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RAPD analysis of genetic diversity in *Entamoeba spp*. isolates from clinical samples

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Abstract

The current study investigated the prevalence and molecular characteristics of Entamoeba spp. from 300 stool samples (200 from patients and 100 from healthy controls) collected between October 2023 and February 2025. Direct microscopic examination revealed *Entamoeba spp*. in 110 patient samples (55%), while culture confirmed positivity in 65 cases (32.5%); all control samples were negative. Infection was more prevalent among males (64.5%) than females (35.4%), with participants' ages ranging from 1 to 70 years. The highest prevalence was recorded in the <10 years age group (38.1%), while the lowest was in the 30–40 years group (9%). Infections were frequently associated with mucus and bloody stools. Seasonal variation showed the highest prevalence in December (18.1%) and the lowest in February (1.8%). Among culture media, Entamoeba medium showed the longest survival period of *Entamoeba* (10 days), followed by TYI-S-33 (8 days), PEHPS (7), CLUPS (5) respectively. while the shortest survival was recorded in Blood.A (3 days). PCR analysis revealed *E. moshkovskii* as the most prevalent species (18%), followed by E. histolytica (8%). RAPD technique demonstrates variations in banding patterns among genetically distinct individuals. The present study identified four distinct *E. histolytica* genotypes, mainly in rural areas, and eight *E. moshkovskii* genotypes with wide geographic distribution across Basrah province.

Keywords: *Entamoeba,* Cultivation, PCR, RAPD strain, Genotype

Introduction

Entamoeba, a protozoan parasite, infects various animals, including reptiles, birds, and amphibians. Seven species have been found in human guts, including *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. polecki,* and *E. hartmanni,* with *E. gingivalis* found in the buccal cavity. (El-Dib and Khater, 2022) .*Entamoeba* infections occur globally, especially in low-income countries with inadequate public health infrastructure, making accurate differential diagnosis crucial to distinguish it from similar species. (Servián *et al.,* 2023). *E moshkovskii*, previously considered a free-living environmental organism, recent evidence confirms its role as a human pathogen with significant prevalence in some populations (Heredia *et al.,* 2012). *E. histolytica* is the most common intestinal protozoan parasite causing amoebiasis, causing diarrhea and dysentery. It can invade the intestinal mucosa, form liver and lungs abscesses, and infect humans, with other species also posing risks. (Roure *et al* .,2019).Laboratory diagnosis for *Entamoeba* spp. in humans primarily involves microscopically examining stool samples, but this method is insufficient for distinguishing between species (Argy *et al.,* 2022). The use of polymerase chain reaction (PCR) for molecular diagnosis is crucial due to potential mistreatment of patients, requiring accurate and global implementation. (Bahrami *et al.,* 2019) The study analyzed genetic variability in *Entamoeba histolytica* using random amplified polymorphic DNA (RAPD) using arbitrary primers due to its intrinsic characteristics (Gomes *et al.,* 2000). The Random Amplified Polymorphic DNA (RAPD) technique, also known as Arbitrarily Primed PCR (AP-PCR), is a cost-effective and efficient method for epidemiological studies, enabling rapid genetic fingerprinting of microbial isolates, facilitating strain differentiation and infection source tracking. (Williams *et al*.,1990;Welsh & McClelland, 1991a) . The prevalence of *E. histolytica, E. dispar,* and *E. moshkovskii* was determined using three diagnostic methods: microscopic examination, cultivation, and PCR and Real-Time PCR technique. (Al-Hilfi,2020) . *E. moshkovskii*, previously thought to be a free-living environmental amoeba, was found in 1961 in a symptomatic patient in Laredo, Texas, causing diarrhea, weight loss, and epigastric pain. (Dreyer, 1961). The aim of this study was to identify *Entamoeba* spp. in human stool samples and assess

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their genetic diversity in Basrah using RAPD genotyping, supported by microscopic, culture, and molecular (PCR) techniques.

Materials and Methods

Samples collection

To obtain *Entamoeba* spp., total of 300 stool samples were collected from various regions of Basrah province between October 15, 2023, and February 28, 2025, including 200 from patients and 100 from healthy individuals. Samples were obtained from hospitals and private clinics, covering ages from <10 to 70 years. All samples were examined microscopically, and DNA was extracted from the 200 patient-derived samples for molecular analyses.

Identification of *Entamoeba* spp.

Microscopic examination

A total of 300 stool samples were examined microscopically using the direct wet smear technique for the detection of Entamoeba spp. in both trophozoite and cyst stages (Garcia & Ash, 1979; Garcia et al., 2018, Hamid et al., 2019).

Identification of parasite stages

Trophozoite stage

Fresh stool samples were examined for trophozoites using the wet mount technique. A small portion of stool was placed on a glass slide, mixed with a drop of physiological saline, and covered with a coverslip. The preparation was observed under a light microscope at 10× and 40× magnifications, which preserved parasite motility and allowed the visualization of trophozoite morphology (Garcia *et al.,* 2018).

Cyst stage

The Cyst stages were identified by direct examination using prepared iodine solution "An iodine solution was prepared by dissolving 1 g iodine and 2 g KI in distilled water, adjusted to 100 ml, and stored in a sealed amber bottle at room temperature until use.

Use of Eosin Y Stain

In selected fresh samples, Eosin Y stain was applied as a complementary tool to improve contrast in trophozoite detection. This non-specific stain provided a pink background, enhancing visualization of unstained or poorly contrasted parasites and aiding the detection of degenerated forms (Tan et al., 2010, Ahmed et al., 2018).

