris-boric acid-EDTA buffer (TBE) and stained with 0.2 mg/ml of ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA), with a 100-bp DNA marker ladder (Promega Corp., Madison, Wisconsin, USA).

Sequencing of PCR products. A single sample of each species was randomly selected and sequenced with species-specific primers in both forward and reverse directions using BigDye terminators and an ABI 3730XL sequencer (Macrogen® Corp., Seoul, South Korea). The nucleotide sequences of forward and reverse reactions were manually edited, and the sequences for each identified species were submitted to the GenBank.

Statistical analysis. The data was analyzed using the IBM SPSS Statistics Server Version 23. Results expressed using descriptive statistics: frequencies, percentages, Fisher exact test, and chi-square. P value < 0.05 was regarded as statically significant.

Results

Sociodemographic factors associated with *Entamoeba* infection. Our simple random sample consisted of 55.2% male and 44.8% female individuals (Table I).

Table I
Statistical analysis of the risk factors associated with microscopic positives for *Entamoeba* species and PCR positives for *E. histolytica* in asymptomatic subjects.

Variants	Total frequency	Frequency and percentages of positive by microscopy	Percentage within each group	95%CI		*p*-value
				Lower	upper	
Gender						
Male	524 (55.2%)	29 (41.4%)	524 (5.5%)	0.036	0.075	0.018*
Female	426 (44.8%)	41 (58.6%)	426 (9.6%)	0.069	0.125	
Residency						
Urban	702 (73.9%)	48 (68.6%)	702 (6.8%)	0.049	0.090	0.322
Rural	248 (26.1%)	22 (31.4%)	248 (8.9%)	0.054	0.125	
Age group						
15–18	87 (9.2%)	4 (5.7%)	87 (4.6%)	0.009	0.090	
19–25	311 (32.7%)	24 (34.3%)	311 (7.7%)	0.048	0.108	
26–35	328 (34.5%)	26 (37.1%)	328 (7.9%)	0.050	0.110	0.76
36–45	157 (16.5%)	13 (18.6%)	157 (8.3%)	0.00	0.100	
> 45	67 (7.1%)	3 (4.3%)	67 (4.5%)	0.00	0.308	
Educational level						
Primary school	298 (31.4%)	25 (35.7%)	298 (8.4%)	0.053	0.115	
Secondary and high school	448 (47.2%)	31 (44.3%)	448 (6.9%)	0.044	0.096	0.73
Bachelor	204 (21.5%)	14 (20%)	204 (6.9%)	0.036	0.107	
Family size						
1–2	92 (9.7%)	5 (7.1%)	92 (5.4%)	0.011	0.105	
3–4	228 (24%)	13 (18.6%)	228 (5.7%)	0.028	0.086	0.563
5–6	301 (31.7%)	24 (34.3%)	301 (8%)	0.050	0.112	
> 6	329 (34.6%)	28 (40%)	329 (8.5%)	0.055	0.116	
Income status						
Poor	355 (37.4%)	39 (55.7%)	355 (11%)	0.078	0.142	
Middle class	594 (62.5%)	31 (44.3%)	594 (5.2%)	0.033	0.069	0.004*
Wealthy	1 (0.1%)	0 (0%)	1 (0%)	0.00	0.00	
Source of water supply						
Chlorinated water	646 (68%)	39 (55.7%)	646 (6%)	0.042	0.079	
Well water	302 (31.8%)	31 (44.3%)	302 (10.3%)	0.068	0.138	0.062
Others	2 (0.2%)	0 (0%)	2 (0%)	0.00	0.00	
Eating out of home						
Never	259 (27.3%)	17 (24.3%)	259 (6.6%)	0.039	0.096	
Sometimes	310 (32.6%)	24 (34.3%)	310 (7.7%)	0.045	0.110	0.857
Always	381 (40.1%)	29 (41.4%)	381 (7.6%)	0.050	0.104	
History of taking medications						
In the last 2 weeks	140 (14.7%)	6 (8.6%)	140 (4.3%)	0.013	0.075	0.161
More than 2 weeks	810 (85.3)	64 (91.4%)	810 (7.9%)	0.059	0.098	
Hygiene practice						
Washing vegetables and fruits	920 (96.8%)	66 (94.3%)	920 (7.2%)	0.055	0.088	0.204
Eating raw unwashed vegetables and fruits	30 (3.2%)	4 (5.7%)	30 (13.3%)	0.029	0.263	

