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Molecular Detection of a Pathogenic *Entamoeba* among Symptomatic Children in Eastern Kurdistan of Iraq

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Abstract

Entamoeba histolytica infects the large intestine of humans, causing a spectrum of clinical appearances ranging from asymptomatic colonization to severe intestinal and extra-intestinal disease. The parasite is identical microscopically to commensal nonpathogenic amoeba. To detect the pathogenic *Entamoeba* and estimate the precise prevalence of the parasite among the symptomatic pediatric population using molecular techniques. 323 fecal samples were collected from symptomatic children admitted to Sulaimani Pediatric Teaching Hospital, Sulaimaniyah Province, Iraq, from June to October 2021. A structured, validated questionnaire was prepared and used to report participants' gender, residency, and drinking water source. Then, stool samples were microscopically examined, and the positive samples were submitted to molecular analysis by amplifying the 18s rRNA gene using nested PCR to differentiate *E. histolytica* from other nonpathogenic *Entamoeba*. Finally, gene sequences were done to confirm the species. Microscopically, 58 positive samples represented *Entamoeba* species infection rate of 18% among symptomatic patients. However, only 18 samples were positive for *E. histolytica* based on molecular methods, which accounts for 31% of the positive by microscopy and 5.6% among the 323 symptomatic populations. NCBI, available in their database, gives the gene sequence and accession number. Patients' sociodemographic data and water sources were directly related to the infection rate. Classical microscopic examination provides a misleading profile about the prevalence of *E. histolytica* in an endemic region that might lead to unnecessary treatments and a lack of appropriate management for patients.

K e y w o r d s: *Entamoeba histolytica*, molecular study, microscopical examination, endemic disease

Introduction

The human gastrointestinal tract (GIT) is the habitat of numerous assemblies of microbes, including bacteria, protozoa, viruses, and fungi (Wilson et al. 2019). The main species of the genus *Entamoeba* that reside in humans GIT are *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba polecki*, *Entamoeba bangladeshi*, and *Entamoeba hartmanni* (Cui et al. 2019).

Morphologically, *E. histolytica*, *E. dispar*, and *E. moshkovskii* look identical; however, *E. histolytica* is the occasional factor of amoebiasis infection (Cui et al. 2019). *E. histolytica* is more prevalent in underdeveloped nations with poor sanitation because it can spread through contaminated food and water, although it is less prevalent in modern nations with better sanitation and hygiene (Marie and Petri Jr 2014). Through boosted travel and immigration to developed countries, infection is becoming widespread in non-endemic areas (Marie and Petri Jr 2014). *E. histolytica* is the reliable protozoa of amoebiasis and persists as one of the main parasites that cause mortality across the board. Usually, the infection is asymptomatic, but the parasite has a potent pathogenic scope. Cystic forms are transmitted by consuming contaminated food, lying in the terminal ileum, and giving rise to invasive trophozoites (Marie and Petri Jr 2014).

E. histolytica is most vivid in the large intestine without causing symptoms, perhaps due to unknown signals. The trophozoites invade the mucosa and epithelium, causing intestinal amoebiasis. It has mastery over different mechanisms of pathogenicity for adhering to the intestinal epithelium and breaking down extracellular matrix proteins, then leading up to tissue lesions that breed to abscesses and a host acute inflammation response (Marie and Petri Jr 2014). Diverse diagnostic appliances are available when amoebiasis is scented, and microscopic stool examination is among the most

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common and applicable. However, the disadvantage is that it is not as accurate as molecular techniques (Gupta et al. 2022). The draft genome sequence of *E. histolytica* (strain HM1: IMSS) was initially released and analyzed in 2005 (Loftus et al. 2005) and later reconstructed and re-annotated (Lorenzi et al. 2010).

In a research conducted in Sulaimaniyah, Iraq, by microscopy in 2010, it was ascertained that 39% of the stool samples were infected with *Entamoeba* species (Ali and Mohammed 2010). Fast forward to 2020, the prevalence rate among symptomatic children was determined to be 16.1% (Mohammed et al. 2022). Before these findings, in 2014, implementing the microscopic wet mount technique with iodine highlighted the presence of *Entamoeba* species infection in 12.9% of the examined cases (Abdullah et al. 2020). No molecular study has been conducted in Sulaimaniyah regarding *E. histolytica* identification.