Preparation of media for *Entamoeba spp.*

A total of 300 stool samples (200 from patients and 100 from healthy controls) were cultured under aseptic conditions using modified media formulations (Gonzalez-Salazar et al., 2018).

1. Modified PEHPS medium

Modified PEHPS medium was prepared by using chicken liver extract (10 g liver blended with 300 ml distilled water, filtered) in place of beef liver/pancreas. The medium contained peptone (10 g), cysteine (1 g), ascorbic acid (0.2 g), glucose (6 g), KH_2PO_4 (0.6 g), and 250 ml chicken liver extract, with the volume adjusted to 1000 ml (pH 6.8–7.0). The medium was sterilized by autoclaving at 121°C for 15 min and stored at 20°C until used (Bradley et al., 1996, Jam et al., 2018).

2. Modified CLUPS medium

Modified CLUPS medium (Gonzalez-Salazar et al., 2018) was prepared with bovine lung extract (10 g/300 ml, filtered) in place of the original lung–pancreas extract. The medium contained peptone (10 g), cysteine (1 g), ascorbic acid (1.26 g), glucose (6 g), KH_2PO_4 (0.6 g), and 250 ml lung extract, adjusted to 1000 ml (pH 6.8–7.0), sterilized at 121°C and stored at 20°C.

3. Modified TYI-S-33 medium

Modified TYI-S-33 medium (Gonzalez-Salazar et al., 2018) was prepared by substituting chicken liver extract (10 g/300 ml, filtered; 250 ml added) for liver infusion broth. Ferric citrate, casein, and K_2HPO_4 were omitted. The final formulation contained peptone (20 g), cysteine (1 g), ascorbic acid (1 g), glucose (10 g), and KH_2PO_4 (0.6 g), adjusted to 1000

ml (pH 6.8–7.0), sterilized at 121°C for 15 min, and stored at 20°C.

4. Entamoeba medium

Was prepared by dissolving 33.0 g of commercial powder in 1000 ml distilled water, boiled until complete dissolution, sterilized at 121°C for 15 min, and dispensed into slant tubes. After solidification, sterile human serum was added to the surface together with 0.01 g of autoclaved rice powder to enhance trophozoite growth, then stored at 4°C (Cleveland and Sanders, 1930).

5.Blood agar

"Blood agar was prepared by dissolving 2.8 g nutrient agar in 90 ml distilled water, adjusting the pH to 6.8–7.0, and bringing the volume to 100 ml. The medium was sterilized at 121°C for 15 min, cooled to 45–50°C, and mixed with 5 ml fresh human blood. The mixture was poured into slanted sterile tubes and stored at 4°C until use.(Maurya, 2010).

Erythromycin and nystatin (5 mg/ml) were sterilized by filtration and stored at 4°C . Two hundred patient samples were inoculated into media with antibiotics (0.25 mg/ml) and 5 ml fresh human serum, incubated at 36°C for 48 h, and parasite growth was confirmed microscopically with trophozoites counted using a Neubauer chamber (Elnazeer et al., 2016)

Molecular identification of *Entamoeba spp.*

DNA extraction

Genomic DNA was extracted from 200 stool samples using the Presto™ Stool DNA Extraction Kit (Geneaid, Taiwan) following the manufacturer's instructions. Extracted DNA was stored at –20°C until analysis.

Identification of Entamoeba spp. by Polymerase Chain Reaction (PCR)

Genomic DNA extracted from each stool sample was subjected to PCR amplification using species specific primers targeting the 18S rRNA gene. For Entamoeba histolytica, amplification was carried out with primers Enta F (5′ ATGCACGAGAGCGAAAGCAT 3′) and EH R (5′ GATCTAGAAACAATGCTTCTCT 3′), generating a 166 bp product (Accession no. AB282658.1) as described by Hamzah et al. (2006). Similarly, Entamoeba moshkovskii was identified using primers Enta F and EM R (5′ TGACCGGAGCCAGAGACAT 3′), producing a 580 bp fragment (Accession no. OP452932.1).PCR reactions were performed in a total volume of 25 µl containing 4 µl of DNA template, 1.5 µl of each primer (forward and reverse), 12 µl of GoTaq Green Master Mix (Promega), and 6 µl of nuclease free water. PCR reactions were carried out in a thermocycler under the following conditions: an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles consisting of

denaturation at 94 °C for 30 seconds, annealing at 56 °C for E. histolytica and 54 °C for E. moshkovskii (30 seconds each), and extension at 72 °C for 60 seconds. A final extension step was performed at 72 °C for 5 minutes .PCR products were verified on 2% agarose gel electrophoresis stained with RedSafe and visualized under UV. The expected amplicon sizes were 166 bp for E. histolytica and 580 bp for E. moshkovskii (Hamzah et al., 2006).

RAPD-PCR Protocol for Genetic Differentiation of Entamoeba Strains.The RAPD-PCR amplification was performed following Al-Barzinjy (2015) with minor modifications. Each 20 µl reaction contained 4 µl of DNA template, 4 µl of four primers (1 µl each), 10 µl of GoTaq Green Master Mix, and 2 µl of nuclease-free water. Thermal cycling was carried out with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 95°C for 1 min (denaturation), 36°C for 1 min (annealing), and 72°C for 2 min (extension), with a final extension at 72°C for 10 min.

