Molecular identification of *Entamoeba* spp. in humans and cattle in Baghdad, Iraq

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**Abstract**

**Background and Aim:** A total of 10% of the global population succumbs to amoebiasis yearly, equating to 50,000–100,000 recorded fatalities. It is closely associated with contaminated food and water supplies due to human feces. The disease’s pathophysiology remains a subject of ongoing debate among experts. Some experts attribute the role of the host’s conditions, parasite species and strain, and infection intensity in eliciting clinical symptoms. The aim of this study was to perform molecular identification of *Entamoeba* species isolated from humans and cattle.

**Materials and Methods:** Stool samples from three hundred patients and one hundred cattle were collected from different regions, age groups, and sexes in Baghdad for microscopic examination. One hundred randomly chosen patient and cattle stool samples underwent microscopic examination and conventional polymerase chain reaction (PCR) targeting the 18S rRNA gene. Phylogenetic tree analyses were performed for *Entamoeba* species identification.

**Results:** The infection rate in humans differed significantly (p < 0.05) between age groups and genders, totaling 38%. The infection rate in cattle, determined by conventional PCR, differed significantly (p < 0.05) between age groups and genders, amounting to 58%. Ten PCR products with positive results were sequenced and deposited in GenBank database. Sequence analysis detected that Eight sequences belong to *E. histolytica* (OM268853.1, OM268854.1, OM268855.1, OM268857.1, OM268858.1, OM268860.1, OM268861.1, and OM268862.1) and two sequences belong to *E. dispar* (OM268856.1 and OM268859.1) in humans, while 10 sequences (ON724165.1 to ON724174.1) belongs to *E. histolytica* in cattle.

**Conclusion:** The increased susceptibility of cattle to *E. histolytica* suggests a considerable role in human infection and substantial public health risks. Further research should be conducted on the many virulence factors such as HM1:IMSS strain, cysteineprotease (Cp1), Gal/lecitin, etc. of *E. histolytica* and *E. dispar*.

**Keywords:** cattle, *Entamoeba* spp., human, phylogenetic, sequence analyses.

**Introduction**

Around the world, 50 million cases of amebiasis lead to 40,000–100,000 annual fatalities [1]. *Entamoeba histolytica* is a unicellular protozoon identified by pseudopodia and noted as the third deadly parasitic origin after Schistosomiasis and Malaria [2]. *E. histolytica* multiplication in the gastrointestinal tract produces cysts, which then progress out with stool and transmission to other healthy persons after consumption of contaminated food and water, causing ulceration and dysentery with bloody diarrhea in some cases and spreading to extraintestinal sites to produce abscesses of the liver, weight loss, colitis, and abdominal pain [2, 3]. *E. histolytica* employs toxins, pores, and adhesions as its primary virulence factors, resulting in tissue damage [2]. In laboratory animals, *Entamoeba dispar* can induce severe intestinal damage [4]. It is considered a chronic commensurable in humans, nevertheless, with no pathogenic traits, producing an asymptomatic carrier state and often being far more prevalent globally than *E. histolytica* [5]. The liver and intestines may suffer damage [6]. Poor sanitation, environmental pollution, overpopulation, inadequate education, and contaminated food and water promote *E. histolytica* transmission [7]. Studies have indicated a higher incidence of *E. histolytica* infection compared to *E. dispar* infection [8–10]. While other studies confirm a higher infection rate for *E. dispar* than *E. histolytica*, Al-Hilli et al. [11] present the opposite finding. The genetic diversity of *Entamoeba* species has received little research attention in Iraq [2, 12, 13]. Alarady and Jasim [14] found that *E. dispar* in cows and sheep was 35.7% and 21.45%, while Alarady and Jasim [14] found that *E. histolytica* was 85.7% and 21.4%, respectively, in Iraq.

Therefore, this study aimed to perform genomic species analyses in *Entamoeba* species isolated from humans and cattle and identify the associated risk.
factors of age and gender on the infection rates to
detect the true pathogenicity.

**Materials and Methods**

**Ethical approval and Informed consent**

Ethical approval was obtained from the guidance of
Research, Publication, and Ethics of the College of
Veterinary Medicine, University of Baghdad, Iraq (No.
BMS/0231/016), which complies with all relevant
Iraqi laws. A verbal consent form was obtained from
the animal’s owners. The families of study participants
and hospital management granted consent. Families
were informed orally about the study’s aim to facilitate
sampling from hospitals and healthcare facilities.

