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Molecular identification of *Entamoeba histolytica* from diarrhetic patients in Baghdad province, Iraq

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Abstract

This study used molecular detection technologies to identify *Entamoeba histolytica*, the amoeba causing amoebiasis, in individuals suffering from diarrhoea. Stool samples were collected from 100 individuals presenting with diarrhoeal symptoms and classified according to age and gender. Microscopic examination showed that 90 per cent of the samples contained *Entamoeba* organisms. The infection rate was higher in females (86.73%) than in males (70.8%), with a statistically significant difference at a P value of 0.05. The findings also indicated higher infection rates in the age groups of 5–11 years and above 50 years (85.23% and 78.81%, respectively), while a lower infection rate was observed in the age group above 2 years (43.45%). Nested multiplex polymerase chain reaction targeting the small subunit gene confirmed an overall *E. histolytica* infection rate of 91%. The prevalence of *E. histolytica* infection was higher in females (89.23%) than in males (82.35%). Both the older age group (>2) and the younger age group (28–48 years) showed an infection rate of 100 per cent attributable to *E. histolytica*. The results were analysed based on gender, age, and nested multiplex PCR findings to determine the proportion of individuals infected with *Entamoeba histolytica*.

Key terms: Diarrhoea, *Entamoeba histolytica,* gene expression, PCR.

1.0 INTRODUCTION

Entamoeba species have been found in the human gut; however, up to 24 species have been identified worldwide. Entamoeba histolytica is a protozoan pathogen responsible for a disease that is the third-largest cause of death worldwide, after malaria and schistosomiasis, in terms of prevalence. Entamoeba histolytica causes the condition known as Entamoebiasis (Laude et al., 2016; Mohammed Alwan et al., 2022).

There are more than 200 million people throughout the world who are afflicted by the condition, and between 50000 and 120000 people every year lose their lives as a direct consequence of the ailment (Solaymani-Mohammadi et al., 2006; Ahmed & Jalil, 2022). Two of the most important factors contributing to the rapid spread of the disease worldwide are the presence of contaminated water and the absence of adequate sanitation systems in developing nations. In addition, it is becoming more common in these areas to disregard the symptoms of parasitic illnesses, even if they may point to a potentially life-threatening condition (Troll et al., 1997).

Because amoebas are the organisms responsible for causing amoebiasis, the condition is classified as a parasitic intestinal disease caused by the parasite E. histolytica, which causes significant mortality and morbidity (Alwan et al., 2021).

The Baghdad region has been the site of a number of investigations into distinct species of *Entamoeba*, including one that was carried out by researchers who utilised the polymerase chain reaction (PCR) method in order to identify and differentiate between *Entamoeba* species. Other researchers have also conducted their research in this region (Núñez et al., 2001; Santos et al., 2007).

As a direct result of the objective of this research is to make use of nested multiplexing in order to determine the rate of infection induced by *Entamoeba histolytica* based on factors such as age and gender.

2.0 METHODOLOGY

Sample Collection

Patients in the Baghdad province who exhibited symptoms of diarrhoea between January 4, 2021, and November1, 2021, were eligible to have their faecal specimens collected, and a total of 100 specimens were collected. These patients were seen at various healthcare facilities in the Baghdad province, including medical labs, medical health centres, General Alzahraa, and a few clinics and hospitals. These people have been examined at a selection of Waist's medical facilities, which are located throughout the province. The samples, collected from the site and placed in Eppendorf tubes, were stored in cold storage containers after transport from the collection region.

Microscopic Detection

In the laboratory, the specimens were cut into two parts, and each portion was assigned a weight that was nearly exactly the same as the weight of the other section (1.5-3 gm). The original sample was placed in an Eppendorf tube and maintained at -20 °C to extract DNA. This has to be done in order to successfully extract the DNA. Mount smear techniques often call for the use of adequate amounts of distilled water, with the typical amount falling between 15 and 25 millilitres.

