Molecular Detection of Entamoeba Species of the Patients with Bloody Diarrhea in Al-Diwaniyah Province

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Abstract
Amoebic dysentery is a human infection caused by Entamoeba histolytica pathogenic parasite. The present study utilized golden standard molecular detection technique a nested multiplex Polymerase Chain Reaction PCR on 112 stoolsamples collected from patients with bloody diarrhea in Al-Diwaniyah Province hospitals and private laboratories after microscopic diagnostic examination. Nested Multiplex PCR showed only 73 (65.2%) positive samples to E.histolytica , 61 (83.6%) of them are mono-infection and 12 (16.4%) are mixed infection with E.dispar and E.moshkovskii 9 (12.3%) and 3 (4.1%) respectively , while the data of Microscopy showed 66 samples are positive to EntamoebaSpecies leading to higher prevalence ofE.histolytica in Al-Diwaniyah Province when the methods of the molecular detection used instead of routine traditional microscopic examination , permitting to distinguish amongmorphologically identical human Entamoeba Species.
Keywords: Entamoeba histolytica, nested PCR, Amoebic dysentery

Introduction

Intestinal amoebiasis and amoebic dysentery considered the second or third most frequent parasitic disease, exceeded only by Malaria and Shistosomiasis according to (1) and contribute with high universal burden of the diarrhea in the regions with poor sanitation and low economic development and also remain to be a great global health crisis through highly spread of mortality and morbidity worldwide (2). Amoebic dysentery caused by extracellular eukaryotic pathogen *E.histolytica*. The pathogenesis with amoebiasis in most cases (perhaps as high as 90%) are generally asymptomatic. The amoebic dysentery differentiated from bacillary dysentery caused by memberes of the genus *Shigella* (3). Amoebiasis may present as diarrhea and dysentery in the colon and abscesses in the intestinal colonic wall or may lead to amoeboma and may reach extra-intestinal organs through abscess formation in hepatic, pulmonary, cardiac and cerebral tissues. *E.dispar*is non-pathogenic , non-virulent , non-harmful and non-invasive but the studies to the future may more investigate with the pathogenicity (4).*E.dispar* characterization has massive effect of prevalence of amoebiasis may lead to the vast majority of asyptomatic illness worldwide (5).*E.moshkovskii* , previously presumed to be a free-living parasite , causing human infectious disease with high prevalence (6). *E.moshkovskii* has a genetic features with *E.histolytica* and *E.dispar*, therefore Cyst and Trophozoites forms are microscopically non-characterized because of morphologically identical but remain hereditarily and biochemically different in the comparison withanother Species.*E.moshkovskii* can cause human infection (7).

In the present study ,aimed to use molecular detection method throng nested multiplex Polymerase Chain Reaction (PCR) used for knowledge of the real prevalence of *Entamoeba* Species among dysentery Patients because of the true prevalence of the infection with amoebiasis among Patients with bloody diarrhea is actually unknown for most areas of the world because of the dependency of routine microscopic diagnosis in which difficulties of identification virulent *Entamoebahistolytica*which is morphologically identical with non-pathogenic *Entamoeba* Species as *Entamoebadispar* and *Entamoebamoshkovskii*(8).

Materials and Methods

The present study included one hundred Twelve (112) Stool samples of patients with dysentery collected (from the beginning of October 2021 till the end of March 2022), The samples collected from (Al-Diwaniyah Teaching Hospital, Afak General Hospital, Women and Children Hospital and Private Clinic) in Al-diwaniyah province. The patients had acute onset of signs of bloody diarrhea. All those patients undergoed with full history. Questionnaire and full information obtained from the patients such as (age, address, the living area (residency), drinkingwatersupply and asking of the clinical symptoms and signs such as vomiting, fever , the confirmation of the presence of the blood in the stool and the period of the bloody diarrhea) . The laboratory investigation included General Stool Examination (G.S.E) by light microscopy.
The specimens of the Stool stored at -20 °C until Genomic DNA Extraction done then the molecular Detection carried by a Nested Multiplex Polymerase Chain Reaction (PCR) Technique.