* presenting statistically significant differences < 0.05

As determined by the microscopic examination, 7.4%, or 70 out of 950 stool samples from asymptomatic individuals, tested positive for *Entamoeba* species cysts and/or characteristic features of the trophozoite. Quadrinucleated spherical cysts and amoebic trophozoites with multiple pseudopodia of *Entamoeba* were observed using light microscopy and identified based on their morphology. A significantly higher ($p < 0.05$) rate of

Table II
Frequency and percentages of positive and negative results for *E. histolytica*, *E. dispar* and *E. moshkovskii* as single and mixed infections.

Entamoeba species	Frequency & percentage of Positives by PCR per total (microscopic) positives	Frequency & percentage of Negatives by PCR per total (microscopic) positives	Frequency & percentage of positives per population	Frequency and percentage of single infection/ positives	Frequency and percentage of mixed infection/ positives	Frequency and percentage of single infection/ total positives	Frequency and percentage of mixed infection/ total positives
E. histolytica	57 /70 (81.4%)	13/70 (18.6%)	57/950 (6%)	29/57 (50.9%)	28/57 (49.1%)	29/70 (41.4%)	28/70 (40%)
E. dispar	41/70 (58.6%)	29/70 (41.4%)	41/950 (4.3%)	13/41 (31.7%)	28/41 (68.3%)	13/70 (18.6%)	28/70 (40%)
E. moshkovskii	3/70 (4.3%)	67/70 (95.7%)	3/950 (0.3%)	0/3 (0%)	3/3 (100%)	0/70 (0%)	3/70 (4.3%)

infection was detected in females (9.6%) than in males (5.5%). Significantly higher ($p < 0.05$) rates were also recorded in low-income participants (11%) than in moderate-income individuals (5.2%).

Nested PCR analysis. DNA was extracted from the 70 positive stool samples; their concentrations ranged from 5 µg/ml to 217 µg/ml, and purity ranged from 2.2–2.8, as measured by a nanospectrophotometer. Nested PCR results indicated that 57 samples tested positive for the 439 bp band for *E. histolytica* (Fig. 1), which is equivalent to 81.4% of the positive samples and 6% of the total number of samples (Table II). However, out of 57 positives, 29 carried a single infection, and 28 carried *E. histolytica* in combination with either *E. dispar* or *E. dispar* and *E. moshkovskii. E. dispar* accounted for 4.3% of the *Entamoeba* infections in the Erbil population (41 positives or 58.6% of the 70 positive samples, as revealed by the 174 bp band in the microscopic analysis (Fig. 2). Of samples testing positive for *E. dispar*, 13 carried *E. dispar* only, and 28 carried mixed infections with either *E. histolytica* or *E. moshkovskii*. Only three samples (4.3%) tested positive for the 553 bp band for *E. moshkovskii* (Fig. 3) as determined by microscopy, which indicated a 0.3% prevalence in Erbil City; all were mixed infections. The negative PCR results for *E. histolytica* (13 samples) represented 18.6% of the positive results for *E. dispar* as a single infection. However, the mixed infection rate for *E. dispar* with *E. histolytica* was 40% of the positive samples as determined by microscopy.

Overall PCR results showed that, out of 70 positive samples, 25 (35.7%) carried mixed infection with both *E. histolytica* and *E. dispar*; 3 (4.3%) samples carried mixed infections with *E. histolytica*, *E. dispar*, and *E. moshkovskii*; 29 (41.4%) samples carried a single infection with *E. histolytica*; 13 (18.6%) samples carried a single infection with *E. dispar,* and none carried a single infection with *E. moshkovskii*.

