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Differential Diagnosis of *Entamoeba* spp. Using the 18SrRNA Gene in Gastroenteritis Patients

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Abstract

In order to accurately diagnose *Entamoeba spp*., this study's major goal was to develop a proof-of-concept method for simultaneously detecting pathogenic and non-pathogenic amoebae using DNA. During amoebiasis, two diagnostic techniques (microscopic inspection and PCR techniques with particular primers) were evaluated. About 100 feces samples from Fallujah individuals who had clinical symptoms were taken. The outcome reveals that only 20 samples have *Entamoeba* spp. infections. According to this study, the two species had distinct infection percentages. *Entamoeba histolytica* was the most prevalent infection, at 85%, followed by *Entamoeba dispar*, which was 15% of all the *Entamoeba*-positive samples. In addition to studying the morphology and genes of the positive samples, the residential environment, age, and gender were also taken into consideration. It showed the infective patients in the rural area had a higher rate of infection, which was 18%, while the infection in the urban area was less, which was represented by 2%. As for the effect of the age factor, the highest percentage was from children who did not exceed ten years old, which was 10%. The lowest percentage was 2% for people over the age of sixteen (16). According to the gender factor, a different percentage appeared in males than females. The result showed a high infection rate for males, which was estimated at about 16%, but for females it was much less, at only 4%.

Keywords: *Entamoeba histolytica*, *Entamoeba dispar*, epidemiology, molecular techniques

التشخيص التفريقي لـ Entamoeba spp باستعمال جين SrRNA18 في مرضى الالتهاب المعوي

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الخلاصة

من أجل التشخيص الدقيق لـ .Entamoeba spp. كان الهدف الرئيسي لهذه الدراسة هو تطوير طريقة إثبات المفهوم للكشف في وقت واحد عن الأميبات الممرضة وغير المسببة للأمراض باستعمال الحمض النووي. خلال داء الأميبات ، تم تقييم طريقتين تشخيصيتين (الفحص المجهري وتقنية تفاعل البوليميراز المتسلسل مع بادئات معينة). تم أخذ حوالي 100 عينة براز من الفلوجة الذين ظهرت عليهم أعراض إكلينيكية. تكشف النتيجة أن 20 عينة فقط لديها .Entamoeba spp الالتهابات. وفقًا لهذه الدراسة ، كان للنوعين معدلات إصابة

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مميزة. كانت إنتاموبيا هيستوليتيكا هي العدوى الأكثر انتشارًا. والتي كانت 85٪ ، تليها Entamoeba dispar ، والتي كانت 15٪ من جميع عينات Entamoeba الإيجابية. بالإضافة إلى دراسة مورفولوجية وجينات العينات الإيجابية ، تم أيضًا مراعاة البيئة السكنية والعمر والجنس. وأظهرت النتائج أن نسبة الإصابة بالمرض في الريف كانت أعلى حيث بلغت 18٪ بينما كانت الإصابة أقل في الحضر بنسبة 2٪. أما بالنسبة لتأثير عامل العمر فقد كانت أعلى نسبة بين الأطفال الذين لم يتجاوزوا العاشرة من العمر والتي بلغت 10٪. كانت أدنى نسبة 2٪ للأشخاص فوق سن السادسة عشرة (16). وفقا لعامل الجنس ، ظهرت نسبة مختلفة في الذكور عن الإناث. وأظهرت النتيجة ارتفاع معدل الإصابة عند الذكور ، حيث قُدِّر بحوالي 16٪ ، بينما كان أقل بكثير للإناث بنسبة 4٪ فقط.

1. Introduction:

The parasite *Entamoeba histolytica* is one of the most common parasites in Iraq and the world and causes amoebic Entamoeba histolytica and dysentery.
The estimated number of infections with this parasite (amoebic and amebic hepatitis) is 50 million in the world [1]. It is the reason for 40,000–100,000 deaths annually [2]. E. histolytica causes amoebic colitis and liver abscesses [3].

There are several *Entamoeba* protozoan species that attack people, but not all of them cause disease. *Entamoeba histolytica*, a pathogenic ameba, is well recognized for causing intestinal and extraintestinal infections. Other Entamoeba species, such as *E. dispar*, *E. moshkovskii*, and *E. bangladeshis*, which are physically similar, despite increasing research into their potential for pathogenicity, are mostly not linked to illness [4]. *Entamoeba histolytica* is a parasitic protozoan that lives worldwide and causes amoebiasis in humans. It is found in the large intestine. Amoebiasis shows up as chronic diarrhea with mucus or blood, along with nausea, fever, flatulence, and stomach discomfort [5].