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Nested MultiplexPolymerase Chain Reaction

Genomic DNA directly extracted from samples using Presto Stool DNA Extraction Kit and carried out according to instructions of Geneaid company. The Nested Multiplex PCR primers utilized for detection *Entamoeba*species designed by (9). in the present study dependent on NCBI-Genbank and primer 3 plus design. These primers from Macrogen company, Korea , as the following table.

Statistical Analysis

The results of the current study analyzed by independent sample t- test utilized for evaluation the difference in mean of numeric variables between Two groups and Chi-sequare test involved Two categorical variables and also included Specificity , Sensitivity and Accuracy(10).

Primers	Sequence 5′-3′		PCR product size
PCR EntamoebaSpecies	F	TAAGATGCACGAGAGCGAAA	900bp
	R	GTA CAAAGGGCAGGGACGTA	
Nested PCR E. histolytica	F	AAGCATTGTTTCTAGATCTGAG	439bp
	R	AAGAGGTCTAACCGAAATTAG	
Nested PCR E. moshkovskii	F	GAAACC AAGAGTTTCACAAC	553bp
	R	CAATATAAGGCTTGGATGAT	
Nested PCR E.dispar	F	TCTAATTTCGATTAGAACTCT	174bp
	R	TCCCTACCTATTAGACATAGC	

Results

The current study included 112 stool samples collected from patients with dysentery. (G. S. E) General Stool Examination (Wet Mount Preparation of fecal samples, Concentration Method) done to all samples investigated under light microscopy. The results showed that 66 (58.9 %) was microscopically positive to *Entamoeba* Species Parasiteswhile 46 (41.1 %) was microscopically negative. The positive cases showed under light microscopy are Cyst only and Cyst with Trophozoite 27 (40.9 %) and 39 (59.1%) respectively.

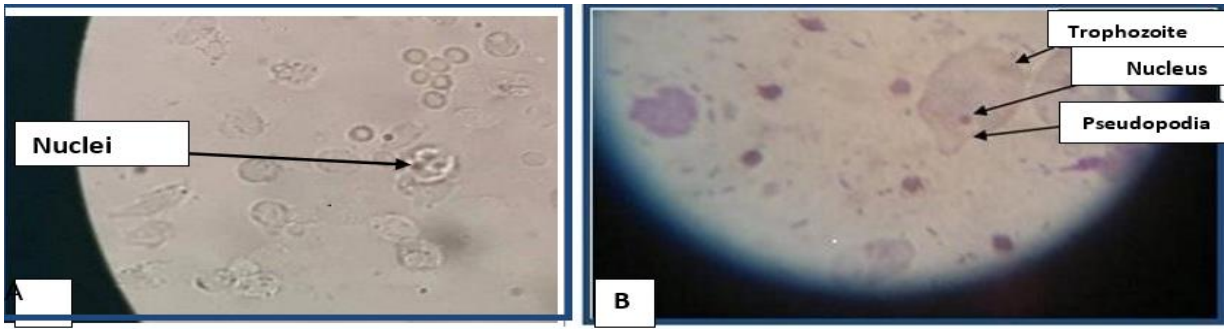
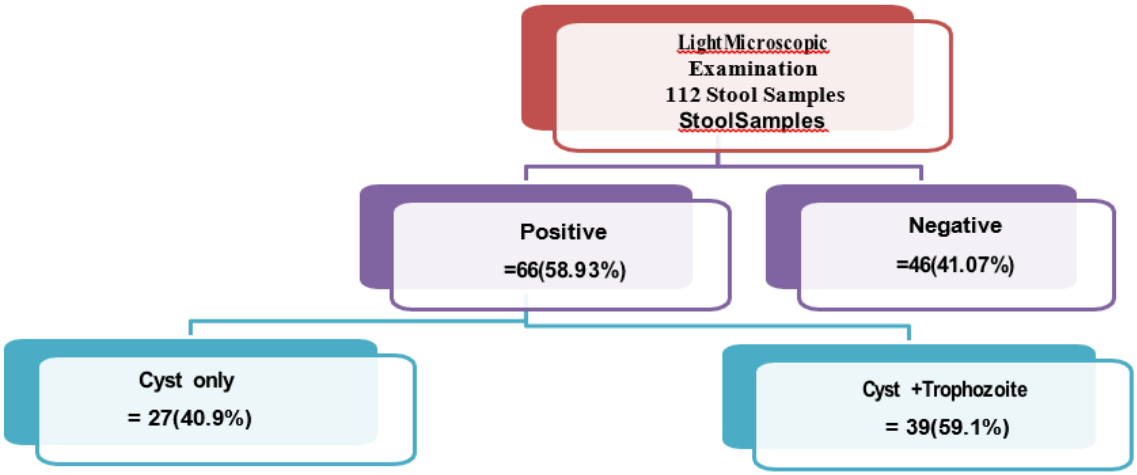