In this study, eight primers were employed for the RAPD-PCR assay to differentiate Entamoeba strains. Four primers For E. histolytica, the primers included NPI-06 (5'-AAGGCGGCAG-3'), NPE-16 (5'-GGTGACTGTT-3'), NPN-07 (5'-GAGCCCGAG-3'), and NPL-05 (5'-ACGCAGGCAC-3'), as described by Al-Barzinjy (2015). Four primers For E. moshkovskii, the primers used were L15996 (5'-CTCCACCATTAGCACCCAAAGC-3'), Qg1-U (5'-CCAATTAGCACCCAAAGCAGACCTCACCCTG-3'), F21U (5'-GATGTAAAAATAGGATTTAGGG-3'), and M13F (5'-GTTTTCCCAGTCACGAC-3'), according to Gomes et al. (2000). These primers were selected for their efficiency in generating polymorphic banding patterns essential for genetic differentiation between

the two species.

Data analysis and phenogram construction

RAPD-PCR products were separated by agarose gel electrophoresis, and banding patterns were analyzed using ImageJ software (NIH, USA). Genetic similarity among isolates was determined based on band-sharing profiles, and similarity coefficients were calculated according to Dice's formula Dice (1945). Phenograms were constructed using the UPGMA method to illustrate the genetic relationships among isolates.

Results

In the current study the prevalence of *Entamoeba* sp. 55%Microscopic examination of 200 stool samples revealed that 110 (55%) were positive for *Entamoeba* spp., while 90 (45%) were negative. Gender distribution showed a higher prevalence among males, with 71 positive cases (64.5%) compared to 39 in females (35.4%) ($\chi^2 = 9.95$, df = 2, P = 0.0069), indicating statistically significant differences.

Regarding age distribution, the highest infection rate was observed among children under 10 years (G1), with 42 cases (38.1%), followed by patients aged 40–70 years (G5) with 26 cases (23.6%). The 20–30 year age group (G3) recorded 20 cases (18.1%), while the 10–20 year group (G2) accounted for 12 cases (10.9%). The lowest prevalence was noted among the 30–40 year group (G4), with only 10 cases (9%). Stool characteristics analysis revealed that mucus-containing stools represented the highest frequency (90 samples; 45%), followed by bloody stools (45 samples; 22.5%), non-diarrheal stools (38 samples; 19%), and diarrheal stools (27 samples; 13.5%).

The highest prevalence was observed in December at 18.1%, followed by November at 12.7%, March at 10.9%, April and October both at 9%, and July at 8.1%. Lower prevalence rates were recorded in May (7.2%), June (6.3%), September (6.3%), and August (4.5%), with the lowest rate found in February at 1.8%. Microscopic examination of positive stool samples revealed different developmental stages using specific stains (Fig. A–C). Cysts were identified with iodine staining showing clear morphology (A). Trophozoites were observed in saline preparations with irregular shape and visible nucleus (B). Eosin staining enhanced visualization of trophozoites, which appeared pink with distinct morphological details (C). (Fig 1)

In this study, 200 clinical stool samples were analyzed by direct microscopy and culture. *Entamoeba* spp. were detected in 110 samples (55%) microscopically, while 65 samples (32.5%) were positive by culture. All 100 stool samples from healthy individuals were negative by both methods.Five culture media (PEHPS, TYI-S-33, CLUP, Entamoeba medium, and blood agar) were compared for *Entamoeba* spp. cultivation. *Entamoeba* medium was the most effective, maintaining viability up to 10 days, followed by TYI-S-33 (8 days), PEHPS (7 days), CLUP (5 days), and blood agar (3 days). Thus, *Entamoeba* medium showed the longest survival period of *Entamoeba* spp. (Table 1).

Table 1. Cultivation performance of *Entamoeba* Spp. on various culture media

Culture Medium	Days with Observed Positive Growth (+)	General Observations
PEHPS	Day 1 to Day 6; some samples up to Day 7	Growth mostly declined after Day 7; moderate performance
CLUP	Day 1 to Day 5; a few samples reached Day 6–7	Good initial growth; reduced viability after Day 5
TYI-S-33 Medium	Day 1 to Day 7; occasionally up to Day 8	Supported longer growth duration than PEHPS and CLUP, higher number of trophozoites and cysts
Entamoeba Medium	Day 1 to Day 10 in almost all samples	Best-performing medium; sustained stable growth up to 10 days
Blood Agar	Day 1 to Day 3 only	Limited support; unable to maintain viability beyond 3 days

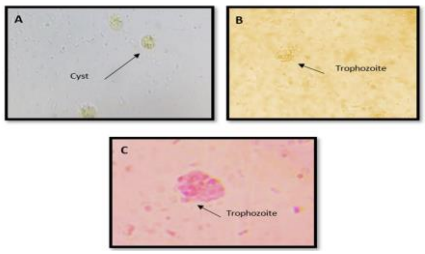

Figure 1. Microscopic images of showing different stages of *Entamoeba* spp in stool sample 100x .(A) Cyst stage observed using iodin stain. (B)Trophozoite stage seen with normal saline. (C) Trophozoite stained with eosin stain , highlighting the trophozoite morphology in pink

DNA extraction

"DNA was extracted from 200 stool samples and verified by Nanodrop to be suitable for further applications

Detection of *Entamoeba spp.* using PCR

Conventional PCR was performed using specific primers targeting the 18S rRNA gene to detect *Entamoeba spp.* The EH and EM primers produced distinct amplification bands of 166 bp for E. histolytica and 580 bp for *E. moshkovskii.*

4.3.3 Molecular identification

Out of the 200 stool samples analyzed, 16 isolates (8%) were identified as *E. histolytica*, while 36 isolates (18%) were confirmed as *E. moshkovskii*. The PCR products were visualized by agarose gel electrophoresis, as shown in) Fig 2& fig 3).