PCR Assay

In order to extract DNA from the stool samples, a Presto TM Stool DNA Extraction Kit was utilised, which was acquired from a company that does business under the name Geneaid/Korea. The kits that are often used in the execution of this method are normally quite a bit more compact than the kit that was used before. The following is a synopsis of the procedures that were carried out in line with the instructions that were given by the manufacturer: After placing 200milligrams of faeces,900 millilitres of ST1 buffer, and a short vortex inside a Bead beating tube containing ceramic beads, the combination was allowed to incubate for five minutes at -70 degrees Celsius, and then it was vortexed for 10 minutes while at room temperature. After that, the substance was centrifuged for two minutes at a speed of 9,000 x g while it was allowed to remain at room temperature (RT).

PCR De-Inhibition

The sample was incubated at 4 °C for 5 minutes. After that, the sample was centrifuged at 15,000 x g for three minutes at room temperature in order to remove any insoluble particles and PCR inhibitors that could have been present in it. The Inhibitor Removal Column was found in the clear supernatant that was left behind after a 2 mL centrifuge tube was used. Within the picture, the ring in purple that represents this column may be seen. A total of one minute of centrifugation at 15,000 x g was performed on the column at room temperature before it was discarded. The centrifugation lasted for one minute. The flow-through that was collected from a centrifuge tube that carried 2 millilitres of material was stored so that it may be used for studies on DNA binding.

Binding and Washings

Following the addition of 800 microliters of ST2 buffer to the flowthrough, the contents were vigorously mixed for 5 seconds. This procedure lasted for the duration of the experiment. The duration of this procedure included the whole of the flow-through.

After that, a 1.5 mL Collection tube was given a green ring, also referred to as a green column, and it was placed inside the tube. The green column was another name for the ring. This process was carried out three times in all. In the green column was a mixture of samples that had a total volume of 800 microliters when they were injected. Microliters served as the unit of measurement for the volume of the injection.

Following that, the flow-through was separated at a velocity of 18,000 x g for one minute, during which time the temperature was held constant at room temperature. Position the green column within the Collection Tube, which has a fluid-holding capacity of 1.5 millilitres and can accommodate the column. After that, the other parts of the sample combination have to be shifted with the help of the green column. In order to properly separate the components, they were centrifuged for one minute at 18,000 x g while the temperature was maintained at room temperature during the process (RT).

To the green column was added the ST2 Buffer, which had a combined capacity of three hundred and fifty litres. After that, they each continued on their own paths for the next thirty seconds while being exposed to an acceleration that was 16,000 times more than their average acceleration caused by gravity in RT.

Following the removal and disposal of the flow-through, the green column was reinserted into the 1.5 ml Collection tube in order to facilitate a second round of collection. This was done so that the sample could

be taken. This was done so that we could collect the sample in its entirety, and it was successful. It was concluded that a total of 500 microliters of Wash Buffer needed to be added to the green column. This was a complete volume increase. After that, they went through an experience that was comparable to 18,000 times their body weight in g, and then for the next thirty seconds, they were kept apart at R.

The green column has to be reinserted into the 1.5 ml Collection Tube as soon as the flow-through has been thrown away. In order to dry the column matrix, the dry green column collecting tube was separated at 18,000 x g for three minutes while the temperature was maintained at room temperature. This was done while the temperature was kept at room temperature.

Qualification of DNA

Using a Nanodrop spectrophotometer, the quality of the genomic DNA isolated from faecal samples was investigated and evaluated. The samples had been taken from different individuals. This operation was carried out as planned. The following techniques, described below, illustrate how to use this instrument to explore and assess DNA quality. This is accomplished by measuring the absorbance of the sample at a variety of wavelengths (270-280 nm). After the Nanodrop program has been activated, choose the application that satisfies your requirements in the way that comes the closest to fulfilling those requirements (Nucleic acid, DNA). During the cleaning of the measuring pedestals, we found we needed to use dry wipes on a number of occasions. To properly reset the instrument, carefully pipette 2 microliters of free nuclease water onto the bottom measurement pedestals. This will ensure that the device is properly calibrated.