Figure (1) Microscopic Results. (A) Cyst and (B) Trophozoite.



Figure(2): The Frequency distribution of Patients with amoebic dysentery according to findings of Light Microscopic Examination.

Table (2) Sensitivity and Specificity of Light Microscopy in detecting EntamoebaSpecies Parasites in the, comparison with Nested Multiplex (18S rRNA gene) PCR as a goldenstandardtechniques								
		PCR		Sensitivity	Specificity	PPV	NPV	Accuracy
		Positive	Negative					
light microscop	Positive	53 (83.9 %)	13 (46.1 %)	72.6	66.7	56.5	80.3	69.7
	Negative	20 (16.1 %)	26 (53.9 %)					
PPV: positive predictive value; NPV: negative predictive value								

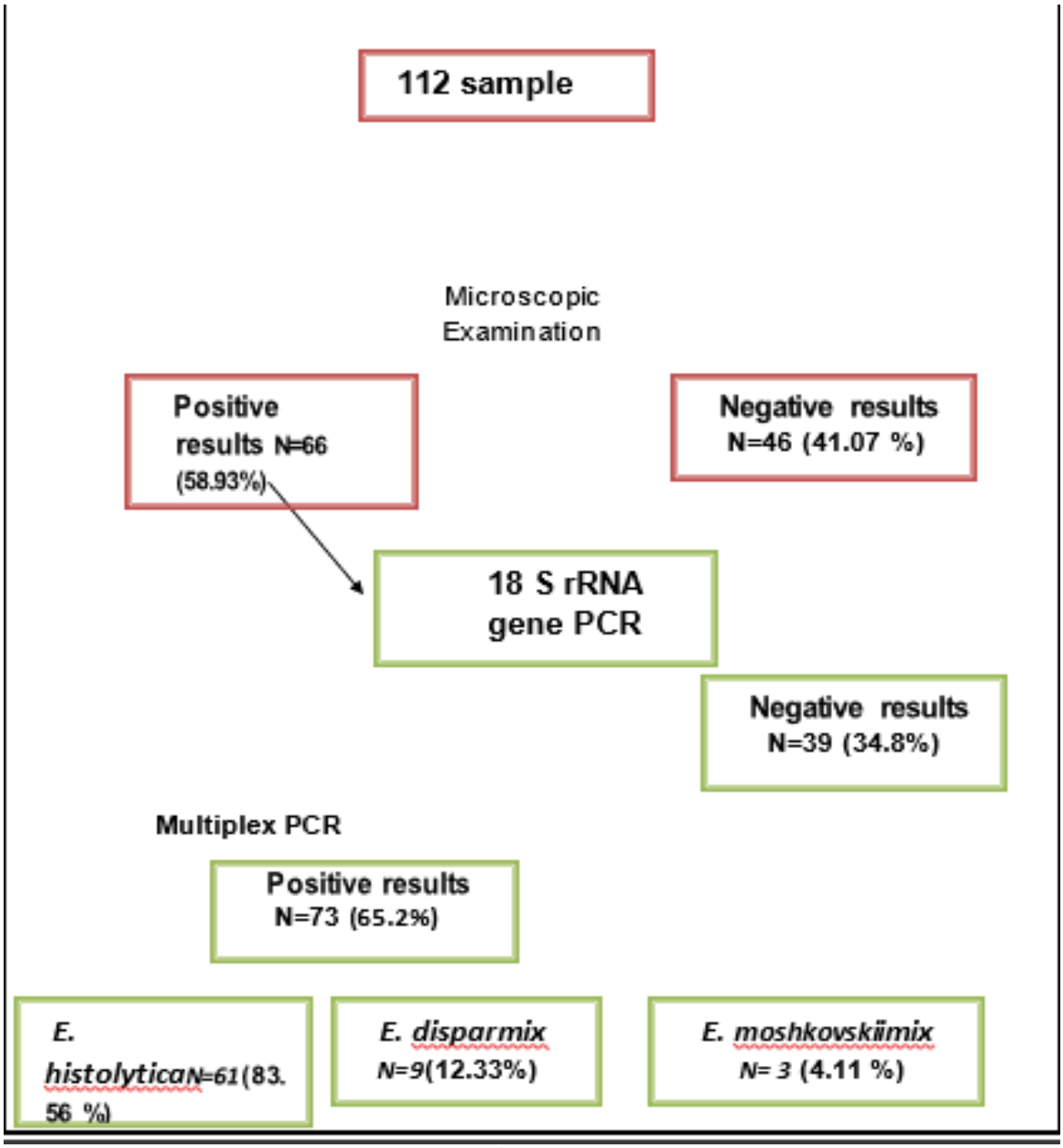


