Original Article

Association between molecularly detected *Entamoeba* species and fecal calprotectin level among a cohort of diarrheic patients

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ABSTRACT

Background: Differentiation between *Entamoeba* species can be molecularly achieved, especially since *E. histolytica* has been linked to high morbidity and mortality. Fecal calprotectin (FC) as an inflammatory marker may be linked to amoebiasis.
Objective: The present study aimed at investigating the association of calprotectin in amoebiasis.
Patients and Methods: Stool samples were collected from 294 patients attending Internal Medicine Outpatient Clinic in Kafrelsheikh University, Egypt; suffering from diarrhea with or without other gastrointestinal manifestations. Samples were subjected to coproscopy, quantification of fecal calprotectin level and multiplex nested PCR for *Entamoeba* species differentiation.
Results: Detection rate of *E. histolytica* complex was 16.6% by microscopic examination and only 14.6% proved to be positive by nested multiplex polymerase chain reaction (PCR): *E. histolytica* (3.7%), *E. dispar* (6.4%) and *E. moshkovskii* (4.5%). Statistical analysis including several variables showed no significance except for the presence of blood and mucus. Fecal calprotectin was positive in 10.5% of the study population and 81.8% in association with *E. histolytica* indicating intestinal inflammation. Frequency of males infected with *E. histolytica* and *E. dispar* was higher than in females who showed a higher infection rates with *E. moshkovskii*.
Conclusion: The association between *E. histolytica* and positivity of FC level is a crucial indicator for disease severity and efficacy of therapeutic regimen.

Keywords: *E. dispar*, *E. histolytica*, *E. moshkovskii*, fecal calprotectin, nested multiplex PCR.

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INTRODUCTION

Species belonging to the genus *Entamoeba* that inhabit the human intestine are comprised of *E. histolytica*, *E. coli*, *E. dispar*, *E. moshkovskii*, *E. hartmanni, E. polecki,* and *E. bangladeshi*. The only pathogenic *E. histolytica* spp.[1] is considered the third leading protozoan parasitic cause of death. It infects 50 million people and causes 40,000-100,000 deaths annually[2]. However, *E. dispar* and *E. moshkovskii* have also been detected in gastrointestinal symptomatic patients[3,4] without any evidence of pathogenicity[5]. It has been documented that 10% of the world's population acquire infection with different *Entamoeba* spp., in which 10% and 90% are infected with the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* respectively[6].

The two non-pathogenic species *E. dispar* and *E. moshkovskii* are morphologically identical to the pathogenic *E. histolytica* but with genetical and biochemical differences[7]. Diagnosis of *Entamoeba* complex (*E. histolytica*/ *E. dispar*/ *E. moshkovskii*) relies on traditional routine microscopic examination either through direct wet mount or fixed smears. Unfortunately, this method cannot differentiate between the three species. A facilitating marker is the presence of ingested red blood cells in trophozoite forms indicating *E. histolytica* spp.[8,9]. Recently, species identification can be achieved by serological[10] or copro immunological techniques[8,11] but with certain limitations because the three species share many identical alleles and similar immunogenic effects. Therefore, the more sensitive and specific molecular techniques are concerned with the species differentiation between pathogenic *E. histolytica* and the other two non-pathogenic species[3,12].

Amoebiasis has intimate correlation with many chronic inflammatory conditions of the intestine. Moreover, amoebiasis can exhibit unpleasant consequences in the course of the disease and its therapy[13]. It was shown that in response to intestinal inflammation FC antibody is produced by responding neutrophils[14]. Elevated levels of FC antibodies are generally attributed to acute or chronic gastrointestinal viral, bacterial or parasite infections, allergic colitis, nonsteroidal

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anti-inflammatory drugs-induced enteropathy and colorectal cancer[15-17]. Recently FC was employed for distinguishing inflammatory bowel disease (IBD) from other non-inflammatory conditions attesting its use as a marker for disease activity and efficacy assessment of treatment regimen[18]. Other researchers recorded 91% specificity and 95% sensitivity of FC in monitoring IBD patients[19,20]. Some intestinal parasitic diseases as those caused by *Dientamoeba fragilis, Giardia intestinalis* and *Schistosoma mansoni* registered elevated concentration levels of FC[21,22]. The present study aimed at assessing relation between the level of FC and the molecularly detected *Entamoeba* species.

PATIENTS AND METHODS

This cross sectional study was conducted at the Medical Parasitology Department, Faculty of Medicine, Kafrelsheikh University during the period from June, 2020 to July, 2021.

Study design: Stool samples were collected from patients attending Internal Medicine Outpatient Clinic, suffering from diarrhea. Samples were subjected to microscopic examination, molecular diagnosis for *Entamoeba* spp, and determination of FC levels.