The expansion of the *Entamoeba* genome has also furnished some pleasant and valuable input for the parasite's growth and spread-out structures (Carrero et al. 2020). The evaluation of the global sense of amoebiasis by the World Health Organization (WHO) designated that about 500 million people were getting the parasite; however, 50 million blokes whole world are infected with *E. histolytica*, with an annual death rate of 40,000 to 100,000 (dos Santos Zanetti et al. 2021). This high infection level is probably an effect of a false positive diagnosis with the morphologically identical, nonpathogenic *E. dispar*/*E. moshkovskii*, and polymorphic nuclear white blood cells and macrophages in the fecal specimens (Petri Jr and Chadee 1996). For that reason, new modes have been improved, making superior identification between pathogenic *E. histolytica* and nonpathogenic *Entamoeba*.

In whatever way, our mastery of human amoebiasis disease burden and epidemiology has encountered a spectacular shift over the last two decades according to molecular interpretation (Carrero et al. 2020) and molecular-based manner, such as polymerase chain reaction (PCR), evolved trial qualitative of the object *E. histolytica* DNA (Paul et al. 2007; Sugeçti 2018). However, there needs to be more molecular records regarding this parasite in Sulaimaniyah City, Iraq. Thus, this study aimed to fill this gap and report the precise occurrence of the pathogenic *Entamoeba* in this endemic region.

Experimental

Materials and Methods

Study design and setting. This cross-sectional study collected stool samples from symptomatic children (n = 323) admitted to Sulaimani Pediatric Teaching Hospital, Sulaimaniyah City, Iraq, from June to October 2021. Individuals suffering from chronic disorders with microbial infections were excluded from the research. Fecal samples were microscopically analyzed and cultivated for harmful gut bacteria such as *Campylobacter*, *Shigella*, *Escherichia*, and *Salmonella* species. Positive samples for such bacteria were removed from the research, but diarrheal specimens (0.5 g) that were microscopically positive for *Entamoeba* were stored at –80°C for further molecular investigation.

Structured questionnaire. Following acquiring verbal and written approval from parents, we devised a structured questionnaire in the Kurdish language. This was then distributed to the participants or their guardians for completion. The questionnaire was designed to gather data on the participants' gender, place of residence, age, and the water they usually consume.

Study protocol. ***Microscopical examination.*** Each stool sample was directly examined microscopically using the iodine and saline wet mount technique to demonstrate *Entamoeba* species trophozoites and cysts.

Molecular detection. Genomic DNA of *Entamoeba* species was extracted from collected stool samples using QiaAmp® Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturing procedure. A purified concentrated DNA was eluted from a spin column silica membrane using the low salt buffer, and a nano-spectrophotometer was used to measure the final concentration of the DNA. Amplification of DNA was performed, targeting the 18S rRNA gene using a nested PCR technique. In the 1st PCR round, the primers amplified 897 bp of the 18S rRNA gene targeting *Entamoeba* species. In contrast, in the 2nd round, the inner primers targeted *E. histolytica* and amplified 439 bp of the gene. The primers targeting the 18S rRNA gene used in the current study were adapted from previous studies (Faqe Mahmood and Mustafa 2020) and established for specificity by the Basal Local Alignment Search Tool (BLAST). Next, amplification of DNA was achieved using a thermal cycler (Techne Ltd., UK) in a reaction volume of 20 µl, which contained 10 µl Hot Start Master Mix (Genetbio Co., Ltd., Republic of Korea), 2 µl DNA template, 2 µl both forward/reverse primers (10 ρmole for each), and 6 µl water. The conditions of PCR running were one cycle of initial denaturation at 95°C for 10 min, followed by denaturation for 30 cycles at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 60 sec with a final extension at 72°C for 5 min. In the second PCR round, the same cycling conditions were run except that the annealing temperature changed to 52°C, and cycle numbers increased to 35 cycles. Negative/positive control for *E. histolytica* DNA (HM-1: IMSS; Kurdistan Biomedical Science University, Sanandaj, Iran) was run in both PCR rounds.

The second PCR products of each sample, negative and positive controls, and a 100 bp DNA marker (Promega Corporation, USA) were electrophoresed in 1% agarose gels, then stained with 0.2 mg/ml of ethidium bromide (Sigma-Aldrich®, Merck KGaA, Germany) using a 1×Tris-boric acid-EDTA buffer (TBE) and visualized with gel documentation under UV light.