Sequencing analysis of PCR products. The BLAST sequence analysis tool (NCBI) showed that the sequence of *E. histolytica* amplicon under accession number MT250837 was 99.7% identical to the available *E. histolytica* GenBank sequence, accession number KY884295.1.1. In comparison, *E. dispar* under accession number MT250839 sequence was 100% identical to the *E. dispar* GenBank sequence, accession number KP722600.1 and the *E. moshkovskii* under accession number sequence MT250838 showed 100% homology to the sequence of *E. moshkovskii* GenBank, accession number KY823428.1.

Discussion

Determining prevalence rates for *E. histolytica* in endemic regions using molecular techniques is a radical solution to light microscopy's shortcomings (Haque et al. 1998; Tanyuksel and Petri 2003). For the first time in Erbil City lying in the north of Iraq, molecular methods were used to estimate the prevalence rates of the pathogenic *E. histolytica*, and nonpathogenic *E. dispar* and *E. moshkovskii* in asymptomatic populations.

The results of the present study, as determined by microscopic examination, showed that 7.4% of individuals residing in Erbil province are asymptomatic carriers of at least one *Entamoeba* species. Several previous studies have recorded the prevalence rate of *Entamoeba* in Erbil City using microscopy. For example, in a study that included 500 diarrheal stool samples from infants and children, *Entamoeba* infections were found in 35% of samples (Al-Sorchee et al. 2013). In another study, the infection rate was 51.7%, but this study did not exclude the commensal protozoa *Entamoeba coli* (Hamad and Ahmed 2011). Unlike the present study, all research that has previously been done in Erbil City was based on samples from symptomatic subjects only, and this may be a reason for the differences in the rate of infections. Additionally, polymorphic leukocytes and macrophages in diarrheal stool samples could be misidentified as *Entamoeba* species and results in false positives.