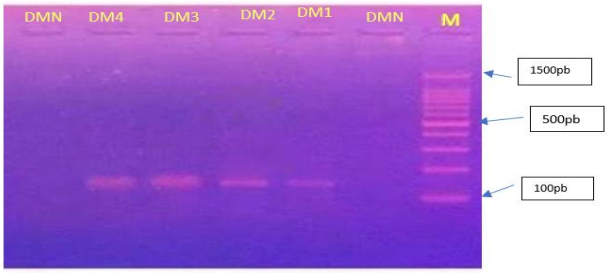

Figure 2. Agarose gel electrophoresis (2%) showing PCR amplicons of the central region of the 18S rRNA gene (166 bp) for E. histolytica using EH primers. Lane M: DNA ladder (100 bp). Four positive bands indicate the presence of E. histolytica isolates, while the DMN lanes are negative.

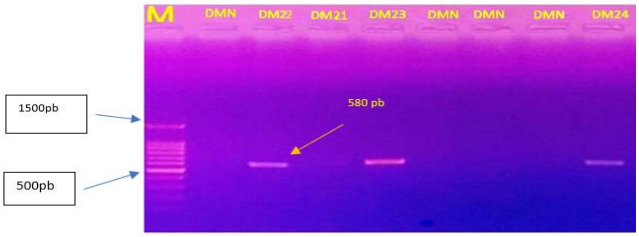

Figure. 3 Agarose gel electrophoresis (2%) showing PCR amplicons of the central region of the 18S rRNA gene (580 bp) for *E. moshkovskii* using EM primers. Lane M: DNA ladder (100 bp). Four positive bands indicate the presence of *E. moshkovskii* isolates, while the DMN lanes are negative.

A- Genetic diversity among *E.histolytica* strain

RAPD-PCR was employed to evaluate the genetic diversity among sixteen E. histolytica strains. The banding profiles revealed four distinct genotypes: Genotype 1 (DM1, DM2, DM4, DM9, DM10, DM11), Genotype 2 (DM5– DM8), Genotype 3 (DM12), and Genotype 4 (DM13–DM16) (Fig. 4; Table 2). Phenogram analysis using UPGMA demonstrated clustering into two major groups with several sub-clusters, reflecting significant intra-species diversity (Fig.5).The genetic distance matrix indicated variable similarity levels among the strains. (Table 3).These findings highlight considerable genetic variability within E. histolytica and confirm the effectiveness of RAPD-PCR for differentiating strains directly from clinical samples .The analysis of E. histolytica strain (DM1–DM16) revealed the presence of four distinct genotypes with differing distributions across geographic locations. Genotype 1 include (DM1, DM2) strains were , observed in urban strain from Al-Qibla , with other urban strain DM9 from Al Hayaniyah and rural strains from Abu Al-Khasib (DM4, DM10, DM11) a, with an estimated 8 bands. Genotype 2 was detected in rural hospital samples (DM5–DM8) from Abu Al-Khasib (Ce), and DM6 urban strain from Al-Jumhuriya showing an estimated 11 bands. Genotype 3 (DM12) was limited to a single strain from Abu Al-Khasib (Abdlyan), with 3 bands. Genotype 4 appeared in four rural strains (DM13–DM16) from Abu Al-Khasib, including Abu Mughirah, and showed 5 bands. Strain DM3 from Al-Fayhaa Hospital (Urban–Educational) was excluded due to a distinct and non-classified profile. (Table 2).

Table 2. Distribution of *E. histolytica* strain by Genotype, Banding Pattern, and Geographic Source.

Genotype	Strain Code	Estimated Band	Geographic Location	Collection Site	Source Type
Genotype 1	DM1	8	Al-Qibla	Private Clinic	Urban
Genotype 1	DM2	8	Al-Qibla	Private Clinic	Urban
Genotype 1	DM4	8	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 1	DM9	8	Al Hayaniyah	Private Clinic	Urban
Genotype 1	DM10	8	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 1	DM11	8	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 2	DM5	11	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 2	DM6	11	Al-Jumhuriya	Private Clinic	Urban
Genotype 2	DM7	11	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 2	DM8	11	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 3	DM12	3	Abu Al-Khasib (Abdlyan)	Private Clinic	Rural
Genotype 4	DM13	5	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 4	DM14	5	Abu Al-Khasib (Abu Mughirah)	Private Clinic	Rural
Genotype 4	DM15	5	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 4	DM16	5	Abu Al-Khasib (Ce)	Private Clinic	Rural
— (excluded)	DM3	Distinct profile	A Karma Ali	Al-Fayhaa Hospital	Rural

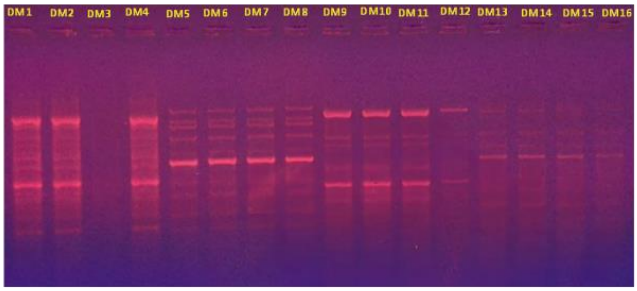

Figure 4. RAPD PCR products from amplification using the primers NPE-16, NPI-06, NPL-05, and NPN-07 for *E. histolytica* (1-16). The gel concentration was 2%.

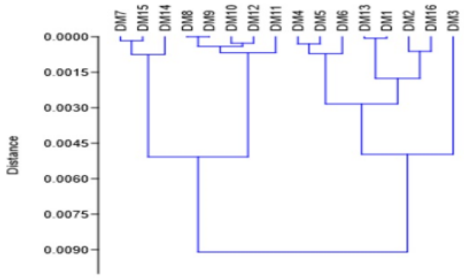

Figure 5. UPGMA phenogram of *E.histolytica* strains based on pairwise band sharing.