Figure (3) Scheme Show the Methods of Diagnosis and the results in the Current Study

Molecular Method

The molecular method, Nested multiplex PCR used in the present study showed 73 (65.2 %) out of 112 stool samples of dysentery patients are amplified for 18SrRNA gene by Nested multiplex PCR (firstround)fordetection the DNA of *Entamoeba* Speciesin stool samples. The Positive result of Nested multiplex PCR (first round) was 73 (65.2 %), while negative result was3 9 (34.8%), as following shown:

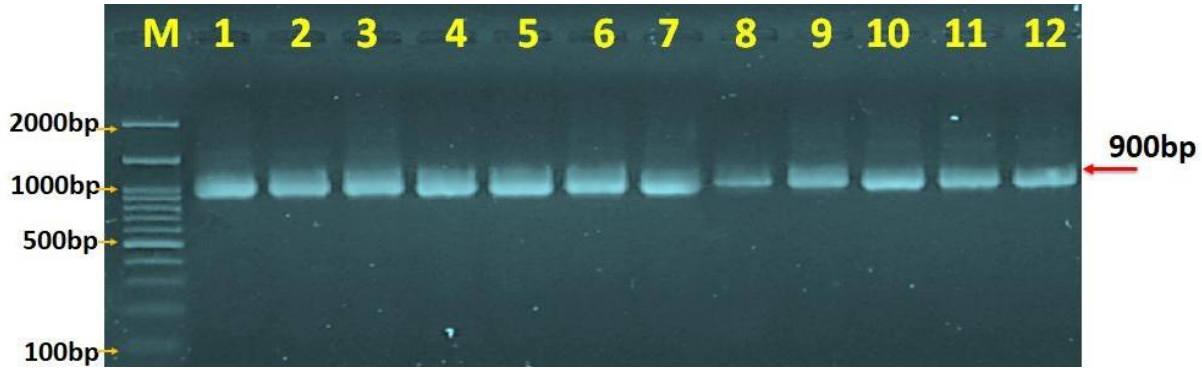


Figure (4)Agarosegelelectrophoresisimagethatshowedthe FirstroundNestedPCRproductanalysisofEntamoebaSpeciesof Human stool samples.