Later on, to ensure that the correct *E. histolytica* gene was obtained, the accuracy and reliability of the molecular results were confirmed by running positive and negative samples in each round of PCR. A single sample was arbitrarily nominated and sequenced with primers in both forward/reverse directions by BigDye terminators and an Applied Biosystems™ 3730xl sequencer (Thermo Fisher Scientific, Inc., USA). The resulting nucleotide sequences were manually edited and submitted to GenBank.

Statistical analysis. The descriptive data was analyzed using Statistical Package for the Social Sciences (IBM® SPSS® Statistics for Windows, Version 23.0, IBM Corp., USA). Results are expressed as frequencies and percentages. Fisher exact test and chi-square test were used to compare variables. A $p<0.05$ was regarded as statically significant, while $p<0.001$ was considered highly significant.

Results

In the current study, among 323 collected samples, 179 (55.42%) were from males, and 144 (44.58%) were from females. Most participants were from urban areas (50.8%), used bottled water (50.8%), and belonged to the age group 1–3 years (43.7%) (Table I).

The Lugole's iodine wet mount staining of a stool sample using direct microscopic examinations showed cystic stage of *Entamoeba* species (Fig. 1) that revealed 58 (18%) positive samples among symptomatic patients. Consequently, infection rates of *Entamoeba* species were higher in male patients (18.4%) compared to females (17.4%) ($p<0.05$). However, higher rates of infections among patients who lived in rural areas (22.6%) were reported than those in urban areas (13.4%) ($p>0.05$). Meanwhile, participants who drank tap water showed higher (20.8%) rates of infection than those who had bottled water (15.2%) ($p>0.05$). Moreover, the results revealed higher infection rates among age group 1–3 years (23.4%), followed by 4–6 years (14.2%), then 7–9 years (11.5%), and the lowest rate of infection was in >10 years, while infants <1-year showed no infection with *Entamoeba* species (Table I).

Table I
Potential risk factors associated with *Entamoeba* species infection among symptomatic children.

Variable	Frequency	Positive	Negative
	Number (%)		
Gender			
Male	179 (55.42)	33 (18.4)	146 (81.6)
Female	144 (44.58)	25 (17.4)	119 (82.6)
p-value	0.884		
Residency			
Urban	164 (50.8)	22 (13.4)	142 (86.6)
Rural	159 (49.2)	36 (22.6)	123 (77.4)
p-value	0.042*		
Water source			
Tap water	159 (49.2)	33 (20.8)	126 (79.2)
Bottled water	164 (50.8)	25 (15.2)	139 (84.8)
p-value	0.246		
Age group (years)			
<1	5.0 (1.5)	0.0 (0.0)	5.0 (100)
1–3	141 (43.7)	33 (23.4)	108 (76.6)
4–6	113 (35)	16 (14.2)	97 (85.8)
7–9	61 (18.9)	7.0 (11.5)	54 (88.5)
≥10	3.0 (0.9)	2.0 (66.7)	1.0 (33.3)
p-value	0.032*		
Total	323 (100)	58 (18)	265 (82)

* – significant difference using chi-square test

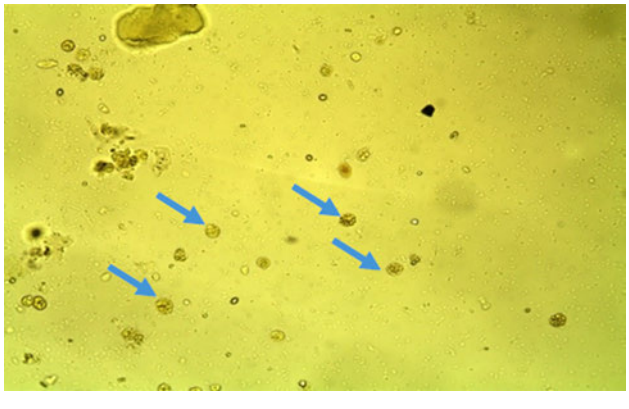


Fig. 1. The Lugol's iodine wet mount of a stool sample showed cystic stage of *Entamoeba* species (blue arrows) applying a light microscope at a magnification of 40×.