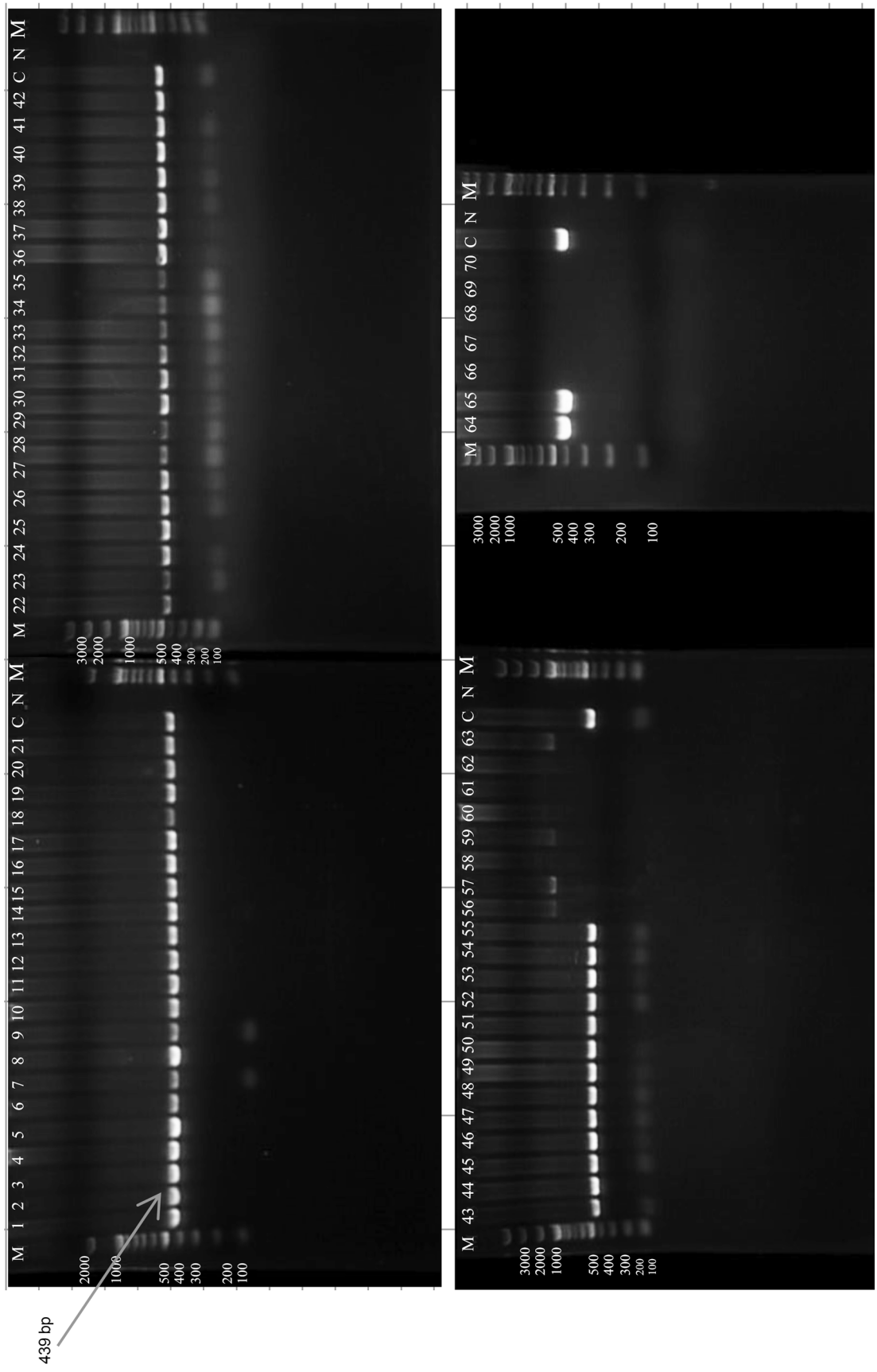

Fig. 1. Agarose gel electrophoresis analysis for nested PCR products, using primers specific for *E. histolytica*, positive samples reveal 439 bp bands. Samples 1 to 55, 64, and 65 showed 439 bands; samples 56 to 63 and 66 to 70 tested negative for *E. histolytica*. Some of these negatives showed 900 bp bands, which are the product of the first PCR and an indicator for the presence of other *Entamoeba* species. C represented positive control, N represented negative control and M the 100 bp DNA marker.