Table (3) Comparison of light microscope results with Nested multiplex (18S rRNA gene) PCR results				
Light Microscope		Nested Multiplex (18S rRNA gene) PCR results		P
		Positive n = 73 (65.2%)	Negative n = 39 (34.8 %)	
All	Positive	53 (72.6 %)	13 (33.3 %)	< 0.001 ¥ HS
	Negative	20 (27.4 %)	26 (66.7 %)	
Cyst only	Positive	22 (30.1 %)	5 (12.8 %)	= 0.001 ¥ HS
	Negative	51 (69.9 %)	34 (87.2 %)	
Cyst and Trophozoite	Positive	31 (42.5 %)	6 (15.4%)	< 0.001 ¥ HS
	Negative	42 (57.5%)	33 (84.6 %)	

n: number of cases: F: Fischer exact test; NS: not significant at P > 0.05; HS: highly significant at P ≤ 0.01

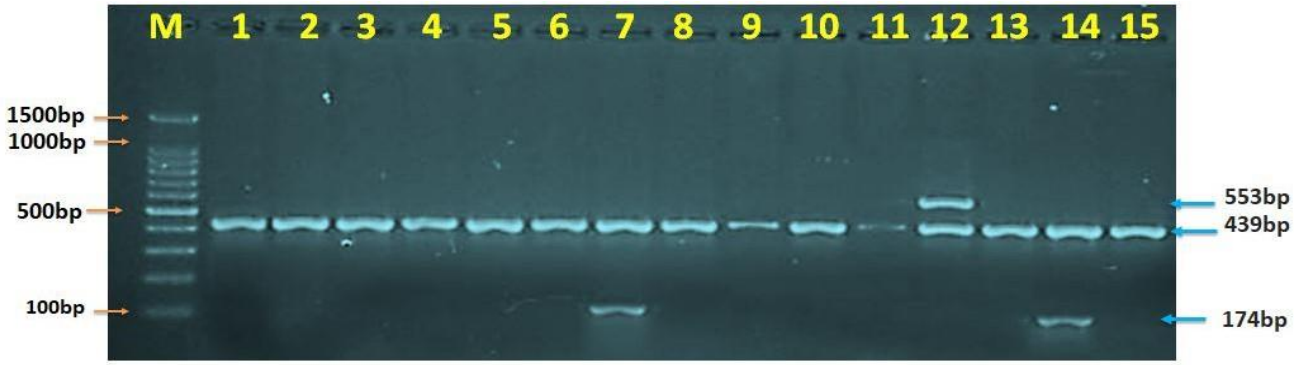


Figure (5) :the Lanes (15) showed Entamoebahistolytica at 439bp PCRproductsize, Lanes(7,14) showed Entamoebadispira at 174bp PCR productsize, and Lane (12) showed Entamoebamoshkovskii at 553bp PCR productsize.

Table (4) Sensitivity and Specificity of Light Microscopy in detecting Entamoeba species Parasite in the comparison with Nested Multiplex(18S rRNA gene) PCRas a goldenstandardtechnique								
		PCR		Sensitivity	Specificity	PPV	NPV	Accuracy
		Positive	Negative					
light microscopy	Positive	53 (83.9 %)	13 (46.1 %)	72.6	66.7	56.5	80.3	69.7
	Negative	20 (16.1 %)	26 (53.9 %)					
PPV: positive predictive value; NPV: negative predictive value								

Discussion

Unfortunately Microscopic examination method cannot differentiate and cannot distinguish morphologically types among species of *Entamoeba*such as *Entamoebahistolytica*, *Entamoebadispar* , *Entamoebamoshkovskii*or *Entamoeba* Bangladeshi but It remains quick , inexpensive and little consumption of time. The truediagnosis of amoebiasis and amoebic dysentery permitting to detect *E.histolytica* from *E.dispar* and *E.moshkovskii* is fundamental for identification the real accurate prevalence of virulent *E.histolytica* among Iraqi population. Many Clinicians in Iraq prescribe systemic medications depending on the results of microscopic examination. Using of Effective identification techniques are necessary to preclude unneedful medications when *E.dispar* , non-harmful species is found. (11).