Molecular amplification of the 18S rRNA gene showed that only 18 (31%) samples among the 58 positive isolates by microscopy were *E. histolytica* (Fig. 2). Infection rates of *E. histolytica* were significantly higher among females than males ($p<0.05$). Similarly, patients who lived in rural regions showed higher infection rates than those in urban areas ($p<0.05$). Higher but not significant rates of infections were reported in children who drank tap water than those who drank bottled water. Infections with *E. histolytica* were higher among the age group 1–3 (9.9%) than those older or younger (Table II). After submitting the obtained DNA sequences to NCBI, *E. histolytica* amplicon was given

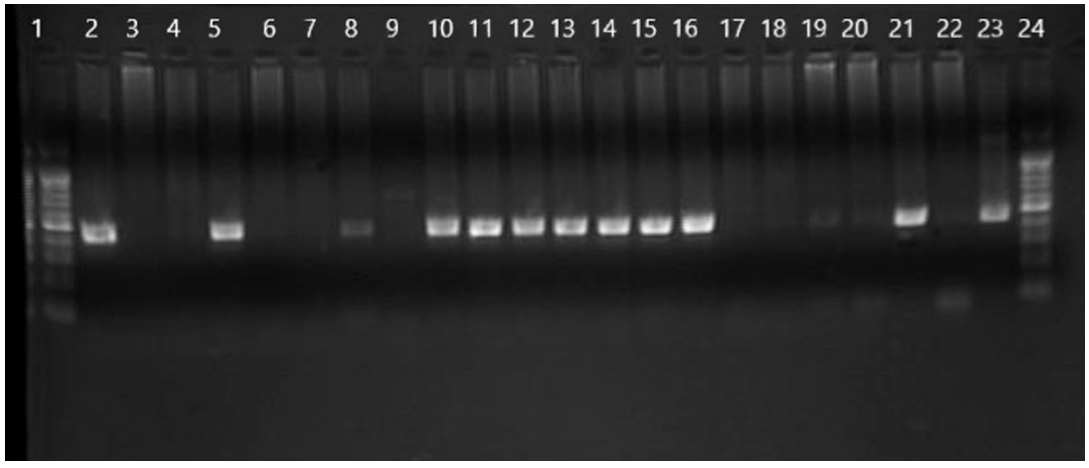


Fig. 2. Shows agarose gel electrophoresis for nested PCR products stained with 0.2 mg/ml ethidium bromide, using primers species specific for *Entamoeba histolytica*. Positive samples reveal 439 bp bands at lanes 2, 5, 8, 10–16, 19, and 21. Lane 23 represented positive control, while lane 22 represented negative control, and lane 1 is the 100 bp DNA marker.

a specific accession number (OR388086). The BLAST sequence analysis from NCBI showed the *E. histolytica* amplified gene sequence product was 99.77% (https://www.ncbi.nlm.nih.gov/nuccore/Mg706986), which matches the *E. histolytica* sequence at GenBank with accession number LC259410.1.

Table II
Potential risk factors associated with *Entamoeba histolytica* infection among microscopic positive symptomatic children.

Variable	Frequency	Positive	Negative
	Number (%)		
Gender			
Male	33 (18.4)	3.0 (1.7)	30 (51.57)
Female	25 (17.4)	15 (10.4)	10 (17.24)
p-value	0.001**		
Residency			
Urban	22 (13.4)	5.0 (3.0)	17 (29.31)
Rural	36 (22.6)	13 (8.2)	23 (39.65)
p-value	0.045*		
Water source			
Tap water	33 (20.8)	11 (6.9)	22 (37.93)
Bottled water	25 (15.2)	7.0 (4.3)	18 (31.03)
p-value	0.299		
Age group (years)			
<1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
1–3	33 (23.4)	14 (9.9)	19 (32.75)
4–6	16 (14.2)	1.0 (0.9)	15 (25.86)
7–9	7.0 (11.5)	2.0 (3.3)	5.0 (8.62)
≥10	2.0 (66.7)	1.0 (33.3)	1.0 (1.72)
p-value	0.004**		
Total	58 (18)	18 (31)	40 (69)

* – significant difference and
** – highly significant difference using chi-square test

Discussion

The screening for symptomatic populations through microscopic examination is valuable. Most nations use microscopic examination as the primary method for diagnosing amoebiasis. This technique identifies hematophagous trophozoites and tetra-nucleated cysts in fecal samples (van Lieshout and Verweij 2010).