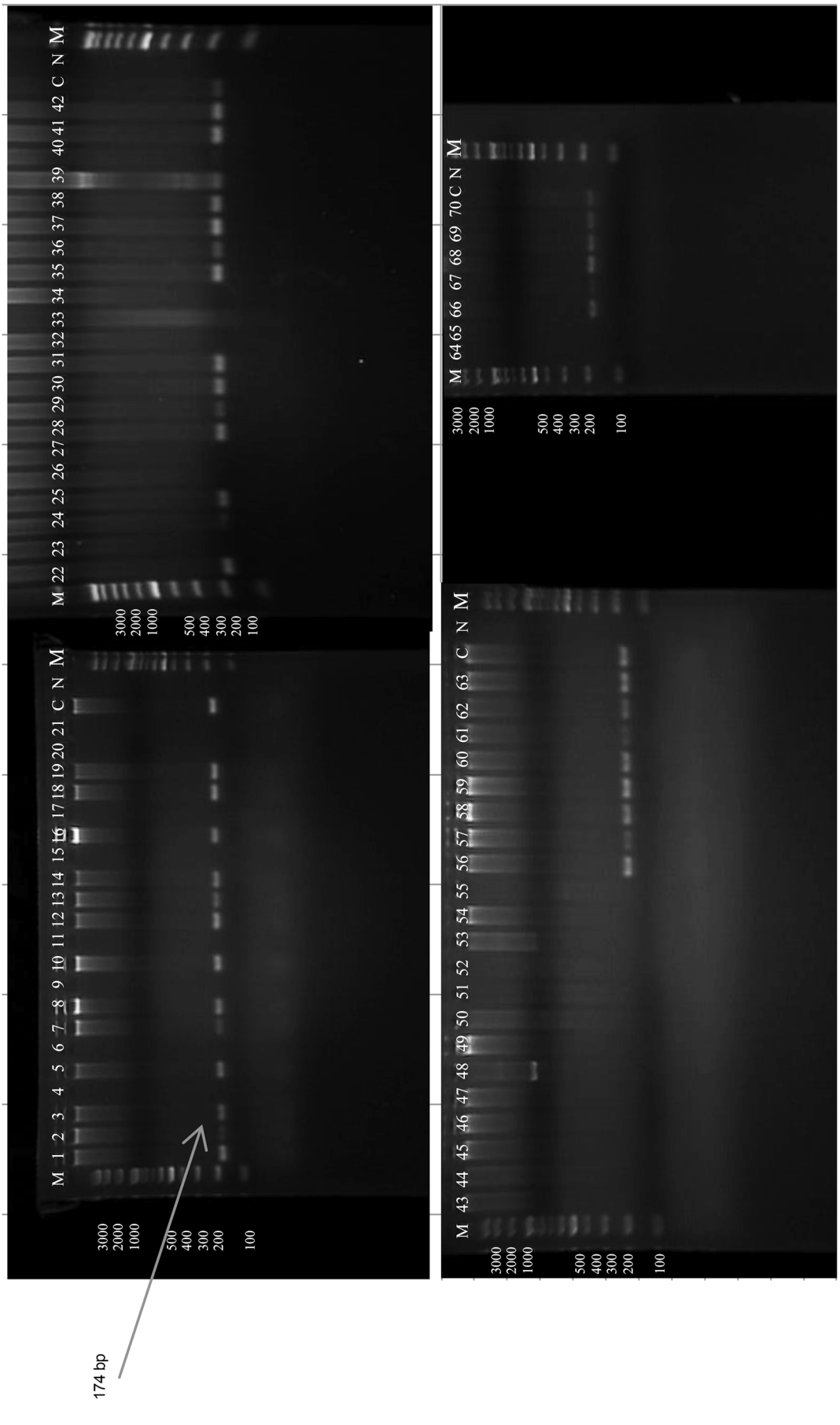

Fig. 2. Detection of *E. dispar* after using species specific primers in the second round of the nested PCR; amplification products were analyzed by agarose gel electrophoresis and the stained gels were visualized under UV light. Positive samples exhibited 174 bp bands, which appeared in sample numbers (1, 2, 3, 5, 7, 8, 10, 12, 13, 14, 16, 18, 19, 22, 24, 25, 28, 29, 30, 31), (35 to 42), (56 to 63), and (66 to 70). Samples (4, 6, 9, 11, 15, 17, 20, 21, 23, 26, 27, 32, 33, 34, 64, 65) and (43 to 55) tested negative for *E. dispar*. C represented positive control, N represented negative control and M was the 100 bp DNA marker.