Amoebiasis that is left untreated, particularly intestinal infections, can cause death. According to World Health Organization projections (WHO), dysentery and infections caused by amoeba cause up to 500 million illnesses and 100,000 deaths globally each year [6]. *E.histolytica* varies by nation, socioeconomic status, sanitary conditions, and population. It is endemic in underprivileged areas across the tropics and subtropics [7]. The transmission and geographic spread of intestinal parasite infections are known to be influenced by environmental, socioeconomic, demographic, and hygiene-related factors [8]. Location, age, consumption of raw vegetables, and drinking water quality were all noted as significant risk factors in a Brazilian study [9].

Brumpt originally suggested *Entamoeba dispar* as a non-invasive species in 1925 when he distinguished between the two species (*E. histolytica and E. dispar*) based on their pathogenicity in people and kittens [10], and Simic added more proof to this hypothesis in 1931. The postulated separation of the two species, however, wasn't supported until 1978 [11], when two *Entamoeba* groups were isolated from symptomatic and asymptomatic patients. Recent epidemiological research on E. histolytica provided correct results by using molecular techniques [12]. The prevalence rate of *E. histolytica* has never been determined using the PCR method up to this point in the city of Erbil. Infections have been documented in several studies. Nearly all Iraqi cities contain *E. histolytica*, but only a handful have used molecular techniques; the majority rely on microscopic investigation [13]. The least-studied category internationally, asymptomatic people have not yet been the subject of any research in Iraq. Furthermore, it is mostly uncertain if the asymptomatic people have contracted either *E. dispara* or the harmless *E. histolytica* conflict [14]. This study was carried out to close this knowledge gap and aims to

ascertain the frequency of Entamoeba in Fallujah City, confirming the existence of and distinguishing between harmful and nonpathogenic amoebae, using microscopic investigation and subsequently molecular methods. The recently updated nomenclature states: The pathogenic strain is *E. histolytica*, whereas the non-pathogenic strain conflicts [15].

2. Materials and Methods

2.1 Feces examination:

100 randomly chosen samples of feces were obtained from both asymptomatic and sick individuals. Specimen donors responded to a detailed questionnaire regarding their background, age, and place of residence. Each participant was given a 60-ml wide-mouth, screw-cap jar with a label, and they were told to bring enough uncontaminated samples to fill it. The samples were immediately wet-mounted and microscopically inspected for the presence of *Entamoeba spp*. trophozoites or cysts. The two species cannot be separated morphologically. However, they may be identified using a variety of experimental techniques [13, 14].

2.2 Genomic DNA extraction

Each sample of positive feces was put into a 2-ml microcentrifuge tube and given three rinses in MilliQ H2O buffer before the DNA was extracted. After that, the samples were centrifuged at 2000 g for 5 minutes to remove the ethanol from them. The QIAamp Fast DNA Stool Mini Extraction Kit's lysis buffer was used with each sample of feces (QIAGEN, Hilden, Germany). The 20 fecal-positive samples (n = 20) were then individually subjected to the manufacturer's recommended DNA extraction procedure. (for use with the QIAamp Fast DNA Feces Mini Extraction Kit). After that, the DNA was extracted and stored for later use at 20 °C.

2.3 Nested multiplex PCR amplification

The work was based on the amplification of the small subunit ribosomal RNA (*18S rRNA*) (Bioneer, Korea) gene of *Entamoeba species*, which was based on an earlier approach reported by Khairnar and Parija [4]. We used three primers. It has two primers, E-1 (5′-TAAGACAGAGCGAAA-3′) and E-2 (5′-GTACAAAGGGCAGGGACGTA-3′), and is 897 base pairs long.