In this study, the total infection rate for *Entamoeba* species was 18%, that is higher than that reported in Sulaimaniyah, Iraq, in 2014 (12.93%) (Abdullah et al. 2020). Interestingly, the lowest infection rate was recorded in 2020, possibly due to the COVID-19 pandemic in Iraq, which caused people to avoid hospitals and health institutions for fear of infection (Flaih et al. 2021). Regarding gender, higher rates of infection were reported in males (18.4%) than females (17.4%); however, in 2021, a resurgence occurred with a positive testing rate of 21.68% (562/2,592) for *Entamoeba* in Duhok, Iraq, and males exhibited a higher infection incidence than women (67.43% vs. 32.56%) (Hasan et al. 2022). This aligns with the results of another recent study in Sulaimaniyah, where males had a higher prevalence of infection (17.8%) than females (14.3%), without significant differences between them (Mohammed et al. 2022). Also, similar findings were observed in Al-Qadisiya province, Iraq, where 58.3% of *Entamoeba* species infections were in males and 41.6% in females (Al-Kaeebi and Al-Difaie 2016). This might be attributable to males' behavioral activities at this age and more exposure to *Entamoeba* species infection sources than females, such as more frequent ingestion of contaminated outdoor food/water and interaction with sick persons. The X chromosome, of which women have two and men only one, contains many immune-related genes. This could give females an immunological advantage over males.

Regarding residency, the infection rate in rural populations was 22.6%, which was much higher than in urban populations (13.4%), which might be due to health awareness and using unclean water sources for drinking. Unexpectedly, in Diyala City, Iraq, 73% of individuals infected with *Entamoeba* species were from the urban population, whereas only 27% were from the countryside (Kadhem et al. 2022). On the other hand, the rate between tap water users was 20.8%, greater than that of bottled water users (15.2%); because of the low socioeconomic condition of the population, unsafe water processing may even occur in this situation. The top-level age of *Entamoeba* species infection was 1–3 years old (23.4%). This might be related to the fact that this age group was the most active in their physical development, with continuous exposure to pathogens and contamination, weak immune systems, and lack of hygiene. This outcome matches the research conducted in Al-Qadisiya, Iraq (Al-Damerchi and Al-Ebrahimi 2016). A meticulous microscopic study conducted in Duhok City, Iraq, revealed that the highest infection rates (54.09%) were predominantly found among children aged 1–10 years (Hasan et al. 2022).

While microscopic diagnosis offers insight into the presence of *Entamoeba* species, it may not accurately distinguish between this pathogen and closely related nonpathogenic species like *E. dispar* or *E. moshkovskii*. In cases where differentiation is crucial, PCR-based assays could provide a more reliable solution (Tanyuksel and Petri Jr 2003). PCR detection for *Entamoeba* species involves amplifying the extrachromosomal rRNA gene, a conserved, highly repetitive gene with around 200 copies per cell. It makes it more suitable for detecting pathogens than single-copy gene fragments in DNA (Faqe Mahmood and Mustafa 2020). The QIAamp® DNA Stool Mini Kit's extraction protocol, specifically designed for extracting protozoan DNA from diarrheic stool samples, offers a simple and cost-effective alternative to conventional methods while avoiding hazardous reagents (Hawash 2014). In the present study, nested PCR was performed on the microscopically positive samples to determine the precise quantity of the illness.

Consequently, 18 out of 58 stool specimens tested positive for *E. histolytica*, yielding a 31% success rate by molecular method over the microscopy (Khairnar and Parija 2007). The incidence of *E. histolytica* infection by molecular was 5.6% in the Sulaimaniyah population. The observed discrepancy in microscopic detection suggests a misdiagnosis, primarily due to the morphological similarities between *E. histolytica* and nonpathogenic species such as *E. dispar*, *E. moshkovskii*, and *E. Bangladeshi*.