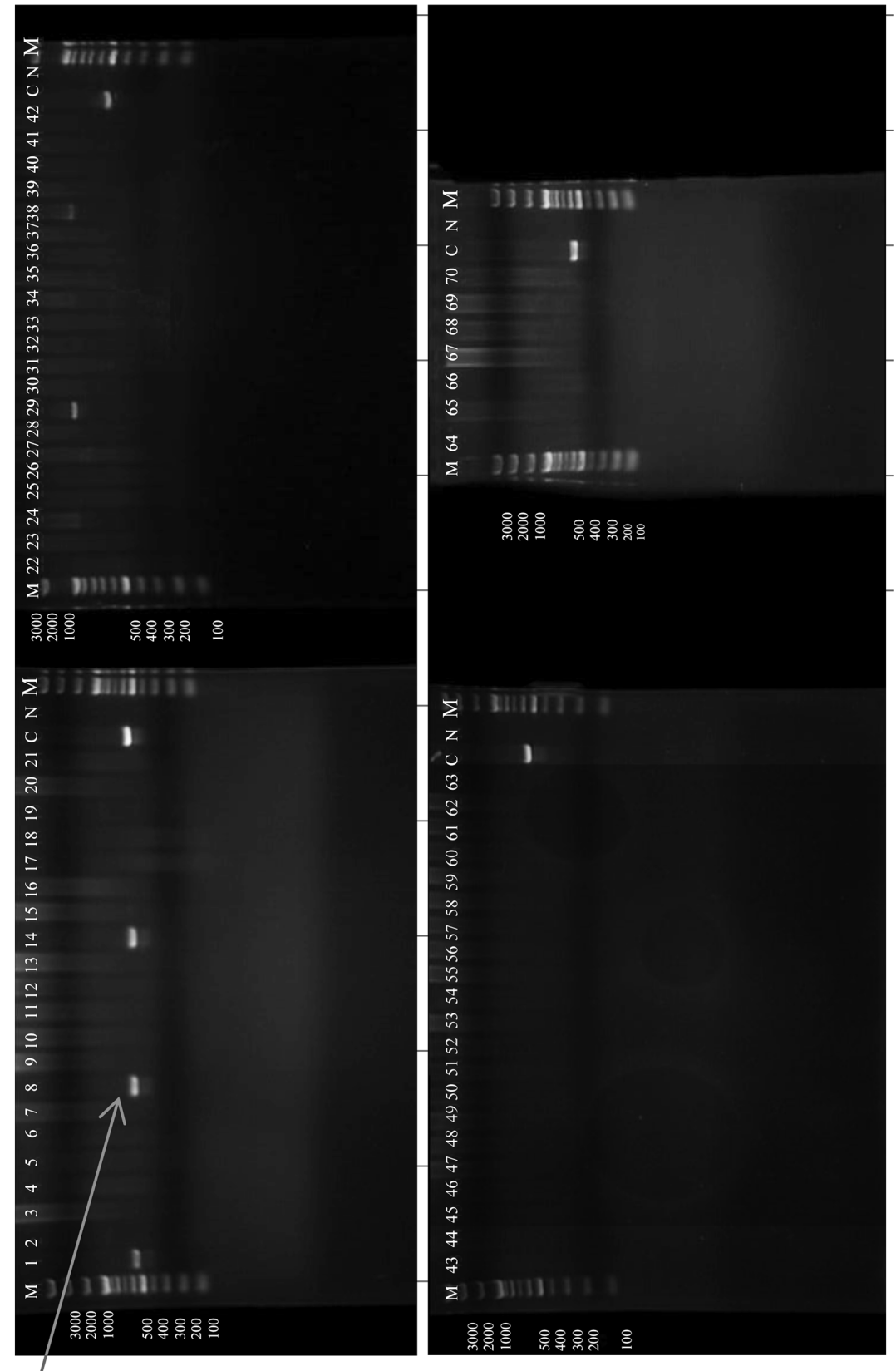

Fig. 3. Nested PCR for identification of *E. moshkovskii,* determined with specific primers for each species, and analyzed by agarose gel electrophoresis. Positive samples amplifying 553 bp amplicon appeared in only three samples (1, 8, and 14); the remaining samples were negative. C represented positive control, N represented negative control, and M was the 100 bp DNA marker.

Statistical analysis of the present study showed a significant difference ($p < 0.05$) in infection by *Entamoeba* species between males and females, revealing a higher rate of infection in females than in males. Similar results were reported in rural Malaysian communities (Ngui et al. 2012). Furthermore, significantly ($p < 0.05$) higher rates of infections were detected in low-income people, who often reside in poor living conditions and have a lower quality of life. These results are consistent with research reported in northeastern India (Nath et al. 2015).

Nested PCR revealed that *E. histolytica* infection was the most common (6%), followed by infection with *E. dispar* (4.3%), and *E. moshkovskii,* which had the lowest infection rate (0.3%) within the Erbil population. These results indicate that around 6% of individuals living in endemic regions are at risk of acquiring an asymptomatic infection caused by pathogenic amoeba. Asymptomatic carriers of *E. histolytica* play a significant role in spreading the parasite, and a prolonged asymptomatic infection can lead to invasive amoebiasis and amoebic liver abscesses (Fotedar et al. 2007a).