In the current study , the prevalence of microscopically diagnosed *Entamoeba*Species parasitic infections of dysentery patients are more in males than females and this may belong to some of the environmental factors such as nature of the work and exposure to pathogens or different functions of hormones because ofSex–dependent behaviour agrees with (12) who explained that oncoming cause of sex difference for infections is differences in endocrine system and immune responses and the effects on host protective immune response , (13) study showed that male patients are more affected with infection than the females , (77 % and 23 %) respectively while our study showed that microscopically diagnosed positive results with prevalence of *Entamoeba*Species parasites for males and females according to light microscopic finding are (51.5 % and 48.5 %) respectively and (p value =768) indicated that there are non- significant association between sex of patients and positive results of light microscopy , the cause for high rates of infection in males in comparison with females and perhaps may be due to inhibition of protective Interferon gamma secretion in natural killer T cells because of effect of Testosterone hormone on natural killer T cells and also Testosterone increase susceptibility to amoebic liver abscess and also Natural killer T cells activated by a lipopeptidophosphoglycan from *Entamoebahistolytica* are critically important to limit amoebic liver abscess as the previous study that showed prevalence amoebic liver abscess and vast majority of more than 80% of all amoebic liver abscess cases occur in adult males in comparison of amoebic liver abscess is rare in women and children (14), protective interferon

gamma activate macrophages to produce nitric oxide that inhibit essential enzymes of *Entamoebahistolytica* (15). Our study result showed that males are more than female also agrees with (15) in Saladin recorded that males (63.64 %) and females(36.36 %)

The rearealsonon-significant association between residency and light microscopic positivity for amoebic pathogenic parasite (P value = 0.412). Inthe current study , the results showed that amoebic parasitic infections are higher prevalence in rural areas more than urban areas but there are statistically non-significant association between rural areas and urban areas. This the study involved survey on some rural areas around Al-diwaniyah province,the number of microscopically positive diagnosed dysentery Patients stool samples with *Entamoeba* Species occur in rural areas is 44 (66.7 %).

The comparison showed that Nested multiplex Polymerase Chain Reaction (PCR) results are 73(65.1 %) positive while 39(34.9 %) negative and this the results are highly significant associated with the light microscopic examination results are 66 (58.9 %) positive and 46 (41%) negative of all of the stool samples of dysentery patients (P value < 0.001).The comparison showed that PCR positive results are highly significant associated with the light microscopic examination of Cyst only (P value = 0.001). The comparison showed that PCR positive results are also highly significant associated with the light microscopic examination of Cyst and Trophozoite (P value < 0.001). The main routine method for examination , detection and eventually diagnosis of intestinal amoebiasis is microscopic examination and this the method is commonly worldwide used , but there are some of disadvantages for example it cannot distinguish among *E.histolytica* , *E.dispar*and*E.moshkovskii*and also with another non-pathogenic species and also skills of laboratory technicians are required and involved with result of the test and also this test method considered less sensitivity and less specificity (72.6 , 66.7) respectively and this also indicate to less accuracy (69.7) in comparison with golden standard highly sensitive technique (PCR) but it may still impractical in routine diagnostic tests because it is difficult of DNA extraction , time consumption and expensive (17).

Conclusions

A lot of the patients with bloody diarrhea are diagnosed with amoebic dysentery. Molecular detection technique a nested multiplex(18S rRNA gene) Polymerase Chain Reaction (PCR) is still a valuable diagnostic method as golden standard tool , can to distinguish among morphologically identical known species of amoebic parasite in contrast with the light microscopic examination is not reliable technique may lead to misdiagnosis of these species because it cannot to differentiate among morphologically identical known parasitic amoebic species complex due to low sensitivity and the requirement for skilled technicians .The lack of the sanitary meaures and decreased hygiene are a risk factors of amoebic infection causing intestinal amoebiasis are more in the rural areas than in the urbanareas .

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