Table 3. Genetic distances between *E . histolytica* species samples

Strains	DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10	DM11	DM12	DM13	DM14	DM15	DM16
DM1	0															
DM2	0.000373	0														
DM3	0.001822	0.00145	0													
DM4	0.000955	0.000583	0.000867	0												
DM5	0.00088	0.000507	0.000942	0.000075	0											
DM6	0.001107	0.000734	0.000716	0.000151	0.000227	0										
DM7	0.001195	0.001568	0.003018	0.002151	0.002075	0.002302	0									
DM8	0.002448	0.002821	0.004271	0.003404	0.003328	0.003555	0.001253	0								
DM9	0.002448	0.002821	0.004271	0.003404	0.003328	0.003555	0.001253	0	0							
DM10	0.002518	0.00289	0.00434	0.003473	0.003397	0.003625	0.001322	0.00007	0.00007	0						
DM11	0.002668	0.003041	0.004491	0.003624	0.003548	0.003775	0.001473	0.00022	0.00022	0.000149	0					
DM12	0.002584	0.002957	0.004407	0.00354	0.003464	0.003694	0.001389	0.000126	0.000126	0.000093	0.000094	0				
DM13	0.000016	0.000357	0.001806	0.000939	0.000863	0.001091	0.001212	0.002465	0.002465	0.002534	0.002685	0.002601	0			
DM14	0.001364	0.001737	0.003187	0.00232	0.002244	0.002471	0.000169	0.001084	0.001084	0.001154	0.001304	0.00122	0.001381	0		
DM15	0.001153	0.001526	0.002976	0.002109	0.002033	0.00226	0.000042	0.001295	0.001295	0.001364	0.001515	0.001431	0.001169	0.000211	0	
DM16	0.000527	0.000154	0.001296	0.000429	0.000353	0.00058	0.001722	0.003	0.003	0.00307	0.003221	0.003137	0.000511	0.001891	0.00168	0

B- Genetic diversity among *E.mashkovskii* strains

Thirty-six E. moshkovskii strains were analyzed by RAPD-PCR, revealing eight distinct genotypes and considerable genetic diversity, demonstrating the method's utility for differentiating clinical isolates (Figs. 6 & 8; Table 4).

The eight genotypes of E. moshkovskii were as follows: Genotype 1 (DM17, DM18, DM19, DM22–DM27), Genotype 2 (DM20, DM28–DM31, DM33), Genotype 3 (DM32, DM34), and Genotype 4 (DM21) (Fig. 6; Table 4).Additional E. moshkovskii genotypes are shown in Fig. 12: Genotype 5 (DM35, DM36, DM37, DM38, DM40), Genotype 6 (DM39, DM46, DM47, DM48, DM51), Genotype 7 (DM41, DM42,

DM43, DM44), and Genotype 8 (DM45, DM49, DM50,DM52). (Fig.8) (table 4)The genetic distance matrix revealed variable levels of similarity among the studied strains. (Table 5).Phenogram analysis grouped the 18 E. moshkovskii strains into two major groups : Group 1 (DM33 and DM34) and Group 2, which included 16 strains further divided into six sub-clusters (A–F). Notably, sub-cluster F (DM24 and DM25) showed the highest genetic similarity(Fig .7)

The RAPD banding patterns of E.moshkovskii strains (DM17–DM34) revealed four genotypes with varied geographic distributions. Genotype 1 was the most frequently detected (in DM17–DM19, DM22–DM27), showing 3 bands, and was largely confined to rural areas of Abu Al-Khasib including Abu Al-Khasib (Ce) ,Al-Bahadria.The DM25 strain, though sharing the same genotype, was isolated from Al-Hayyaniyah, an urban region, suggesting a broader distribution of this genotype. Genotype 2 appeared in six strains (DM20, DM28–DM31, DM33), all from rural private clinic and hospitals different regions in Abu Al-Khasib, with 6 bands.Genotype 3 was exclusively found in urban Al-Qibla (DM32 and DM34), with 4 bands.. Genotype 4 was observed only once in a sample from Khamsah Mile (DM21), an urban private clinic, also showing 2 bands (table 4).

"Among E. moshkovskii strains DM35–DM52, four additional genotypes were identified (Fig. 8). Genotype 5 (DM35–DM38, DM40) showed 3 bands and was collected from various locations including., including Abu AL-Khasib (Al-Bahadria, Abu AL-Khasib CE, Abu Mughirah) and Al-Qibla.Genotype 6 included strains DM39, DM46, DM47, DM48, and DM51, all showing 1 bands, predominantly from rural and urabn hospitals and private clinics in Abu Al-Khasib, CE Al-Qibla and El--Ablah . In contrast Genotype 7 was the most frequently detected strains DM41 to DM44 with 2 bands. These strains were mostly from private clinics and hospitals in Khamsah Mile, Junaina, and Abu AL-Khasib CE areas. Genotype 8 consisted of strains DM45, DM49, DM50, and DM52, each showing 3 bands. These strain collected from hospital and private settings All strains existed in different areas in including Abu Al-Khasib CE, Al-Bahadria, Abdalyan . (table 4.)