Previously, there have only been four molecular-based studies that have reported the prevalence of *E. histolytica* in Iraq. Only one study targeted non-pathogenic *E. dispar* and *E. moshkovskii*. The studies that detected *E. histolytica* by molecular methods were conducted in Diwanyha (south-central), Baghdad (central), and Al-Najaf (southwest of Iraq) provinces. Reported prevalence rates were 44.3%, 7%, and 24%, respectively, among symptomatic patients (Al-Hameedawi 2014; Hussein et al. 2015; Al-Khalidi 2016). The high rates of infections reported in Diwanyha and Al-Najaf cities, which share internal boundaries, could be due to the small sample sizes of their respective studies, the differences in the study design (they studied the symptomatic population whereas the present work studied the asymptomatic population), the differences in environmental conditions and hygienic practices in these regions, and the higher population density in Al Najaf city (whose shrine receives thousands of visitors). Amoebiasis is regarded as one of the primary food and water-borne diseases; the high rates of infections could be attributed to poor nutrition and sanitation and contaminated water supply (Jackson 2000). It has been documented that about 0.5 million tons of sewage a day are dumped into Iraqi rivers, resulting in water supply contamination. This especially concerns southern cities that use the rivers as their primary water sources (Korzeniewski 2006).

The prevalence rate of the pathogenic *E. histolytica* is higher than the non-pathogenic *E. dispar* and *E. moshkovskii* in the present study. Similar results were reported in asymptomatic individuals in Yemen, Mexico, and Japan (Tachibana et al. 2000; Ramos et al. 2005; Al-Areeqi et al. 2017); the latter two studies did not estimate the rate of *E. moshkovskii* infection. Similarly, the prevalence rate of *E. histolytica* was higher than the infection rate with nonpathogenic species in symptomatic subjects in the United Arab Emirates, Malaysia, and northeast India. Additionally, the studies conducted in populations living in south-west Iran, Cairo, Gaza Strip, and Barcelona did not determine the rate of *E. moshkovskii* infection (Al-Hindi et al. 2005; Pestehchian et al. 2011; Ngui et al. 2012; Rodulfo et al. 2012; Anuar et al. 2013; Elbakri et al. 2013; Nath et al. 2015; Roshdy et al. 2017).

The only study which discriminated among the three species of *Entamoeba* in Iraq was conducted by D'asheesh (2016) in Diwanyha city, south-central Iraq; the study involved symptomatic diarrheal patients. D'asheesh's results differed from the present study by reporting the higher prevalence rates of *E. dispar* than *E. histolytica*. Similar results were recorded in the central and north-west regions and the Kurdistan province of Iran; Izmir, Turkey; Australia; and north--west Ethiopia (Dagci et al. 2007; Fotedar et al. 2007b; Mojarad et al. 2010; Fallah et al. 2014; Yimer et al. 2017; Bahrami et al. 2019).

The present study reported the lowest rate of infections by *E. moshkovskii*; similar results were documented in western Iran, northeast India, Malaysia, Diwanyha, and south-central Iraq (Ngui et al. 2012; Nath et al. 2015; D'asheesh 2016; Bahrami et al. 2019).

In conclusion, the current study finds that 7.4% of individuals who live in Erbil City, where amoeba infections are endemic, carry intestinal *Entamoeba* species, asymptomatically. The incidence rate of *E. histolytica* was higher than the incidence rate of *E. dispar* or *E. moshkovskii* among asymptomatic carriers. In the present study, *E. histolytica* and *E. dispar* were reported as single or mixed infections; only three cases of *E. moshkovskii* were documented as mixed infections with both *E. histolytica* and *E. dispar*.

ORCID
Shler Akram Faqe Mahmood https://orcid.org/0000-0001-5635-1422

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki – Ethical Principles for Medical Research, revised in 2008, and was approved by the Ethics Committee of Hawler Medical University.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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