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Molecular and Microscopic Diagnosis of *Entamoeba histolytica* in Human Stool Samples from Al-Kut, Iraq

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Abstract

Entamoeba histolytica, a protozoan parasite, is responsible for amoebiasis, a significant public health issue, particularly in developing regions where sanitation and sanitary infrastructure are frequently insufficient. It is imperative to have a precise and expeditious diagnosis in order to effectively manage the patient and prevent the transmission of the infection. Our objective in this investigation was to assess and contrast the diagnostic efficacy of two methods for the detection of Entamoeba spp. in stool samples: conventional microscopic examination with Lugol's iodine staining and molecular identification via multiplex polymerase chain reaction (PCR) that targets the 18S rRNA gene. At the Al-Kut and Al-Karama hospitals in Wasit Province, Iraq, a total of 67 stool samples were obtained from patients who were experiencing gastrointestinal symptoms, particularly diarrhea. The positivity rate for Entamoeba spp. was 67.2%, as evidenced by the microscopic examination, which revealed spherical cysts with clearly defined nuclei. On the other hand, conventional PCR-based molecular analysis identified E. histolytica in 80% of the tested samples, indicating a greater diagnostic yield. Significant differences between the two methodologies were verified through statistical analysis ($P \leq 0.05$). The findings emphasize the superior sensitivity and specificity of PCR for accurate detection and demonstrate the limitations of microscopy in differentiating morphologically similar species within the Entamoeba complex. This research advocates for the inclusion of molecular diagnostic methods, such as multiplex PCR, in routine parasitological screening, particularly in endemic regions. In addition, the results establish a basis for enhanced diagnostic strategies and public health interventions that are designed to manage amoebic infections.

Keywords: *Entamoeba histolytica*, PCR, Molecular diagnosis, Stool samples, Microscopic examination.

التشخيص الجزيئي والمجهري للأميبا الحالة للنسج في عينات براز بشرية من الكوت، العراق

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الملخص

الأميبا الحالة للنسج، وهي طفيلي من الطفيليات الأولية، مسؤولة عن داء الأميبا، الذي يُمثل مشكلة صحية عامة خطيرة، لا سيما في المناطق النامية حيث غالبًا ما تكون مرافق الصرف الصحي والبنية التحتية للصرف الصحي غير كافية. من الضروري إجراء تشخيص دقيق وسريع لإدارة المريض بفعالية ومنع انتقال العدوى. هدفنا في هذه الدراسة هو تقييم الفعالية التشخيصية لطريقتين للكشف عن أنواع الأميبا في عينات البراز: الفحص المجهري التقليدي باستخدام صبغة اليود لوغول، والتعرف الجزيئي عبر تفاعل البوليميراز المتسلسل المتعدد (PCR) الذي يستهدف جين 18 .S rRNAفي مستشفيي الكوت والكرامة بمحافظة واسط، العراق، تم الحصول على 67 عينة براز من مرضى يعانون من أعراض الجهاز الهضمي، وخاصة الإسهال. وبلغ معدل الإيجابية لأنواع المتحولة الأميبا 67.2%، كما يتضح من الفحص المجهري، الذي كشف عن أكياس كروية ذات نوى محددة بوضوح. من ناحية أخرى، حدد التحليل الجزيئي التقليدي القائم على تفاعل البوليميراز المتسلسل (PCR) وجود المتحولة الحالة للنسيج (E. histolytica) في 80% من العينات المختبرة، مما يشير إلى عائد تشخيصي أكبر. تم التحقق من الاختلافات المهمة بين المنهجيتين من خلال التحليل الإحصائي ($P \leq 0.05$). تؤكد النتائج على الحساسية والنوعية الفائقة لتفاعل البوليميراز المتسلسل للكشف الدقيق ويدعو هذا البحث إلى إدراج طرق التشخيص الجزيئي، مثل تفاعل البوليميراز المتسلسل المتعدد(multiplex PCR) ، في الفحص الطفيلي الروتيني، وخاصة في المناطق الموبوءة. بالإضافة إلى ذلك، تُرسي النتائج أساسًا لاستراتيجيات تشخيصية مُحسّنة وتدخلات صحية عامة مُصممة لإدارة العدوى الأميبية.

الكلمات المفتاحية: المتحولة النسيجية، تفاعل البوليميراز المتسلسل(PCR) ، التشخيص الجزيئي، عينات البراز، الفحص المجهري.

