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Differential detection of some *Entamoeba* spp. in fresh stool specimens by PCR

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ABSTRACT. The current study aimed to collect stool samples from patients visiting Sharqat General Hospital and primary healthcare centers in Salah ad-Din Governorate to diagnose some the species of *Entamoeba* parasite using the molecular PCR technique, and by designing specialized primers to detect parasite DNA. The study spanned a period of time extending from the beginning of November 2023 to the end of March 2024. Stool samples were collected from individuals infected with amoebic parasites after they were clinically diagnosed by doctors based on their symptoms. Patient data was obtained and classified to determine if they were infected with parasites. Microscopic analyses were carried out on the stool in order to determine if individuals were infected with *Entamoeba*. PCR was utilized in molecular studies and every case identified using microscopy was confirmed by the molecular assay. We conclude the PCR technique is very accurate in diagnosis species of organisms, and this was observed through what was obtained from the current study.

Keywords: *Entamoeba hitolytica*; *Entamoeba dispar*; PCR; DNA.

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Introduction

Parasitic diseases also pose a great international health challenge (Rosa et al., 2020; Beavogui et al., 2021; Iannacone et al., 2021). Intestinal parasites continue to be quite widespread due to a variety of environmental, social and geographical conditions, and they are more prevalent in tropical and subtropical nations (Oliveira et al., 2020; Silva et al., 2021a; Rahman et al., 2022; Ramos et al., 2023; Neto et al., 2025). According to Silva et al. (2021b), developing countries are characterized by poor sanitation conditions, such as a lack of sewage systems, waste collection, public cleaning services, and water services, along with low levels of education and hygiene among the population, leading to the occurrence of intestinal parasitoses. Intestinal parasites are estimated to infect about more than one million people (Karim et al., 2024). According to the World Health Organization, *E. histolytica* is the third leading parasitic cause 100000 death year^{-1} (Cuellar-Guevara et al., 2019).

Life cycle is direct and transmitted by water and food contamination by mature cyst and excystation occur in bowel lumen in order to release trophozoites, which have the ability to colonise mucosa of large bowel, the severity of symptoms vary depending on the site of ulceration and its severity, such as colicy pain & diarrhea sometimes mixed with blood & mucus accompanied with loss of appetite, nausea, vomiting, and weight loss, general debilitation, loss of electrolyte, potassium. The intestinal protozoan is, one of the greatest causes of malnutrition and anemia (AL-Agha & Teodorescu, 2000; Alelign et al., 2024).

The condition may be present with no signs or lead to serious complications. There are instances when certain species of amoeba, including *E. histolytica*, spread beyond the intestine and cause infections elsewhere in the body, resulting in the development of amoebic abscesses (Wesel et al., 2021).

The clinical disorder amoebiasis results from infection by *E. histolytica* although the genus *Entamoeba* contains six species (*E. coli*, *E. histolytica*, *E. dispar*. *E. moshkovskii*, *E. bangladeshi*, *E. hartmanni*, and *E. poleki*) that colonize the human large intestine (Flaih et al., 2021).

If left untreated, signs of infection with *Entamoeba histolytica* may continue for several days (Sharif et al., 2022). The prevalence of this disease is higher in developing countries because of poor sanitation and water contamination (Vasquez-Rios et al., 2016). Furthermore, failing to address parasitic infections in these nations contributes to the rise of more infections (Abuseir, 2023).

The common microscopic methods of detecting amoebiasis use the observation of tetranucleated cysts or hematophagous trophozoites and its sensitivity is limited by the expertise of the laboratory workers in identification of trophozoites which without their movement might be misidentified as leukocytes and macrophages and tissue cells. Additionally, rapid sample handling is required (20-30 min), as the trophozoites are destroyed, resulting in false negatives. Or immunological techniques, such as antigen or antibody detection. Unluckily, the possibility of other enteropathogenic infecting species of *Entamoeba* (with the same morphology of cysts as *E. histolytica*) or the impossibility of distinguishing between a recent and earlier infection causes these methods to be inappropriate for specific diagnosis. To this end, molecular detection of parasite nucleic acids through the polymerase chain reaction and its modifications (nested PCR, multiplex PCR, and real-time PCR). These methods solve some issue of identification, taxonomy, epidemiology, and clinical significance; as well as a source of information on the genetic variety of *Entamoeba* species that are implicated with pathogenic ambiguity. the context of such knowledge offers directions in the proper clinical management of amoebiasis (Calle-Pacheco et al., 2022). However, nucleic acid detection methods based on polymerase chain reaction (PCR) have demonstrated higher sensitivity compared to microscopic and antigen detection tests (Madden et al., 2019).