Patients: This study included 294 patients complaining of diarrhea with or without other gastrointestinal manifestations including abdominal pain, flatulence, and vomiting. Patients receiving anti-diarrheal treatment were excluded. Among the patients enrolled in our study, eleven were previously diagnosed in Internal Medicine Outpatient Clinic as IBD. Data were recorded using a previously designed questionnaire including demographic data, age, gender, the presence, and type of gastrointestinal symptoms.

Sample collection and processing: The fresh fecal sample of each participant was divided into two parts. The first was microscopically examined, while the other was stored frozen at -20°C for molecular diagnosis and determination of FC levels.

Microscopic examination: This was assessed using unstained and Lugol's iodine stained direct wet mount of each collected sample before and after formol ether concentration[23] for detection of *Entamoeba* complex trophozoites and/or cysts.

DNA extraction: Part of the frozen fecal samples were subjected to genomic DNA extraction using Favor Prep Stool DNA Isolation Mini Kit (Favorgen Biotech Corporation, Taiwan) according to manufacturer's instructions. The extracted genomic DNA was then amplified using nested multiplex PCR targeting 16S like ribosomal RNA to detect *E. histolytica* and differentiate it from *E. dispar* and *E. moshkovskii*.

Multiplex nested PCR: Primary PCR used specific primers for the detection of *Entamoeba* genus; E-1 (5'-TAA GAT GCA GAG CGA AA-3') and E-2 (5'-GTA CAA AGG GCA GGG ACG TA-3'). The reaction components and the cycling conditions of both primary and secondary reactions were adjusted according to Ngui *et al*.,[8]. In each run, negative and positive control samples were included. The amplified PCR products were subsequently subjected to secondary PCR reaction for *E. histolytica*, *E. dispar* and E. moshoviskii characterization. Amplification was achieved using primer sets as in table (1). Ethidium bromide gel electrophoresis was used to analyze the 2ry PCR products which were then visualized using UV light.

Quantification of FC level: The rest of the frozen collected fecal samples were then tested using DRG: HYBRiDXL Calprotectin Kit (Calprest1, Eurospital, Trieste, Italy) which is a kind of solid phase sandwich ELISA, performed according to manufacturer's instructions. Quantitative FC results above 200 µg/g were considered positive[13].

Statistical analysis: The recorded data was tabulated and analyzed using the statistical package SPSS version 21 (Chicago, IL, USA). Data were described as frequency and percentage with $P<0.05$ considered significant.

Ethical consideration: The study was conducted after approval of Kafrelsheikh, Faculty of Medicine ethical committee under the number KSU 18-5-2020. A verbal consent was taken from each patient before filling the questionnaire or collecting stool samples.

RESULTS

Coproscopic and molecular data evaluation: Microscopic analysis of 294 fecal samples detected 16.6% (49/294) overall detection rate of *Entamoeba* complex's cysts, but only 14.6% (43/294) was proved

Table 1: The used primers' sequences[8].

Entamoeba spp.	Primer sequence	Amplified segment
E. histolytica	**EH-1:** 5'-AAG CAT TGT TTC TAG ATC TGA G-3' **EH-2:** 5'-AAG AGG TCT AAC CGA AAT TAG-3'	439 bp
E. dispar	**ED-1:** 5'-TCT AAT TTC GAT TAG AAC TCT-3' **ED-2:** 5'-TCC CTA CCTATT AGA CAT AGC-3'	174 bp
E. moshkovskii	**Mos-1:** 5'-GAA ACC AAG AGT TTC ACA AC-3' **Mos-2:** 5'-CAA TAT AAG GCT TGG ATG AT-3'	553 bp

by nPCR. The contribution of *E. histolytica*, *E. dispar* and *E. moshkovskii* were 3.7%, 6.4% and 4.5% respectively (Fig. 1). A high prevalence of *E. histolytica*, *E. dispar* and *E. moshkovskii* was recorded in the age group 2-12 years. Record of *E. histolytica*, *E. dispar* infections was higher in males; but *E. moshkovskii* was apparently higher in females with no statistical significance difference.

Moreover, there was significant association between the presence of blood and mucus and infection with *E. histolytica* (P=0.003 and >0.001, respectively) with insignificant difference between each one of them and infection with *E. dispar* or *E. moshkovskii*. Besides, there was no statistical significance difference between any of the clinical manifestations and infection with any of the three species (Table 2). There was significant statistical correlation between the molecularly detected *E. histolytica* and cases previously diagnosed as IBD (54.4%).

FC level evaluation: The FC antibody was positive in 10.5% (31/294) of the population studied; and 4.8% (14/294) was associated with *Entamoeba* species of which 81.8%, 15.7% and 15.3% correlated with molecularly detected *E. histolytica*, *E. dispar* and *E. moshkovskii* infection, respectively. Statistical significance (P>0.001) was recorded with *E. histolytica* only (Table 3).