1. Introduction

Entamoeba histolytica, or E. histolytica, is a unicellular parasitic protozoan from the Entamoebidae family that induces parasitic illnesses in animals [1]. Increased diarrhea, gastrointestinal discomfort, dyspepsia, gas, and fever are the symptoms of intestinal amoebiasis, the most common form of amoebiasis [2]. The parasite Entamoeba histolytica infects over 50 million people yearly and kills about 100,000 of them. It has been found in many parts of the world. In cases of parenteral amoebiasis, the amoeba travels from the intestines to other parts of the body via the bloodstream, potentially infecting other organs including the brain, liver, or lungs and causing abscesses there [3, 4]. The illness is more common in regions with less developed sanitation and food security, including the tropics and subtropics, than in regions with more severe weather. Liver infections, caused by amoebas, are a leading cause of death and disability in many parts of the world. If these parasites are able to infiltrate injured blood vessels, they will most likely cause an amebic liver abscess, which may spread throughout the body [5].

Among the nine species of intestinal amoebae that may infect humans, E.histolytica is the only one that can cause illness. Its life cycle is simple: contaminated food or water is the first vector for transmission, and the parasite can live and multiply in the gut until it releases its cysts into the feces [6]. Most infections don't show any signs, but certain strains of E. histolytica may get through the gut wall and produce amebic dysentery, which is characterized by bloody stools and severe ulceration [7].

Entamoeba histolytica has a simple two-stage life cycle that includes an infectious cyst and a motile trophozoite. Tissue invasion is caused by the amoeboid trophozoite, which is 10–60 μm in size and consists of a single nucleus with a central Karyosome. The cyst form comprises four nuclei and a diameter of 10–20 μm [8]. Entamoeba histolytica has a simple two-stage life cycle that includes an infectious cyst and a motile trophozoite [9]. The cyst form contains four nuclei and is between 10 and 20 μm in diameter. The amoeboid trophozoite, which is 10 to 60 μm in size, is the thing that invades tissue. It has a single nucleus with a central Karyosome [10].

The cysts may persist for weeks in a moist environment and exhibit relative resistance to desiccation and chlorination; infection occurs via ingestion of food or drink contaminated with feces [3]. The quadrinucleate cyst undergoes nuclear and cytoplasmic division inside the lumen of the distal small intestine, resulting in the formation of eight trophozoites [11]. Ninety percent of persons who become infected become asymptomatic cyst passers, which means they conclude the organism's life cycle [12].

Trophozoites cause colitis in the last 10 % of cases by infiltrating the intestinal epithelium. When trophozoites get into the circulation, they may move via the blood and infect other organs, most often the liver [13]. In developing nations, microscopy continues to be the standard diagnostic technique for *E. histolytica*, despite its lower reliability compared to more specific and sensitive methods, In collaboration with the Pan American Health organization (PAHO) and the World Health Organization (WHO) [14]. Red blood cell-ingesting trophozoites in a stool sample are a strong diagnostic indicator of *E. histolytica* infection when performed under microscopy, and to identify parasite antigen in the faeces via PCR amplification or the detection of parasite DNA [15].

The aim of this study is to assess the efficacy and dependability of two diagnostic methods for the detection of Entamoeba spp. parasites: microscopic identification through Lugol's iodine staining and molecular detection through multiplex Polymerase Chain Reaction (PCR) technique. The goal is to improve diagnostic accuracy and facilitate the differentiation of Entamoeba species that are morphologically similar.

3. Materials and Methods

3.1 Collection of stool samples

The tool samples were collected from patients who were getting medical care at Al Kut Hospital and Al-Karama Teaching Hospital, both of which are located in the city of Kut|Iraq. Between the months of December 2023 and March 2024, a total of sixty-seven stool samples were collected while exhibiting symptoms of bloody and mucous diarrhea. The samples are taken from children of both sexes who are of various ages and who have diarrhea. To keep the samples from drying out and to preserve their moisture content, they were collected in 80 ml plastic bottles that were sterile, well-sealed, and clean. Until it was needed, each stool sample was kept in 2.5% potassium dichromate at 4 oC. Following their numbering, the samples were sent to Wasit University's College of Science laboratory for analysis.

4. Results and Discussion

4.1. Detection *Entamoeba Spp.* by using microscopic examination test

The present investigation involved the examination of 67 stool samples from patients who were experiencing normal diarrhea, bloody diarrhea, and mucous diarrhea. A glass slide was divided into two sections, one of which contained iodine dye and the other of which contained physiological saline solution, and they were examined microscopically. The results indicated that the percentage of individuals infected with amebiasis was 67.2%, with 45 positive samples out of 67, while the percentage of non-infection was 32.8%, with 22 samples out of 67 being negative, as in table (3-1). The findings indicated that *Entamoeba spp.* cysts were spherical, of red color with a blue background, and contained four nuclei, as shown in figure (1,2).