PCR is a proven method for identifying and distinguishing parasites due to its high accuracy and sensitivity. It is a globally reliable molecular technique for rapid diagnosis with high accuracy (Ögren et al., 2020). The principle of this method relies on the use of the DNA polymerase enzyme and occurs in vitro. Therefore, tens of billions of copies of specific parts (which may be a specific gene or sequence) of the extracted DNA can be generated (Kadri, 2019).

There are two phenotypically similar species of *Entamoeba*, *E. histolytica* (the pathogenic species) and *E. dispar* (the non-pathogenic species) (López-López et al., 2017; Costa et al., 2018). As an example, in 1926, Brumpt postulated the existence of *E. dispar*, a species that could not be distinguished using light microscopy with *E. histolytica*. Nevertheless, *E. dispar* has characteristic physiological, biochemical and ultra-structural features, the latter of which have been discussed most recently. Through the application of PCR, *Entamoeba* species have been detected in human beings, despite the fact that organisms exhibit the same morphology similar to that of *E. histolytica* and even share virulence components. Also, the method identifies complex infections that comprise *E. histolytica* and *E. dispar* or *E. moshkovskii* (Calegar et al., 2016; Calle-Pacheco et al., 2022; Servián et al., 2023). Therefore, the current study aimed to isolate *E. histolytica* from infected patients, diagnose it molecularly, and attempt to distinguish it from the *E. dispar* parasite by designing a gene primer specific to both of them.

Materials and methods

This study was conducted over a period extending from the beginning of November 2023 to the end of March 2024. Samples containing *Entamoeba* parasites, both feeding and encysted, were obtained from the feces of parasitized individuals who visited the general hospital and primary healthcare centers in Sharqat city.

Examination of stool specimens

Visual examination

The examination of stool samples was performed by observing the shape of feces, as well as, consistency and color. Trophozoites are often found in liquid or the soft and cysts are seen in semisoft samples (Sastry & Bhat, 2018).

Microscopic examination

Preparation of direct wet mount

Stool samples were taken from patients with diarrhea and investigated microscopically by direct wet mount using physiological saline and Lugol's iodine solution (Sastry & Bhat, 2018).

Method of sedimentation

The concentration method was used for parasites because of their small numbers in stool samples taken from patients and their inability to visual them using a direct wet mount. Therefore, the sedimentation method is followed to detect parasites according to Sastry & Bhat (2018).

Collection of parasite samples

Fresh fecal samples were collected from infected individuals using sterile, sealed plastic containers. They were examined under a microscope within 30 minutes of arrival at the laboratory. A portion of the samples was preserved with a 500-microliter preservative of polyvinyl alcohol, known as shelled DNA, until DNA extraction was initiated and used in PCR technology.

The primers

The work was based on the amplification of the small-subunit ribosomal RNA (18S rRNA) gene of *Entamoeba* species, which was based on an earlier approach reported by Farhan et al. (2024) as shown in the Table 1.

Table 1. The primers of *Entamoeba* species and sequence it.

Primer name		Sequence (5'-3')	Size bp
E. histolytica	Eh-1 F (forward primer)	5'-AAGCATTGTTTCTAGATCTGAG-3'	439
	Eh-2 R (reverse primer)	5'-AAGAGGTCTAACCGAAATTAG-3'	
E. dispar	Ed-1 F (forward primer)	5'-TCTAATTTCGATTAGAACTCT-3'	174
	Ed-2 R (reverse primer)	5'-TCCCTACCTATTAGACATAGC-3'	

DNA extraction

DNA of *Entamoeba* parasites was extracted from stool samples taken from patients infected with the same parasite using the Presto Minig DNA Extraction Kit, supplied by Geneaid company.

Estimation of DNA concentration and purity

Extracted DNA was examined using a Nanodrop spectrophotometer, which measures DNA concentration (nanograms/microliter) in laboratories of Faculty of Applied Sciences. DNA purity was verified by reading the absorbance at a wavelength of 260/280 nm and the values (1.7-2.0) is considered as standard for pure DNA according to the method Bruijns et al. (2022).