Table 4. Distribution of *E. mashkovaskii* strain by Genotype, Banding Pattern, and Geographic Source

Genotype	Sample Code	Estimated Band	Geographic Location	Collection Site	Source Type
Genotype 1	DM17	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 1	DM18	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 1	DM19	3	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 1	DM22	3	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 1	DM23	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 1	DM24	3	Abu Al-Khasib (ABahadria)	Private Clinic	Rural
Genotype 1	DM25	3	Al-Hayaniyah	Private Clinic	Urban
Genotype 1	DM26	3	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 1	DM27	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 2	DM20	6	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 2	DM28	6	Abu Al-Khasib (Abu Mughirah)	Private Clinic	Rural
Genotype 2	DM29	6	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 2	DM30	6	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 2	DM31	6	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 2	DM33	6	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 3	DM32	4	Al-Qibla	Private Clinic	Urban
Genotype 3	DM34	4	Al-Qibla	Private Clinic	Urban
Genotype 4	DM21	2	Khamsah mile	Private Clinic	Urban
Genotype 5	DM35	3	Abu Al-Khasib (Al-Bahadria)	Private Clinic	Rural
Genotype 5	DM36	3	Abu Al-Khasib (Ce)	Private Clinic	Rural
Genotype 5	DM37	3	Al-Qibla	Private Clinic	Urban
Genotype 5	DM38	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 5	DM40	3	Abu Al-Khasib (Abu Mughirah)	Hospital	Rural
Genotype 6	DM39	3	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 6	DM46	1	El-Ablah	Hospital	Urban
Genotype 6	DM47	1	Abu Al-Khasib (Muhaijeran)	Private Clinic	Rural
Genotype 6	DM48	1	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 6	DM51	1	Al-Qibla	Private Clinics	Urban
Genotype 7	DM41	2	Khamsah mile	Private Clinic	Rural

Genotype 7	DM42	2	Junaina	Private Clinic	Urban
Genotype 7	DM43	2	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 7	DM44	2	Abu Al-Khasib (Ce)	Hospital	Rural
Genotype 8	DM45	3	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural
Genotype 8	DM49	3	Abu Al-Khasib (Abdlyan)	Private	Rural
Genotype 8	DM50	3	Abu Al-Khasib (Al-Bahadria)	Private Clinic	Rural
Genotype 8	DM52	3	Abu Al-Khasib (Ce)	Abu Al-Khasib Hospital	Rural

Abu Al-Khasib Center= ce

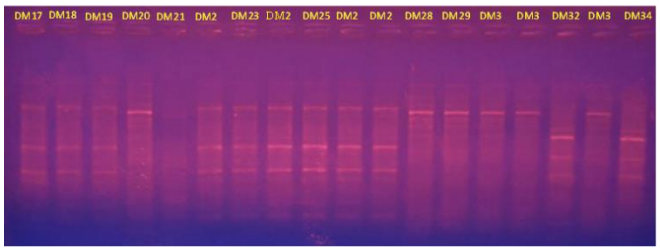

Figure 6. RAPD PCR products of the amplification using these L15996, M13F, Qg1-U , F21U for *E. mashkovskii*(17 -34)The gel was 2% and the DNA dye is RedSafe

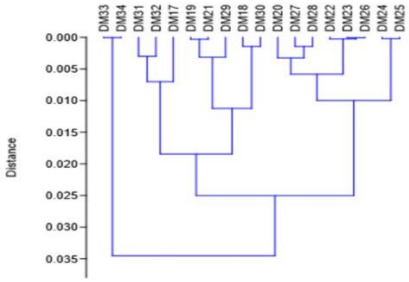

Figure 7. UPGMA phenogram of E. mashkovaskii strains based on pairwise band sharing.

Table 5. Genetic distances between *E. mashkovskii* strains

Strains	DM17	DM18	DM19	DM20	DM21	DM22	DM23	DM24	DM25	DM26	DM27	DM28	DM29	DM30	DM31	DM32	DM33	DM34
DM17	0																	
DM18	0.003919	0																
DM19	0.006491	0.002572	0															
DM20	0.009777	0.005858	0.003286	0														
DM21	0.006562	0.002643	0.000071	0.003215	0													
DM22	0.010757	0.006838	0.004266	0.00098	0.004195	0												
DM23	0.010703	0.006784	0.004212	0.000926	0.004141	0.000054	0											
DM24	0.012577	0.008658	0.006086	0.0028	0.006015	0.00182	0.001874	0										
DM25	0.01253	0.008611	0.006038	0.002753	0.005968	0.001773	0.001827	0.000047	0									
DM26	0.01069	0.006771	0.004199	0.000913	0.004128	0.000067	0.000013	0.001887	0.00184	0								
DM27	0.009181	0.005261	0.002689	0.000596	0.002618	0.001577	0.001523	0.003397	0.003349	0.00151	0							
DM28	0.008839	0.00492	0.002348	0.000938	0.002277	0.001918	0.001864	0.003738	0.003691	0.001851	0.000341	0						
DM29	0.007266	0.003347	0.000774	0.002511	0.000704	0.003492	0.003438	0.005311	0.005264	0.003424	0.001915	0.001573	0					
DM30	0.00426	0.000341	0.002231	0.005517	0.002302	0.006497	0.006443	0.008317	0.00827	0.00643	0.00492	0.004579	0.003006	0				
DM31	0.001298	0.002621	0.005194	0.008479	0.005264	0.00946	0.009405	0.011279	0.011232	0.009392	0.007883	0.007541	0.005968	0.002962	0			
DM32	0.002006	0.001913	0.004485	0.007771	0.004556	0.008751	0.008697	0.010571	0.010524	0.008684	0.007174	0.006833	0.00526	0.002254	0.000708	0		
DM33	0.003437	0.007356	0.009929	0.013214	0.009999	0.014195	0.014141	0.016014	0.015967	0.014127	0.012618	0.012276	0.010703	0.007697	0.004735	0.005443	0	
DM34	0.003429	0.007348	0.00992	0.013206	0.009991	0.014186	0.014132	0.016006	0.015959	0.014119	0.012609	0.012268	0.010694	0.007689	0.004727	0.005435	0.000008	0