Table (1): Detecting *Entamoeba spp.* by using microscopic examination.

Microscope examination of *Entamoeba Spp*	**Results**	
	No	**%**
Specimen positive for *Entamoeba spp*	45	67.2%
Specimen negative for *Entamoeba spp*	22	32.8%
Total	67	100%

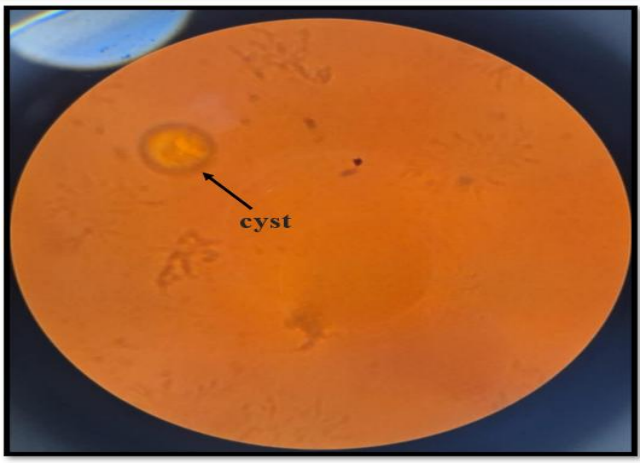

Figure (1): Direct examination of *Entamoeba spp.* cysts using Lugol's Iodine method. Cysts have a spherical shape with a single, 40x nucleus.

This study aimed to assess the prevalence of *E.histolytica* and *Giardia lamblia*, a total of 398 stool samples (215 from men and 183 from females) were obtained from both children and adults visiting Heet General Hospital and several private laboratories associated with Heet, An infection rate of 64.3% was recorded overall, compared to *Giardia lamblia* infection (7.5%), the rate of *E.histolytica* infection was greater (56.7%). When looking at the impact of the seasons on infection rates, the highest rate of *E. histolytica* was recorded in August (14.6%), while the lowest rate was recorded in September (9%), Age-related infections predominantly occur in individuals over 35 years (26.63%), followed by those under 15 years (16.83%), the infection appears to be non-gender specific, with a little increase in prevalence among males (29.4%) compared to females (27.3%), Seasonal infections were thought to be much more common in May and June (2%) than in July, August, and September (125%, 1.25%, and 1%, respectively), with a rate of virus retention that was the same for people of all ages or between women (3.2%) and men (4.3%) [16].

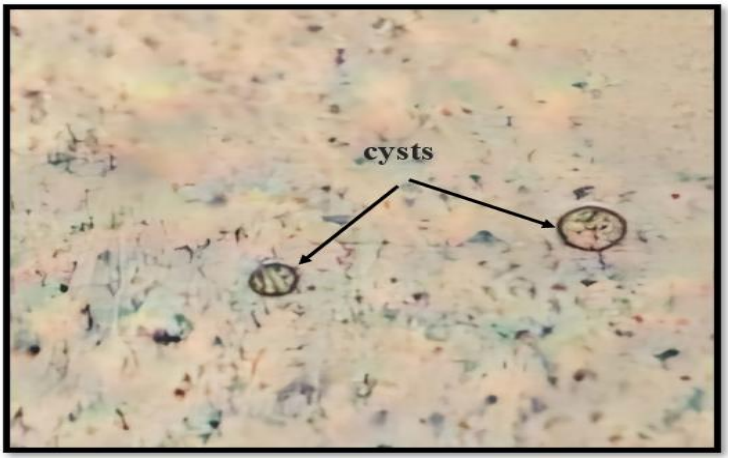

Figure (2): Direct examination of Entamoeba spp. cysts using a conventional saline method. Cysts are characterized by a spherical shape and a single 10x nucleus.

4.2 Detection of *E. histolytica* in experimental animals by using conventional PCR technique.

The present study revealed an 80% revalence of *E. histolytica* in the PCR data, demonstrating a higher detection rate compared to microscopy (80% vs 67.2%). In this study, 45 This cross-sectional study involved the collection of stool samples from symptomatic patients presenting with gastrointestinal complaints at Al-Kut and Al-Karama hospitals between December 2023 and March 2024. The samples were obtained from patients infected with the parasite and were taken from Al Karama Hospital and Al Kut Hospital. The infection rate for the *E. histolytica* parasite was 80%, with 36 positive samples identified. Nine samples tested negative, as shown in the table (3-1):

Table (2): percentage of overall parasite infections.