Multiplex-PCR

PCR was performed using a thermocycler device equipped by Applied Biosystems, a Singapore-based company. The method included the following:

Primer dilution

All primers were prepared by Macrogen, a Korean company, in the form of lyophilized powder. A stock solution was prepared by adding 250 µL of nucleic acid-free water to each primer to obtain a stock solution with a concentration of 100 pmol. A working solution was then prepared by withdrawing 100 µL of the stock solution and diluting it with 90 µL of nucleic acid-free water to obtain a final working solution concentration of 10 pmol.

Solutions and buffers

I used the PCR Master Mix Kit, supplied by Germany/r-biopharm. It consists of 96 test strips in small 0.2 ml tubes. Each tube contains a set of materials prepared in a volume of 20 µL.

PCR protocol program

The protocol of PCR technique was used to identify the two species of *Entamoeba* (*Entamoeba histolytica* and *Entamoeba dispar*) as shown in the Table 2.

Table 2. The protocol of PCR technique for *Entamoeba* species.

The steps	Temperature (°C)		Time (min)	The cycles
	E. histolytica	*E. dispar*		
First denaturation	94	94	5	1
Denaturation	94	94	30	
Annealing	53	51	30	35
Extension	72	72	30	
Final extension	72	72	10	1
Holding	4	4	Infinity	

Electrophoresis on agarose gel

DNA samples were electrophoresed on a 1% agarose gel to confirm the presence and integrity of DNA according to the method of Ysea et al. (2022).

Estimating the molecular weights of parasitic DNA

Once electrophoresis was finished, all the samples were carefully loaded into a gel electrophoresis unit. The molecular weights of the DNA were determined by the locations of the bands formed in the gel compared to a molecular weight marker. It generated bands with specific sequences and molecular weights across the range of 100 to 1200 pb in the gel. From the results obtained, a species and genus of parasites were determined in each of the collected faecal samples based on the type of PCR primers used.

Results and discussion

The primers used in the current study demonstrated their specialized effectiveness in the migration process and detection of DNA bands of the aforementioned sizes. The specialized primer pairs (EhF-EhR) for *E. histolytica* and (EdF-EdR) for *E. dispar* showed bands of 439 pb and 174 base pairs, respectively. The DNA size buffer indicator (Lader DNA) showed graded bands of size (100-1200 pb). All samples under study contained both *E. histolytica* and *E. dispar*. The resulting bands were imaged using a state-of-the-art Gel Documentation device connected to an imaging system, as shown in Figures 1 and 2.

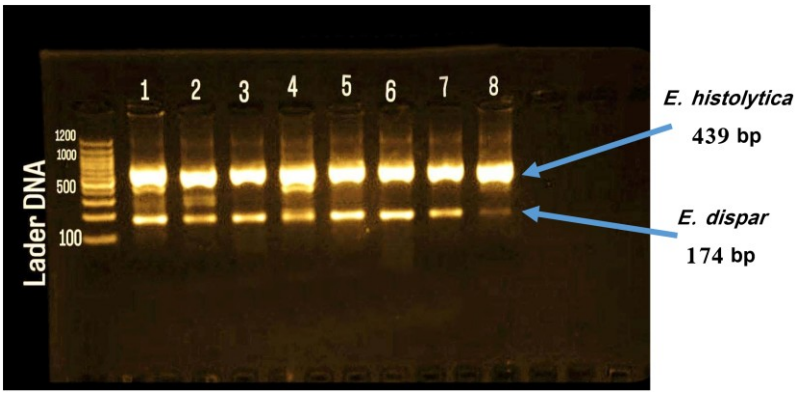

Figure 1. PCR diagnostic results for samples (1-8) of *E. histolytica* and *E. dispar*.

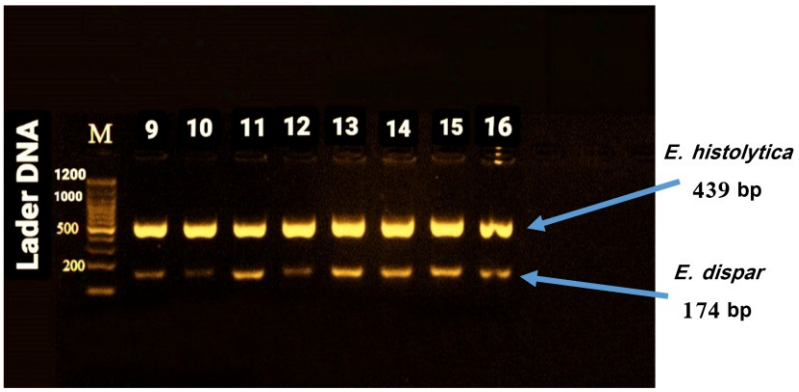

Figure 2. PCR diagnostic results for samples (9-16) of *E. histolytica* and *E. dispar*.