PCR Reaction

Gene Aid, a firm that has its headquarters in Korea, was kind enough to provide the three extraction kits that were necessary for the process of extracting DNA from human faeces and urine. In addition, forward and reverse primer pairs were employed in order to complete the amplification of the 19S rRNA gene by employing nested multiplex PCR. This was done so that the gene may be replicated more than once. This was done in order to establish beyond a reasonable doubt the existence of the gene.

The initial primer pair was created in such a way that it allowed for the identification of 900 base pairs (bp) of the 19S rRNA gene in *Entamoeba*. Primers were used in tandem, and then that tandem was put to use in the analysis of (*E. histolytica*). This research, which used a one-plus primer design, drew its information from the NCBI-Genebank as its primary source of data. This primer, which contains 800 base pairs and may be used in nested PCR investigations, including *E. histolytica* as well as nested PCR tests comprising *Entamoeba* spp (532 bp), was kindly donated by the Macroge Corporation in Korea.

Primers and probes are two examples of additional PCR reaction components that may be kept in a standard Maxime PCR PreMix container. These components are called primers and probes, respectively. In addition, the PCR component that is outlined in the table positioned directly above this one is included inside this tube. In addition, the PCR component that is outlined in the table may be found included inside this tube (dNTPs, pH: 9.0, KCl, MgCl2, stabiliser, tracking dye, Tris-HCl and Taq DNA polymerase).

Following that, a PCR tube was inserted inside an Exispin vortex centrifuge, and the centrifuge was used to spin the tube at a speed of 4000 rpm for a length of three minutes. This process was repeated three times. This procedure was carried out a total of three times.

Following that, the samples were put into a PCR thermal cycler to be processed. PCR is the abbreviation for "polymerase chain reaction". The components of the PCR master mix that are described in the table located above are then placed in a typical Maxime PCR PreMix, which is an example of a mixture that includes all of the additional components required for the polymerase chain reaction. Following this step, the PCR master mix is ready to be used.

After the PCR master mix has been assembled, this next step may then be carried out. The table that is located just above this one has an explanation of the many components that make up the PCR master mix (dNTPs, pH: 9.0, KCl, MgCl2, stabiliser, tracking dye, Tris-HCl and Taq DNA polymerase). After that, the PCR tubes were spun for three minutes at a speed of four thousand revolutions per minute inside an Exispin vortex centrifuge. Following that, the samples were loaded into a PCR thermal cycler and allowed some time to complete their respective processes.

Estimation Analysis

After all of these steps had been carried out, the PCR's findings were analysed using agarose gel electrophoresis, and everything was finished. To produce agarose gel with a concentration of one per cent, 2X TBE was dissolved in a water bath maintained at one hundred degrees Celsius for fifteen minutes prior to the temperature being lowered to fifty degrees Celsius. This step was performed in order to manufacture the gel. This procedure was carried out so that the gel could be produced successfully. Following that, three microliters of ethidium bromide dye were added to the agarose gel solution so that it could be coloured. This was done so that the gel could be used.

After finishing the meticulous process of positioning the comb in the tray, the agarose gel solution was then poured into the tray. This procedure was repeated a number of times until the tray could hold no more stuffing. Once the comb was allowed to first set for fifteen minutes at room temperature while the agarose gel solution was being made, it was carefully removed from the tray after the initial setting period had concluded. This was done at the same time as the solution for the agarose gel was being prepared. This therapy was administered a total of three times over the course of the investigation. Position 6 on the gel tray that was placed inside the electrophoresis chamber was adjusted in order to make room for the inclusion of a 2X TBE buffer. The chamber accommodates the gel tray within its confines. The PCR product was added to each of the wells in the comb at a concentration of 10 microliters, with the exception of the first well, which got only four microliters of the PCR product. Four microliters of the PCR product. This was due to the fact that the first well served as a control for the experiment (120bp Ladder).

After that, there was an electrical current source that lasted for the whole of one full hour, had 120 volts, and 90 AM. This current source was there after the event in question. In order to get a vision into the findings of the PCR, a transilluminator that emits ultraviolet (UV) light was used (Al-Eodawee et al., 2023).