**Study period and location**

The study was conducted from September 01,
2021, to March 2022, at the Laboratory of Medical
Laboratory Techniques, Institute of Medical
Technology Al-Mansour, middle Technical University.

**Samples collection**

Three hundred patients (consisting of
187 females and 113 males, aged from <10 to more
than 40 years) provided 25 g stool samples from
Al-Zahra Teaching Hospital (80), Ibn -Al-Baladi
Hospital (100), and Al-Kadhimyia Hospital (120) in
Baghdad province. One hundred cattle stool sam-
ples, <1 year to over 3 years in age and comprising
56 females and 44 males, were gathered from Abu-
Ghraib (25), Al-Mahmodyia (25), Al-Shualah (25),
and Al-Nahrwan regions (25). In cold bags, clean
plastic containers containing stool samples, each with
a sequential number and a pair of disposable gloves
were transported to the laboratory. The data included
sex and age information for all samples. For micro-
scopic diagnosis of *Entamoeba* spp., 15–20 g of each
collected stool sample was preserved in 10% formalin
and stained with Lugol’s iodine (1%) and 0.9% saline,
while 1–2 g was used for further tests such as ELISA
and molecular [15].

**DNA extraction**

The DNA in stool samples was extracted using a
Bioneer (Korea) kit. The DNA was extracted using a
stool lysis protocol with proteinase K, following
the manufacturer’s instructions. The nanodrop spec-
trophotometer (Thermo Fisher, USA) was used to
test the PCR products. The nanodrop protocol included freeze-dried pellets of Taq DNA
polymerase IU, tris HCL (PH 9.0) 10 Mm, dNTPS
250 µm, Mg CL21.5 Mm, KCL 30 Mm, Tracking dye
and stablizers), and the polymer chain reaction master
mix was achieved based on kit instructions in 20 µL
total volume by adding 1 µL of 10 pmole of forward
primers and 1 µL of 10 pmole of reversed primers and
5 µL of purified g DNA, then added deionizer premix
by PCR water to increase volume to 20 µL and mixing
with Exispin vortex centrifugation (Bioneer). The
reaction was accomplished in a thermocycler (Mygene,
Bioneer) as follows: The reaction undergoes initial
denaturation for 5 min at 95°C, followed by 30 cycles
then 95°C denaturation cycles of 30 s each, annealing
of 30 s each at 58°C, followed by extension
cycles of 1 min each at 72°C, and ended with a final
extension of 5 min at 72°C. 1% agarose gel electro-
phoresis and ethidium bromide staining under ultravi-
iolet light were used to test the PCR products.

**DNA sequence methods**

Identification of *Entamoeba* spp. was based
on phylogenetic tree analysis of the 18S rDNA gene
sequences. The 18S rDNA gene (590 bp PCR product)
was purified using an EZEZ-10 spin column and then
sent to Bioneer in Korea for DNA sequencing with 18S
rDNA forward primers and AB DNA sequence system.
Phylogenetic analyses were carried out based on
NCBI Basic Local Alignment Search Tool (BLAST)
alignment results and the Neighbor Distance method in
Mega version 6 (https://www.megasoftware.net).

**Statistical analysis**

The data’s values are presented in both percent-
age and numerical form. Chi-square test was con-
ducted to analyze the percentage discrepancies using
Statistical Package for the Social Sciences Statistics
22 software (IBM Corp., NY, USA). A p < 0.05 was
considered statistically significant [16].

**Results**

18S ribosomal RNA gene primers were used in
PCR to distinguish *Entamoeba* species from human
and cattle stool samples. Results from agarose gel
electrophoresis demonstrated distinguishable bands
at 590 pb for *Entamoeba* species in human and cattle
stool samples (Figures-1 and 2).