Additionally, 439 bp Primer sets made specifically for the *Entamoeba histolytica* genus include E-1 (5′-AACATTGTTTCTAGATCTGAG-3′) and E-2 (5′-AAGAGGTCTAACCGAAATTAG-3′). The third PCR was created for *Entamoeba dispar* and has a forward EH-1 (5′-TCTAATTTCGATTAGAACTCT-3′) and reverse EH-1 (5′-TCCCTACCTATTAGACATAGC-3′) primer pair. Its size is 174 bp. With every set of samples evaluated, there were also positive responses. All three *Entamoeba* species had distinctly different sizes, with *E. histolytica* and *E. dispar* having species-specific product sizes of 439 and 174 bp, respectively [18].

3. Results:

100 male and female participants made up our small random sample. As established by the microscopic analysis, 20% of 100 feces samples from sick people tested positive for *Entamoeba species* cysts and/or distinctive characteristics of the trophozoite. *Entamoeba* quadrinucleated spherical cysts and amoebic trophozoites with many pseudopodia were seen with a light microscope, and their shapes were used to figure out what they were. Rural participants (18%) had rates that were significantly higher (p 0.05) than urban participants (2%).

Depending on the quality of the smears, some of their diagnostic morphologic characteristics overlap, making differentiated diagnostic identification difficult. The screening method for the identification of *E. histolytica*/E. dissimilar complex/genus and the additional *Entamoeba*

discovered in human stools should still be microscopy, but at this moment molecular methods can be used to quickly confirm *E. histolytica* infections. A number of PCR-based techniques and antigen tests have been created over the past ten years for the identification of *E.dispar* contrast and *E. histolytica*. However, when considering their application in a multiplex format, most of these systems have certain drawbacks. *Entamoeba spp*. identification using multiplexing might support the amebiasis diagnosis. A recent multiplex PCR-bead approach that only comprised one species of *Entamoeba* produced a sensitive diagnostic screen for a wide array of intestinal parasites, *E.histolytica* . We created a Luminex test for detection and discrimination to achieve this objective. *E. histolytica* and *E.dispar*. Such tests could eventually be approved for use in clinical diagnosis. In our investigation, biotinylated primers were used to create the PCR products, which were then combined with microspheres attached to probes built on *18S rRNA* sequences that could distinguish between *Entamoeba species.*

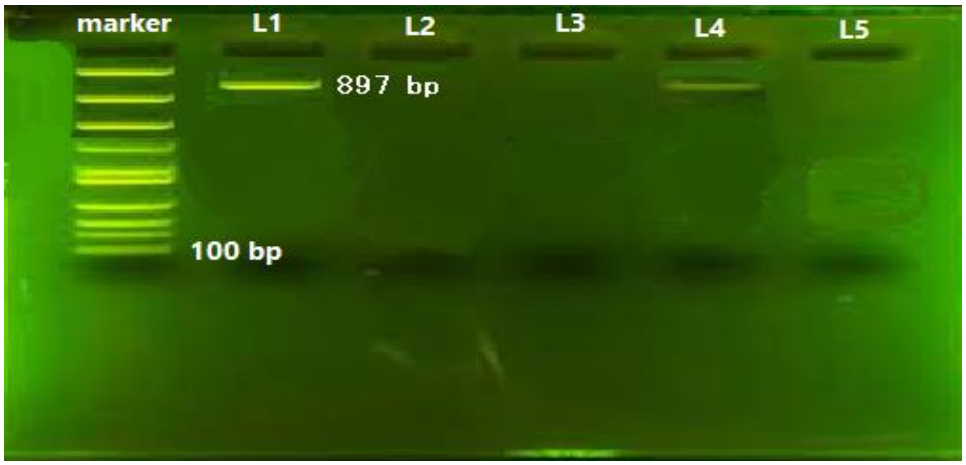

Figure 1: Detection PCR product: 1.5% agarose gel electrophoresis revealed 897 bp for *Entamoeba spp.* fragment amplification L1. marker DNA ladder 1000 bp