3.0 FINDINGS AND DISCUSSIONS

Microscopical Findings

Our study demonstrated that the percentage of infections caused by *Entamoeba spp.* was significantly higher in females (86.73%) in comparison to males (70.8%). Because of the way nature works, females are more likely to handle food preparation and household chores, which can expose them to water or food that has been contaminated by Entamoeba cysts. This difference in infection rate between females and males can be attributed to a wide variety of factors, including hormones and the fact that nature works in such a way that females are more likely to handle food preparation and household chores.

The most recent revision's findings revealed that the highest number of infections occurred in the age groups of 5-11 years old and 50+ years old. *Entamoeba* species were found to be responsible for these findings. A rate of infection of 85.9 per cent was found in both of these age groups. The age group of less than 2 years old had a lower infection rate than the other age groups, which had a percentage of 63.23. When viewed under a microscope, trophozoites of the parasite Entamoeba *histolytica* could be seen in both female and male-positive samples (Figures 1, 2, 3).

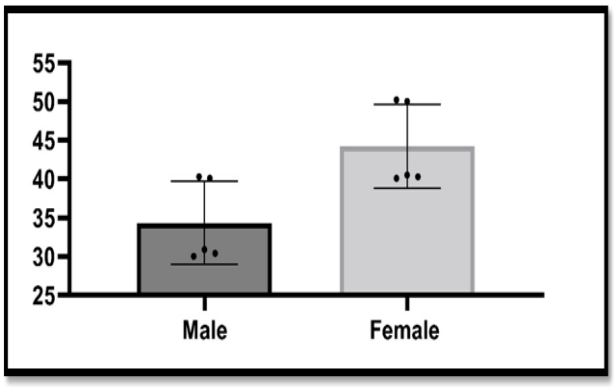

Figure 1: Microscopic Detection *Entamoeba Histolytica* Could Be Seen in Both Female and Male-Positive Samples

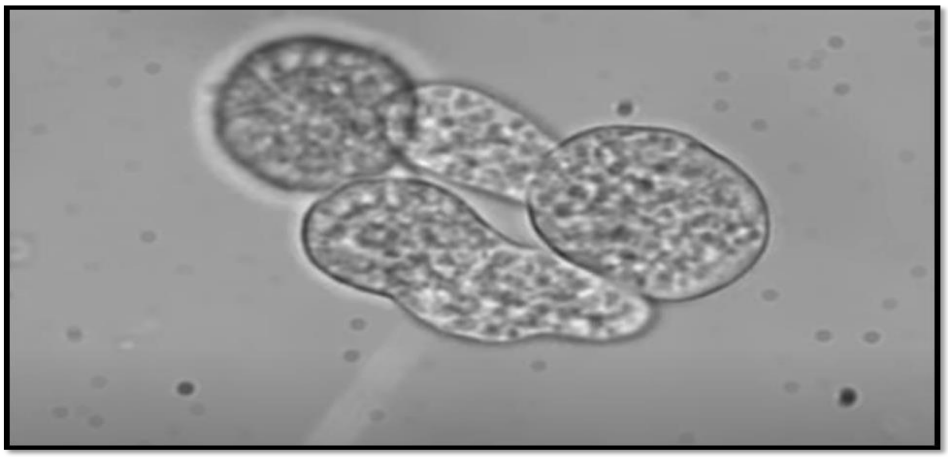

Figure 2: Microscopic Detection *Entamoeba Histolytica* Could Be Seen in Male-Positive Samples