Thirty-eight out of 100 tested samples were posi-
tive using conventional PCR, yielding a 38% infection
rate in humans. Age groups <10 years and ≥40 years had
65% and 55% infection rate, respectively ( ), followed
by age groups 20–30 years (40%), compared to the lowest
(10%) in age groups 30–40 years with a significant
difference at p < 0.05 (Table-1). About 52% of females
had the infection compared to 24% of males, with a sta-
tistically significant difference at p < 0.05 (Table-2).
DNA sequence in humans 18S ribosomal (RNA) gene sequencing was performed on isolated Entamoeba species and related Entamoeba spp. from NCBI-GenBank. Humans appeared closely related to E. histolytica (MK332025.1) in the phylogenetic tree analysis of genomic relationships with Entamoeba species (No.1, NO.2, NO.3, NO.5, NO.6, NO.8, NO.9, and No.10). Humans were found to be closely related to E. dispar (MK559465.1) according to NCBI-BLAST analysis with Entamoeba species No.4 and No.7. Isolated human Entamoeba species (No.1, NO.2, NO.3, NO.5, NO.6, NO.8, NO.9, and No. 10) demonstrated genomic homology ranging from 99.09% to 100% with the E. histolytica reference sequence MK332025. No.4 and No.7 Entamoeba species show 100% genomic homology with NCBI-BLAST E. dispar (MK559465.1). The human Entamoeba spp. isolates submitted to NCBI GenBank with accessions OM268853.1 to OM268862.1 (Figure-3 and Table-3) were verified.

The infection rate in cattle was 58%, as indicated by 58 positive samples from 100 tested. The percentage for age groups <1–6 months (77.14%) and 6–12 months (66.66%) was significantly higher than that for age groups 1–3 years (28.12%), as shown in Table-4 (p < 0.05). The infection rate was significantly higher in males (79.54%) than in females (41.07%) (p < 0.05; Table-5).

DNA sequence in cattle 18S rRNA gene sequences were obtained from NCBI-GenBank for both Entamoeba species and their related strains, and DNA sequencing was conducted for genomic species type analysis. E. histolytica (MK332025.1) was the closest phylogenetic match to the genomic relationships of the isolated Entamoeba spp. cattle (No. 1–No. 10). The genomic identity of isolated cattle Entamoeba spp. ranged from 99.09% to 100%. 1–10, plus NCBI-GenBank entry MK332025.1, corresponds to Ent NCBI GenBank verified the submission of isolated cattle Entamoeba spp. using the accessions numbers ON724165.1 to ON724174.1 (Figure-4 and Table-6).

**Discussion**

The rate of Entamoeba spp. infection in humans, as determined by PCR, was 38%, which is higher than 9.3% in Salah Al-Din, Iraq [17] and 12% in Iran [18]. These differences may be due to suppression of the immune system and inadequate personal hygiene measurements, bad environmental conditions, retardation due to the lack of toilet management, direct contact from person to person [11], overcrowding, socioeconomic conditions, and malnutrition. In our study, age group of <10 years had the highest rate compared to the lowest in age group of 30–40 years. Barakat [19] reported the highest rate in age group of 1–30 years which was 78.79%, while Al-Hilfi et al. [11] reported the lowest rate in age group of 60–90 years which was 2.02% with a significant difference at p < 0.05. The highest infection rate in age group of 1–10 years may be due to this age spending more of their time outdoors playing, eating, discarded food, and staying put on the street and foraging in garbage dumps, touching with sands, and eating with dirty hands [20]. The school children involved in this study living in poor houses constructed with poor quality materials and no drainage [21]. Mahmood and Mustafa [9] reported the
highest rate in age group of 36–45 years (8.3%), while Bahrami et al. [22] observed the maximum rate in age group of 30–50 years (28%). Another study recorded a low rate in age of 10–20 years and this may be due to young ages becoming more sanitation and hygienic associated with their looks, compared to those of lower age groups and accelerated ability to avoid contact as possible, which lead to get infection [23] or maybe extension of using metronidazole [24], and albendazole is given to school children in the campaign of national deworming and has been recorded that single dose of albendazole (400 mg) decrease *E. histolytica* infection in more than 50% of children of 7–15 years [25].

**Total infection rate of Entamoeba spp. in humans according to gender by PCR**

In ThiQar, females had a higher prevalence of *Entamoeba* than males, as previously reported by Mahmood and Mustafa [9] and Flaih et al. [10]. These studies are not agree with previous studies in Iran [2, 11, 22], which pointed the highest rate in males compared to females, and this may be attributed

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**Table-3:** The NCBI-BLAST homologous sequencing identity percentage between isolated human *Entamoeba* spp. and NCBI-BLAST closed genomic relationship the isolated *Entamoeba* species.