All 16 samples under study examined microscopically showed a positive result by PCR.

The success of the specialized primers used in the study to detect and amplify the parasite DNA is due to the fact that the primer used was designed to amplify specifically repeated sequences in the ribosomal gene region in an extrachromosomal circular rDNA region in the parasite DNA (Santos et a., 2007).

The results of the current study are consistent with the results of the study of Parija & Khairnar, (2007) and the results of the study of Farhan et al. (2024), where they used the same target gene and the same primers to diagnosis *E. histolytica* and *E. dispar,* and the results of their study were positive after extracting the DNA of *E. histolytica* from urine samples and liver abscess by Parija & Khairnar (2007), and stool samples by Farhan

et al. (2024). It is consistent with Rivero et al. (2021) reported in Venezuela where they found only three samples positive for *E. histolytica* and *E. dispar,* and the low rate of positivity to *E. dispar* was only four samples out of the nine that PCR was performed, same target gene and the same primers to diagnosis *E. histolytica*.

The results of the study did not agree with what was recorded by Haghighi et al. (2009) after examining 16 samples, as no positive result was shown for *E. histolytica* parasite, while all of them recorded a positive result for *E. dispar* parasite.

The divergence in the result of the PCR test in this study, as compared to other studies might be because of the differences in the way the stool DNA was extracted and also how a PCR test is administered as this might make one test more sensitive than the other or it could be simply because of the different values of parasites present in the stool. The reason these parasites aren't found in all areas of the world may be because they thrive in specific environments and cultural practices (Hooshyar et al., 2012).

Some studies show inconstant results when applying PCR to samples that are found to be positive using microscopy or using PCR instead of microscopy. This may be due to the presence of inhibitory substances in the stool samples, such as DNA polymerases, bile salts, hemoglobin degradation products, and bilirubin, which were not adequately removed during DNA extraction. Alternatively, other *Entamoeba* species, such as *E. coli*, *E. hartmanni*, and *E. polecki*, may be more susceptible to microscopic examination (Ngui et al., 2012), or the reason is due to the time of taking and analyzing the samples, as many of the samples from which DNA is extracted and used in the PCR test are taken from feces stored for long periods at low temperatures, which leads to the decomposition of these samples and gives false negative results (Furrows et al., 2004).

One of the reasons some authors give negative results when using PCR is the presence of only a trophozoite, which breaks down over time, leading to the failure of DNA replication, and the reason for the difference in the results recorded in the current study compared to other studies may be attributed to the difference in the geographical distribution of parasite infection or the use of a more sensitive test to detect the parasite molecularly, such as the Nested-PCR test (Kathryn & Charles, 2007). The explanation for the occurrence of co-infection in the current study is that infection occurred as a result of swallowing cysts contaminated with a mixture of parasites or different groups of parasites, such as water contaminated with mud or sewage water (Amar et al., 2002). Also, the reason for the negative results in other studies may be attributed to the mismatch in the sequence of the primer that was designed to bind to certain conserved regions in the gene, such as being too long, which leads to the failure of amplification of the gene in stool samples (Lalle et al., 2009). The negative results in other studies can also be explained by the loss of DNA when trying to purify it with phenol chloroform after freezing and thawing. This is attributed to the fact that the proteins were not completely removed from the sample and remained bound to the DNA. When the washing solution was added to the sample for purification, the proteins were removed along with the DNA bound to them, or their removal led to damage to the DNA, thus losing its ability to dissolve in water, as chloroform is an organic solvent with a polar nature that works to separate water from organic materials (Utaminingsih & Sophian, 2022), in addition, the low concentration of DNA in the sample may be a result of DNA damage during the extraction process or sample preservation, which leads to false results (David et al., 2011).

Conclusion

The present results showed that all microscopically preserved stool samples were positive for diagnosis by PCR. The PCR technique is more accurate than light microscopy in diagnosis and does not allow for confusion in determining the genus and species of organism.

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