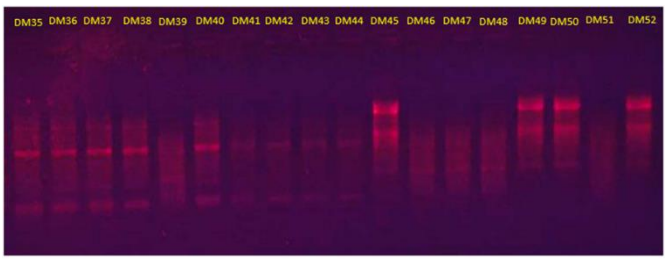

Figure 8. RAPD PCR products of the amplification using these L15996, M13F, Qg1-U , F21U for E. mashkovskii(35 -52)The gel was 2% and the DNA dye is RedSafe

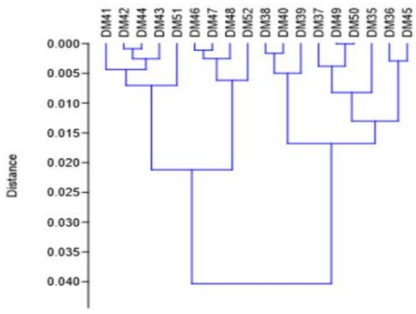

Figure 9. UPGMA phenogram of *E. mashkovaskii* strains based on pairwise band sharing.

Table 6. Genetic distances between *E. mashkovski* strains

Strains	DM35	DM36	DM37	DM38	DM39	DM40	DM41	DM42	DM43	DM44	DM45	DM46	DM47	DM48	DM49	DM50	DM51	DM52
DM35	0																	
DM36	0.002593	0																
DM37	0.001338	0.003931	0															
DM38	0.004235	0.006828	0.002897	0														
DM39	0.0056	0.008193	0.004262	0.001365	0													
DM40	0.004616	0.00721	0.003278	0.000382	0.000983	0												
DM41	0.013554	0.016147	0.012216	0.009319	0.007955	0.008938	0											
DM42	0.014275	0.016869	0.012937	0.010041	0.008676	0.009659	0.000721	0										
DM43	0.014975	0.017568	0.013637	0.01074	0.009375	0.010358	0.001421	0.000699	0									
DM44	0.01448	0.017073	0.013142	0.010245	0.00888	0.009863	0.000925	0.000204	0.000495	0								
DM45	0.001907	0.000686	0.003245	0.006142	0.007507	0.006523	0.015461	0.016182	0.016882	0.016387	0							
DM46	0.01002	0.012613	0.008682	0.005785	0.00442	0.005403	0.003534	0.004256	0.004955	0.00446	0.011927	0						
DM47	0.009759	0.012352	0.008421	0.005524	0.004159	0.005142	0.003795	0.004517	0.005216	0.004721	0.011666	0.000261	0					
DM48	0.009299	0.011892	0.007961	0.005064	0.003699	0.004682	0.004256	0.004977	0.005676	0.005181	0.011206	0.000721	0.00046	0				
DM49	0.002238	0.004831	0.0009	0.001997	0.003362	0.002379	0.011316	0.012038	0.012737	0.012242	0.004145	0.007782	0.007521	0.007061	0			
DM50	0.002229	0.004823	0.000891	0.002005	0.00337	0.002387	0.011325	0.012046	0.012745	0.01225	0.004136	0.00779	0.007529	0.007069	0.000008	0		
DM51	0.015995	0.018588	0.014657	0.01176	0.010395	0.011378	0.002441	0.001719	0.00102	0.001515	0.017902	0.005975	0.006236	0.006696	0.013757	0.013765	0	
DM52	0.008239	0.010833	0.006901	0.004005	0.00264	0.003623	0.005315	0.006036	0.006735	0.00624	0.010146	0.00178	0.001519	0.001059	0.006002	0.00601	0.007755	0

Discussion

The present study reported a prevalence of *Entamoeba* spp. infection of 55%, which is comparable to previous findings in Iraq, such as 53.18% in Baghdad (Nayef *et al.,* 2011) and 58.3% in Anbar (Salah *et al.,* 2017). However, the prevalence was slightly lower than that reported in Basrah by Al-Hilfi (2020) (60%) and Al-Asadi (2007) (65%). Variations in prevalence between studies may be attributed to environmental, nutritional, socio-economic, and geographical factors, as well as differences in diagnostic methods and sample size (Obaid, 2013).

In the present study, a significant difference in infection rates was observed between male and female patients, indicating that gender is an important factor influencing susceptibility. Higher prevalence was observed among males, consistent with findings by Nisar (2024) and Al-Hilfi (2020), who also reported male dominance in infection, although Farhan (2012) documented higher rates in females. The predominance in males may be attributed to greater exposure to unhygienic outdoor environments and reduced immune protection compared to females, where estrogen enhances resistance to intestinal parasites (Klein, 2000; Sayyari *et al.,* 2005). Furthermore, agricultural practices and the use of human feces as fertilizer may increase exposure risk for both sexes (Kean *et al.,* 1979).

The highest prevalence of *E. histolytica* infection was recorded in the age group under 10 years (38.1%), while the lowest was observed in the 30–40 years group (9%).