<table>
<thead>
<tr>
<th><em>Entamoeba</em> spp. isolate</th>
<th>Accession number</th>
<th>Homology sequence identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ-Human No. 1 OM268853.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 2 OM268854.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 3 OM268855.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 4 OM268856.1</td>
<td><em>Entamoeba dispar</em> MK559465.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 5 OM268857.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 6 OM268858.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>99.12</td>
</tr>
<tr>
<td>IQ-Human No. 7 OM268859.1</td>
<td><em>Entamoeba dispar</em> MK559465.1</td>
<td>100</td>
</tr>
<tr>
<td>IQ-Human No. 8 OM268860.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>99.13</td>
</tr>
<tr>
<td>IQ-Human No. 9 OM268861.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>99.09</td>
</tr>
<tr>
<td>IQ-Human No. 10 OM268862.1</td>
<td><em>Entamoeba histolytica</em> MK332025.1</td>
<td>100</td>
</tr>
</tbody>
</table>

NCBI-BLAST=National Center for Biotechnology Information-Basic Local Alignment Search Tool
to weak immunity and exhibit to infection, these variations due to ecological and physiological factors and hormonal sex-specific behaviors, also variation in endocrine immune system and males sexually mature and more susceptible to disease due to the sex steroids of hormones alternative the genes and behaviors that control stimulation and resistant to disease [26].

Total infection rate of *Entamoeba* spp. in cattle according to age groups by PCR

Many studies have been conducted in cattle to detect the prevalence of *Entamoeba* spp., which is in accordance with current results (58%), such as 57.41% [27] and 54% [28] of *E. histolytica* in cattle. A lower infection rate, 45.6% was detected by Al-Areeqi et al. [8]. These differences are due to farmers’ disregard for culture and health, lack of commitment to health standards in the development of farms and animal breeding, ignorance during the movement of animals or use of water-polluted feed diseases, and differences in the geography and temperature of the region [29], environmental conditions, sample sizes, and immunity.

A study conducted by Naguib et al. [30] agree with our findings, which reported 62.79% for the 6–12 month age group and 34.28% for the age group above 12 months, with significant differences (p < 0.05; According to Naguib et al. [30], this discovery is linked to the adult cattle’s physiological condition due to their adaptive immunity against past infections. The various factors, including sample size, age groups, living conditions, management, season, and collection location, can influence the infection rate.

**Table-4:** Total infection rates of *Entamoeba* spp. in cattle according to age groups by conventional PCR.

<table>
<thead>
<tr>
<th>Age/years</th>
<th>No. of samples examined</th>
<th>No. of positive samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1–6 month</td>
<td>35</td>
<td>27</td>
<td>77.14</td>
</tr>
<tr>
<td>6–12 months</td>
<td>33</td>
<td>22</td>
<td>66.66</td>
</tr>
<tr>
<td>1–3 years &gt;</td>
<td>32</td>
<td>9</td>
<td>28.12</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>58</td>
<td>58</td>
</tr>
</tbody>
</table>

Chi-square 18.007, p = *0 (HS)

*HS=Highly significant difference at p < 0.05.
PCR=Polymerase chain reaction

**Table-5:** Total infection rates of *Entamoeba* spp. in cattle according to gender by conventional PCR.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of samples examined</th>
<th>No. of positive samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>56</td>
<td>23</td>
<td>41.07</td>
</tr>
<tr>
<td>Males</td>
<td>44</td>
<td>35</td>
<td>79.54</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>58</td>
<td>58</td>
</tr>
</tbody>
</table>

Chi-square 14.97, p = *0 (HS)

*HS=Highly significant difference at p < 0.05.
PCR=Polymerase chain reaction

**Figure-4:** Isolated local cattle *Entamoeba* spp. was employed in phylogenetic tree analysis dependent on the partial sequencing of the 18S ribosomal RNA gene using genomic species typing analysis. Unweighted Pair Group Methods with Arithmetic Mean (UPGMA tree) were used to construct the phylogenetic tree (MEGA 6.0 version). 1–10 were identified as being closely linked to the *E. histolytica* (MK332025.1) strain by NCBI-BLAST, with total genomic alterations of 0.06%–0.01%.