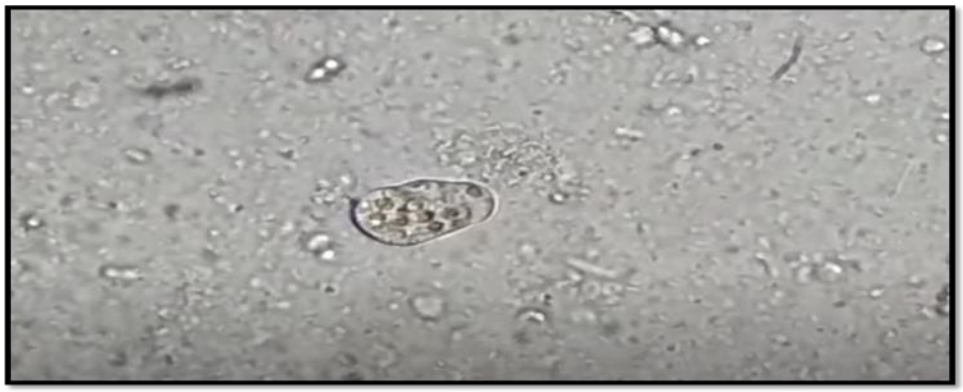

Figure 3: Microscopic Detection *Entamoeba Histolytica* Could Be Seen in Female-Positive Samples

Molecular Finding

The first round of PCR for DNA samples showed that 79 out of 100 stool samples successfully amplified the 19sRNA gene using nested multiplex PCR. A success rate of 79 per cent can be calculated from this. In spite of the fact that Entamoeba infections are typically mild, there are certain strains that have the potential to attach themselves to the bowel wall, which can lead to severe extra-bowel pathogens and amoebic colitis. In spite of this, amoebic dysentery continues to be observed in clinical settings (Table 1, Figure 4).

Table 1: Molecular Detection *Entamoeba Histolytica* Could Be Seen in Both Female and Male-Positive Samples

Gender	Samples of study	Infected results (+)	Total Percentage
Male	54	59.2	70.8%
Female	46	64.23	86.73%
Significance	P. value = 0.002		

P. value <0.005

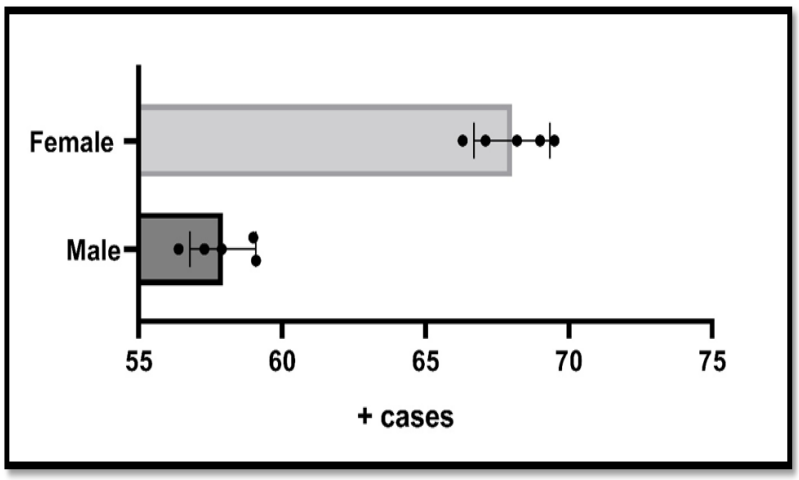

Figure 4: Molecular Detection *Entamoeba Histolytica* Could Be Seen in Both Female and Male-Positive Samples

Discussion

The findings of our study are in line with those that were recounted, which indicated that the West province in Iraq had a higher ratio of females to males (Haque et al., 1998). On the other hand, the findings of Baghdad revealed that females had a significantly higher infection rate than males (86.73 per cent), as compared to the results of the test conducted on males (70.8%) (Ngui et al., 2012). Despite the findings of a recent study, which demonstrated that the percentage of *Entamoeba* spp. Infections were more prevalent in females (44.1%) compared with males, and the researchers concluded that males were equally susceptible to contracting the parasite (22.3%) (Verweij et al., 2004).

Females are more likely to be exposed to water or food that has been contaminated by *Entamoeba* cysts when they are cooking or cleaning (Stark et al., 2011). This difference in infection rate between females and males may be due to a number of factors, including hormones or the fact that nature works differently for females, which means that females are more likely to become infected when they are exposed to water or food that has been contaminated by *Entamoeba* cysts (Madden et al., 2019).