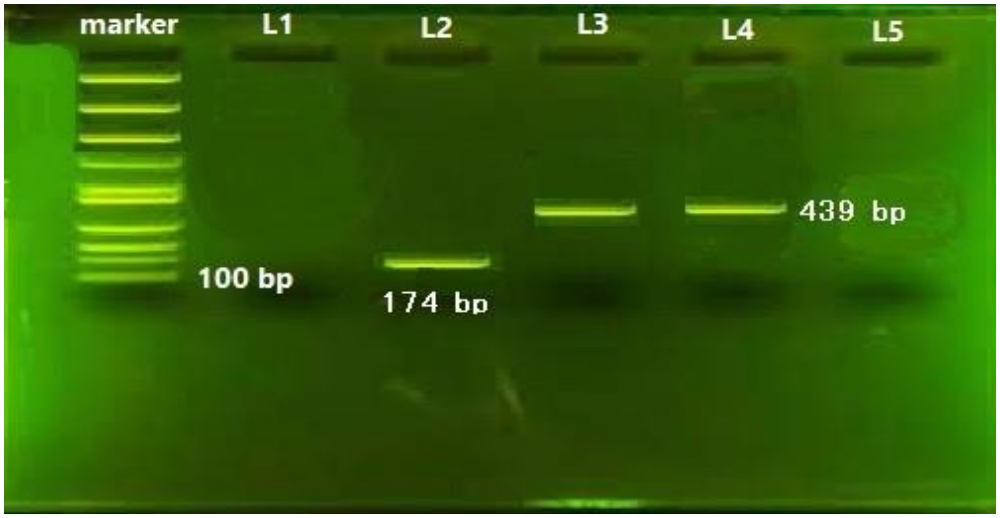

Figure 2: Detection PCR product: 1.5% agarose gel electrophoresis revealed 439 bp and 174 bp for *E.histolytica* and *E. dispar*, respectively. L1 fragment amplification. 1000 bp marker DNA ladder

4. Discussion

From 100 samples, there were 20 positive samples with confirmed cases of *E. histolytica* and *E. dispar* infections in Fallujah city. The high incidence rate of men is the opposite of that of women. It is inferred that the reason may be different personal hygiene habits, like in a previous study [19]. Although it cannot distinguish between *E. histolytica* and *E.dispar*,

microscopic examination is still the most accurate way to identify intestinal *Entamoeba* infections [20]. In developed countries, *E. histolytica* infection is common among travelers and new immigrants [18, 19], as per the findings of this study. The transferred cases of *E. histolytica* from outside the epidemic area showed a high incidence.

Globally, developing countries are plagued by an epidemic of intestinal illnesses brought on by intestinal pathogens [23]. Additionally, children aged 6 to 10 years old had the highest rates of parasite infection; however, there were no differences between infected and uninfected children in the sociodemographic variables examined. Intestinal parasitic infections are closely associated with specific sociodemographic risk factors in the community environment; particularly, no statistically significant differences ($p > 0.05$) were found between the age or gender of children who were parasitized and those who were not. This shows that the children's primary infection sources may not be within the home. School is the other location where kids spend most of their time. We anticipated finding some significant disparities in the parasitized state of the children since the schools revealed variances in the cleanliness index, which is comparable to the study [24]. It is vital to research infections that endanger human health globally because epidemiological studies on the frequency of parasitic intestinal infections in various places often try to pinpoint diseases that affect human populations and at-risk communities [25]. Various ecological, behavioral, socioeconomic, and health-related variables can directly or indirectly impact parasite infestations. Additional important factors that influence the spread of infection, the transmission of disease, and mortality include income, work status, and educational attainment [26]. There were 18 rural amoebic patients in this research. This outcome is comparable to that reported for Iraq by Hamza et al. [27] and Al-Damerchi & Al-Ebrahimi [28]. The coronavirus disease 2019 (COVID-19) outbreak in Iraq, which made people wary of hospitals and healthcare facilities, may have contributed to the lowest infection rate throughout the research period in 2020 [29]. Our findings concur with those of several investigations, including those conducted in Iraq by Saida [25], Al-Taei [30], and Al-Saqur et al. [31], in India by Nath et al. [32], and in Jordan by Al-Dalabeeh et al. [33]. The study's findings, which are consistent with earlier research by Nath et al. [32], Al-Damerchi & Al-Ebrahimi [28], and Al-Dalabeeh et al. [33], which included just two patients, show that there were more infections in rural than urban regions. There were fluctuations in the proportion of infections by month over the course of the six-year research period. Although environmental factors are usually thought to alter over time, such as temperature, drinking water pollution, health services, the frequency of parasitic illnesses, and dietary practices [30], these findings concur with those of Saida [25] and Nath et al. [32].

5. Conclusion

In addition to the microscopic method, the molecular method using DNA extraction is also useful in distinguishing between different types of *Entamoeba.*

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