Furthermore, the trophozoites stage of these organisms may occasionally resemble polymorphonuclear cells and macrophages, contributing to potential diagnostic inaccuracies. Therefore, the variation in these ratios could be attributed to these factors. Currently, in Iraq, molecular studies for the identification of *E. histolytica* and its prevalence among symptomatic patients were reported in Erbil (6%), Diwaniyha (44.3%), Baghdad (7%) (Hussein et al. 2015), and Al-Najaf (24%) (Yousif 2014; Hussein et al. 2015; Al-Khalidi 2016; Faqe Mahmood and Mustafa 2020). The reasons behind these variations in the infection rate might be several tested samples in the screening study, time of sample collection, different environmental conditions, diagnostic methods used, and variations of temperature between the seasons.

Significant differences were observed according to gender, as males showed a 1.7% lower infection rate than females (10.4%), which agrees with a study done in rural Malaysian areas (Ngui et al. 2012). These outcomes might be related to the fact that females consume more water than men, and the source of water supply may be unsafe (Atabati et al. 2020). It is hypothesized that invasive amoebiasis and infection rate is influenced by host factors, including host gene expression (Thibeaux et al. 2013). Also, the host immune response to infections and sex-related hormone variations in males and females might cause differences in the infection rates reported in the current study among the genders (McClelland and Smith 2011). Additionally, inadequate maternal knowledge and handling of domestic animals may react to this condition, as having close contact with domestic animals raises the risk of *E. histolytica* infection (Afzali et al. 2018).

Depending on residency, the prevalence of *E. histolytica* infection varies. According to several research studies, rural regions have the highest frequency of infections. In this study, the frequency of *E. histolytica* infection was 8.2% vs. 3% compared to 42% vs. 23% in a study done in Bangladesh (Ferdous et al. 2014). Rural areas in emerging nations like Nigeria, India, and Brazil observed similar results that might be due to inadequate waste management and limited availability of clean water, and rural regions are more prone to have dispersed frequencies. However, the prevalence is lower in industrialized nations where access to improved sanitation and clean water is more common.

A stinting water supply is a prohibition factor for *E. histolytica* infection, so the dispersal of the parasite is related to tap water and bottled water (Jaeffer 2011). The extreme average was announced among patients who consumed tap water (6.9%), while 4.3% was found in patients who used bottled water as a drinking source, which showed the minimum infectivity rate. Compared with that research in Al-Qadisiya, Iraq, in 2008, the rate was 17.2% for users of unsafe water supply (Hadi and Faraj 2008). *E. histolytica* cysts have a highly prospective chance for dissemination through drinking water

because they resist the harsh environment. Also, cysts can penetrate physical barriers in remediated water fountains (Sukprasert et al. 2008).

Significant differences were reported regarding age group, as the highest infection rate was recorded in the age groups of ≥ 10 years and 1–3 years. In contrast, those aged < 1 year had the lowest rate, consistent with the study conducted in Saudi Arabia (Al-Shammari et al. 2001). These outcomes are mainly related to the fact that parents are responsible for their hygiene (Al-Saeed and Issa 2006), as the highest infection rate may be ascribed to defecation practices because these groups of children are fully independent in outdoor activities and feeding, in addition to their poor level of education (Omar et al. 1991). The study was completed despite several challenges, such as the limited sample size, time constraints, and hurdles in obtaining information from participants.

Conclusions

Patients' gender, age, and residency are directly correlated to the infection rate of *E. histolytica.* Males in the age group 1–3 years and those living in urban areas are more vulnerable to infection than others. Also, the water source is considered a risk factor for enhancing the appearance of disease among the population. Moreover, molecular methods are gold standard techniques for tentative detection and diagnosis of *E. histolytica*. We suggest identifying *E. histolytica* in fruits and vegetables to stop the spread of invasive amoebiasis and identify the virulent strains of *E. histolytica*. Patients with acute amoebiasis should dispose of their waste appropriately, especially in rural regions. More molecular study is required to choose specific virulence genes, such as the lectin, CP1, CP5, and the *Entamoeba* pore genes, and compare their presence in samples with and without symptoms.

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Ethical statement

The proposal of the current study was approved by the Ethics Committee at the College of Medicine, University of Sulaimani, Sulaimaniyah, Iraq, with approval number (No. 300 on October 05, 2022). All parameters were done according to the Declaration of Helsinki. Participants'/their guardians' written consent was obtained before starting the study.

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Author contributions

SJA: Conceptualization, methodology, data analysis, writing the original manuscript. SAA: Research administration, validation, resources, original manuscript editing, supervision. Both authors agreed to submit the manuscript.

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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