This variation in infection rates among age groups likely reflects multifactorial influences, including immunological changes, behavioral patterns, environmental exposure, and geographical conditions, rather than being limited to specific age groups, the distribution of infection is not confined solely to the youngest or oldest age groups but results from multifactorial influences affecting exposure likelihood and host susceptibility. (Al-Mekhlafi *et al.,* 2007) . Four types of stool samples were examined (bloody, mucus-containing, diarrheal, and non-diarrheal), with *Entamoeba* infections most prevalent in mucus and bloody stools. High mucus content reflects intestinal mucosal inflammation caused by *E. histolytica* trophozoite invasion, which damages the colonic epithelium and stimulates goblet cells to secrete excess mucus. Such stool characteristics may provide preliminary diagnostic indications of invasive amoebiasis (Masilo, 2020; Serrano-Luna *et al.,* 2013).

In the present study, the highest prevalence of infection was observed in December (18.1%), while the lowest prevalence was recorded in February (1.8%), Seasonal variation in *E. histolytica* prevalence in Basrah shows conflicting patterns across studies. Nassar *et al.* (2019) reported higher infection in December and September, whereas Ibrahim (2012) observed peaks from May to September, and Al-Hilfi (2020) reported highest prevalence in July. Such differences are influenced by environmental conditions, including temperature, humidity, cyst survival, fly activity, as well as population behaviors, hygiene practices, and local sources of infection (Shah, 2002).

Entamoeba medium was the most effective for

supporting *Entamoeba* growth due to its rich composition, including liver infusion, proteose peptone, sodium alpha-glycerophosphate, and sodium chloride, which provide essential nutrients, phosphate, and energy while mimicking the parasite's natural anaerobic intestinal environment. TYI-S-33 medium also supported good growth because of its nutrient richness, including amino acids, vitamins, and reducing agents like cysteine and ascorbic acid. In contrast, other media such as PEHPS, CLUPS, and Blood agar lacked optimal nutrient balance or key components (e.g., yeast extract, reducing agents), resulting in shorter growth periods. Thus, *Entamoeba* medium and TYI-S-33 are preferred due to their ability to closely simulate the intestinal conditions and nutrient requirements necessary for prolonged *Entamoeba* trophozoite survival. (Fotedar, 2007).

Microscopic examination alone cannot reliably differentiate *Entamoeba* species due to morphological similarities (Van Wyk *et al.,* 2013). Therefore, PCR was used in this study for accurate detection, revealing *E. moshkovskii* (18%) and *E. histolytica* (8%) as the most prevalent species in Basrah . In Yemen, *E. moshkovskii* was reported at 18.2% (Al-Areeqi *et al.,* 2017), in Australia 61.8% (Fotedar *et al.,* 2007), in South Africa 15.9% (Samie, 2020), in Bangladesh 21.1% (Ali *et al.,* 2003), in the UAE 3.3% (El-Bakri *et al.,* 2013), and in Peninsular Malaysia 1% (Anuar *et al.,* 2012).Although previously considered non-pathogenic, *E. moshkovskii* has been increasingly associated with gastrointestinal symptoms, indicating its emerging pathogenic potential (Heredia *et al.,* 2012; Sardar *et al.,* 2023). Misidentification by microscopy can lead to unnecessary anti-amoebic treatment, contributing to drug resistance and inaccurate epidemiological data (El-Bakri *et al.,* 2013; Calegar *et al.,* 2016). PCR-based detection is thus essential for distinguishing pathogenic from non-pathogenic *Entamoeba species* and guiding appropriate clinical management.

Random Amplified Polymorphic DNA (RAPD) is a widely used, simple, and cost-effective technique for genetic characterization across diverse organisms. Developed by Williams *et al.* (1990), it uses arbitrary primers to detect genetic variability without prior sequence knowledge. Given the strain variation in *Entamoeba histolytica,* a pathogenic protozoan causing amebiasis, RAPD is employed to differentiate strains of *Entamoeba* species.

The study identified four genotypes of *E. histolytica* with distinct geographic distributions. Genotype 4 predominated in rural regions, particularly Abu Al-Khasib, where all genotypes were detected. Genotype 1 occurred in both urban (Al-Qibla, Al-Hayaniyah) and rural areas, suggesting broader adaptability, while Genotypes 2 was mostly rural with one urban occurrence in Al-Jumhuriya. Genotypes 3 and 4 were exclusive to rural settings, indicating potential geographic or environmental constraints. These genotype variations may influence phenotypes and explain differences in parasite virulence (Gomes *et al.,* 2000)

RAPD analysis of *E. moshkovskii* strains revealed distinct geographic patterns. Genotype 1 predominated in rural Abu Al-Khasib, with one urban strain (DM25) sharing the same profile, suggesting possible strain migration or wider environmental spread. Genotype 2 was strictly rural, Genotype 3 confined to urban Al-Qibla, and Genotype 4 found only in urban Khamsah Mile, indicating geographic specificity. Genotype 5 was distributed across rural and urban areas, Genotype 6 showed mixed distribution, Genotype 7 appeared in both rural and urban regions, and Genotype 8 was mostly limited to rural Abu Al-Khasib, reflecting localized transmission clusters and varying adaptability.

Conclusion

The study revealed a high prevalence of Entamoeba infections in Basrah, with children being the most affected. RAPD-PCR analysis demonstrated substantial genetic diversity among E. histolytica and *E. moshkovskii* strains, highlighting the presence of multiple genotypes and intraspecific variation. These findings emphasize the importance of molecular tools for accurate species differentiation, understanding transmission dynamics, and guiding effective control and management strategies for amoebiasis.

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