Entamoeba species discovered in. According to the findings of the most recent round of research, the age range of 5–11 years old and the age range of more than 49 years old each had a percentage of 65 per cent in terms of the number of infectors that were discovered within their respective populations (Evangelopoulos et al., 2000). The age group of less than 2 years old had a lower infection rate overall, with a percentage of 45.3 per cent. They found a higher prevalence of infection among children aged 5 to 14 (31%) but a lower prevalence among children aged 0 to 2 years old. This is in agreement with what we already knew (11%) (Rivera et al., 1998).

Similarly, many researchers reported a higher prevalence of *Entamoeba* spp. Infection in young children in Iraq and Yemen, recording a high infection rate of (50.2%) in children under ten years of age. However, their findings did not align with a lower infection rate of (7.7%) observed in individuals over 45 years old (Cnops & Van Esbroeck, 2010). This was the case despite the fact that they recorded a lower infection rate in people over 45 years (Hamzah et al., 2006). It would be described by the effect of various aspects on the children as a result of their activities, which are the result of their caring less about their own sanitation (Bahrami et al., 2019). These activities are the result of the children spending less time cleaning up after themselves (Haque et al., 2007). Additionally, the areas of their bodies that are exposed have the highest concentrations of nutrients, which come from a wide variety of sources (Blessmann et al., 2002). Others may be resistant to infectious organisms due to the use of medicines that target a variety of pathogens (Rivera et al., 1998). While a high infection rate in older age may be the cause of the detail, as the elderly individuals show types of infections among them, like weakened immune systems, this may not be the case for all elderly people (Leiva et al., 2006). Despite the fact that a high infection rate in older age may be the root cause of the detail, this holds true (Al-Eodawee et al., 2023; Roy et al., 2005).

In the first round of PCR for DNA samples, the 19sRNA gene was successfully amplified in 86.8 per cent of the stool samples using nested multiplex PCR, as indicated by the results of that round of testing (Freitas et al., 2004). Even though *Entamoeba* infection is not typically dangerous in the majority of cases, there are strains, some of which can attach to the bowel wall, resulting in severe extra-bowel pathogens and amoebic colitis (Kebede et al., 2004). These conditions can be caused by certain strains of *Entamoeba*. In terms of the clinical picture, amoebic dysentery is always present.

Using nested PCR, the researchers found that there were 55 per cent positive samples out of a total of 80 samples in Baquabah city (Lebbad & Svärd, 2005). These findings are consistent with the findings that were found in Iraq, which recorded a lower percentage than ours did in this city (Lau et al., 2013), despite the fact that the nested multiplex PCR method recorded an even higher percentage (88%) (Fotedar et al., 2007).

4.0 CONCLUSION AND RECOMMENDATIONS

Conclusion: The most recent report found that patients who suffered from diarrhoea had a higher rate of infection with Entamoeba histolytica. This information was found in accordance with the findings of a previous study. According to the findings of the research, the female participants had a significantly higher infection rate with E. histolytica than the male participants did; however, when the two groups were compared using the $P < 0.05$ threshold, there was no significant difference found between them. Even though the microscopic examination method is a reliable test for the detection of *Entamoeba* spp. in diarrheal patients, there was a high probability of incorrectly diagnosing amoebic dysentery as being caused by Entamoeba histolytica. This was due to the fact that the microscopic examination method relied on the observation of a small number of organisms. This was because the bacteria were much too small to be seen by the human eye, even when viewed through a microscope. According to the findings of this study, the infection rate of E. histolytica was high among people aged 2 years or less and 25-49 years. The median age of the participants in this study was 25 years old.

Recommendations: Given the high probability of misdiagnosis using microscopic examination alone, further diagnostic methods should be considered to accurately identify *Entamoeba histolytica* in patients. It is recommended that additional diagnostic techniques, such as molecular methods, be incorporated to improve detection accuracy. Additionally, healthcare providers should be mindful of the varying infection rates across different age groups, particularly in individuals aged under 2 years and between 25 and 49 years, when diagnosing and treating amoebic dysentery.

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