**Total infection rate of* Entamoeba* spp. in cattle according to sex by PCR**

The study showed that 62.5% of males and 46.15% of females had significant differences (p < 0.05), which is consistent with Al-Maliki [28]. Female mammals exhibit greater resistance to parasite
infections than males due to differences in exposure and immunosuppressive properties of testosterone. Male livestock carried higher parasite loads than their female counterparts.

**Phylogenetic analyses and sequencing**

The current results are in agreement with many studies that found that *E. histolytica* was higher than *E. dispar* as 6% and 4.3% [9], 31.3% and 17.5% [10]; 66.03% and 11.32% in humans [28]. The number of patient samples assessed in the study and the time of specimen collection are contributing factors to the disparity in *E. histolytica* infection rates. The parasite may be acquired through ingestion of fecal matter from dogs, cats, sheep, and rodents. Both high temperatures and water contamination significantly contribute to the spread and completion of this parasite’s life cycle. Another factor is one’s economic standing.

Khan et al. [31] reported a different prevalence. *E. dispar* had higher prevalence (57.5%) than *E. histolytica* (47.5%). Another study reported a prevalence of 10% with *E. dispar* and 5% with *E. histolytica* in humans [11]. Like Alarady and Jasm [14], studies have reported *D. diaper* to be present in cows and sheep at respective levels of 35.7% and 21.45%, and *E. histolytica* at 85.7% and 21.4%. Environmental conditions, hygiene practices, population density, and DNA extraction from stool samples could account for observed differences.

In areas with extremely low standards of living and poor sanitation, *E. histolytica* thrives. The inadequate sanitary system may contribute to the high prevalence rate of *E. histolytica*. Contracting *E. histolytica* infections can be increased by keeping pets due to the parasite’s prevalence in animals and its spread through contamination. Therefore, it is proposed that more research should be conducted to identify and determine the genetic diversity of these parasites, as well as to determine the true pathogenicity and risk factors associated with *Entamoeba* species [31].

PCR-positive samples were identified as *E. histolytica* subtype 1 by sequence alignment (99% similarity) with accession number KB823016 [2].

According to the results of the BLAST analysis, six *E. dispar* amplicons (KY823418-KY823423), are 100% identical in sequence to accession number KP722600.1 in GenBank for *E. dispar*. The sequences KY823424 to KY823427 and KY884295 were identical to KP233840.1 (99%–100% homology). The phylogenetic analysis of *E. histolytica* gene sequences suggested that Iraqi isolates [22] are more closely related to Japanese isolates (AB282660.1 and AB485592.1) than to isolates from other countries. Accession numbers KT253450, KT253451, KT253452, KT253453, and KT253454 correspond to distinct isolates of a novel strain [12].

Phylogenetic sequence alignment of the local Iraqi isolate of *E. histolytica* revealed 100% identity with strain KF429800.1, which is more similar to *E. histolytica* than other *Entamoeba* species.

About 100% of the *E. histolytica* Iraq local isolates were identified as distinct from other *E. histolytica* isolates from AB282660.1 (Japan), KJ870211 (Cameron), Y11272.1 (India), and GQ423749.1 (Philippines).

The five *E. histolytica* isolates from Iraq shared 98% identity with *E. dispar* (AB282661.1) and 100% identity with *E. histolytica* (AB282660.1 Japan); yet, significant genetic diversity exists among parasites exhibiting different morphologies. Morphological differences among *Entamoeba* species may not impact species-level variation, as some species infect multiple hosts [12].

**Conclusion**

Phylogenetic analysis identified eight human isolates as *E. histolytica*, two as *E. dispar*, and 10 from cattle as *E. histolytica*. The high infection rate of *E. histolytica* in cattle compared to humans implies that the predominant species of this parasite in cattle significantly contributes to the transmission of this disease to humans and poses significant public health concerns.

**Authors’ Contributions**

SMKA, HHA, and EJA: Conceptualization and data duration. SMKA: Formal analyses and drafted and revised the manuscript. HHA and EJA: Methodology. All authors have read, reviewed, and approved the final manuscript.
Acknowledgments

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Competing Interests

The authors declare that they have no competing interests.

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