Multiplex PCR for *E.histolytica*	Results		P value	df
	No.	%		
Specimen Positive for *E.histolytica*	36	80%	0.0201 *	1
Specimen Negative for *E.histolytica*	9	20%		
Total	45	100%		
* (P≤0.05).				

The results of conventional PCR are illustrated in Figure (1-1), which illustrates the analysis of the small subunit ribosomal rRNA gene for the detection of *E.histolytica* in human stool samples. M (Marker cascade 2000-100bp). Some positive *E.histolytica* samples were observed in lane (1-15) at a PCR product size of (515bp). The picture indicates that lane (1,2,3,4,5,8,9,10,11,12,13, and 15) is positive, while lane (6,7, and 14) is negative.

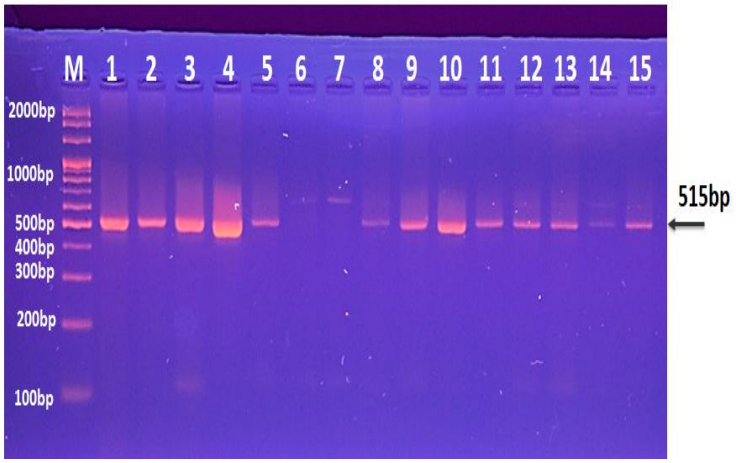

Figure (3): A picture of agarose gel electrophoresis displaying the PCR product analysis of the small subunit ribosomal rRNA gene for the detection of *E. histolytica* in human stool

samples. M (Marker ladder 2000-100 bp). Lane (1-15) Demonstrated positive *E. histolytica* samples with a PCR product size of 515 bp.

Ngui, R., et al. [17] reported that PCR detected 75.0% of *E.histolytica* infections, which is consistent with our findings. Nested PCR successfully amplified 52 (69.3%) of 75 microscopy-positive samples, enabling identification of *Entamoeba spp* based on amplicon size. PCR analysis confirmed that 351 microscopy-negative samples did not have *Entamoeba* infections. Repeat PCR testing confirmed that all 23 samples that were positive by microscopy but negative by PCR were still negative. *E. histolytica* infection (75.0%; 39/52) was the most prevalent outcome among the 52 PCR-positive results.

The current study's findings are in line with those of a previous one that examined the prevalence of *E. histolytica* in stool samples from children in Nasiriyah city, Dhi Qar Governorate, Iraq. In that study, a total of 2,639 stool samples were collected from the children, and PCR analysis of 90 of those samples revealed a high infection rate of 74.44% for *E. histolytica*, while 25.56% of the samples tested negative. These findings shed light on the frequency of *E. histolytica* among diarrhea patients in the research region over the allotted time frame [18].

In the West Bank, Shareef's (2022) research revealed that 74% of positive samples were confirmed by PCR, which is consistent with the current study [19]. Roy *et al.* (2005) found a PCR rate of 72.0% for *E.histolytica*, which is consistent with previous findings

The results of our study conflict with those of Alkhuzaey (2019). *E. histolytica* was found to be 38.1% prevalent in Thi-Qar. In addition to disagreeing with Salim (2016), he reported that the *E. histolytica* prevalence in Thi-Qar was 5.0% [20]. In contrast to the findings of Ngosso *et al.* (2015) in Dar es Salaam, which found that out of 144 stool samples, 48 (33.3%) of them had *E. histolytica*, the findings of the current investigation contradict those findings [21].

4. Conclusion

This study indicates that while Lugol's iodine stain is still a useful and accessible diagnostic tool for Entamoeba spp. identification, it lacks species differentiation and sensitivity. However, multiplex polymerase chain reaction (PCR) can accurately identify Entamoeba histolytica and distinguish it from morphologically identical non-pathogenic species. In clinical settings, where correct distinction is critical for treatment choices, PCR approaches for amoebiasis diagnosis are reliable due to their statistical significance and increased detection rate. By adding molecular diagnostics to parasitological investigations, especially in endemic countries like Iraq, early identification, epidemiological monitoring, and disease management might improve. This study suggests that future research should assess the cost-effectiveness of large-scale diagnostic application in resource-limited environments, increase the sample size, and include other Entamoeba species.

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