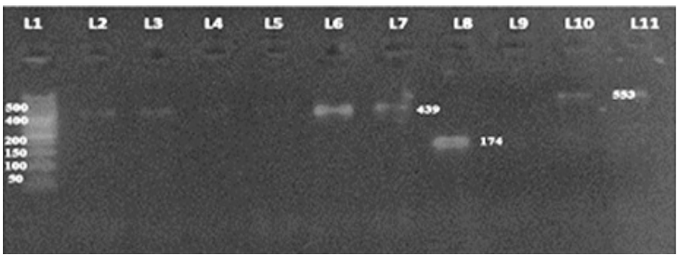

Fig. 1. Gel electrophoresis of amplified DNA products. **L1:** 50 bp ladder. **Lanes 2-7:** *E. histolytica* positive samples. **Lane 8:** *E. dispar* positive sample. **Lane 10 and 11:** *E. moshkovskii* positive samples. **Lane 9:** Negative sample.

Table 2. Clinical data, microscopic, and molecular results.

		Positive PCR = 43 (14.6%)					
		Positive N(%) *E. histolytica*		Positive N(%) *E. dispar*		Positive N(%) *E. moshkoviskii*	
		11 (3.7)	*P*	19 (6.4)	*P*	13 (4.5)	*P*
Age group	<2	0	0.420	1	0.186	1	0.783
	>2-12	7		13		6	
	>12- 20	0		2		1	
	>20-40	2		1		2	
	>40-60	2		2		3	
Gender	Male	9 (81.2)	0.030*	10 (52.6)	0.500	5 (38.5)	0.286
	Female	2 (18.8)		9 (47.4)		8 (61.5)	
Blood	Yes	4 (36.7)	0.003*	0	0.289	1 (7.7)	0.568
	No	7 (63.3)		19 (100)		12 (92.3)	
Mucus	Yes	9 (81.2)	<0.001*	4 (21)	0.234	2 (15.4)	0.535
	No	2 (18.2)		15 (79)		11 (84.6)	
Abdominal pain	Yes	7 (63.3)	0.509	12 (63.1)	0.431	8 (61.5)	0.426
	No	4 (36.7)		7 (36.9)		5 (38.5)	
Flatulence	Yes	4 (36.7)	0.479	5 (26.3)	0.408	3 (23)	0.367
	No	7 (63.3)		14 (73.7)		10 (77)	
Vomiting	Yes	1 (9.1)	0.128	4 (21)	0.413	1 (7.7)	0.074
	No	10 (90.9)		15(79)		12 (92.3)	
IBD	Yes	6 (54.5)	<0.001*	2 (10.5)	0.449	1 (7.7)	0.730
	No	5 (45.5)		17 (89.5)		12 (92.3)	
Microscopy	Positive	6 (54.5)	0.004	6 (31.5)	0.077	4 (30.7)	0.155
	Negative	5 (45.5)		13 (68.5)		9 (69.3)	

***:** Statistically significant (P<0.05), **IBD:** Irritable bowel disease, **FC:** Fecal calprotectin.

Table 3. Relation between FC and *Entamoeba* spp.

Entamoeba spp.		FC positive [14/294 (4.8 %)]	Statistical analysis
Species	Number	No. (%)	*P* value
E. histolytica	11	9 (81.8)	< 0.001*
E. dispar	19	3 (15.7)	0.323
E. moshkovskii	13	2 (15.3)	0.407

***:** Statistically significant (P<0.05), **FC:** Fecal calprotectin.

DISCUSSION

Diarrhea is globally considered as a leading cause of morbidity and mortality and *E. histolytica* is one of the most common protozoa causes[24]. For routine diagnosis of *Entamoeba* species, microscopy is the most commonly used method, but it lacks sensitivity and specificity as it is dependent on the proficiency of the examiners and the intermittent shedding of cysts; and it cannot differentiate between the morphologically similar species[25]. Recently, more sensitive, and specific techniques have been used for detection and differentiation of *Entamoeba* species complex that depend on detection of species-specific nucleic acids[8].

Detection rates of *E. histolytica* vary widely among different countries, depending on many factors that include environmental, demographic, socioeconomic, and personal hygiene[10,26]. Our nPCR results demonstrated 14.6% overall detection rate of *Entamoeba* species that was composed of 3.7%, 6.4% and 4.5% for *E. histolytica*, *E. dispar* and *E. moshkovskii* respectively. However, this overall rate proved to be lower than that recorded by microscopic examination (16.6%). Samples that were microscopically positive but proved to be negative by nPCR might be another species of *Entamoeba* as reported by Santos *et al.*[27]. Microscopic misinterpretation of *E. coli* cysts cannot be excluded. We confirmed that *E. dispar* is more frequent than *E. histolytica* which agrees with the reported worldwide distribution of *Entamoeba* species[28]. Our results are within the range of overall detection rate of *Entamoeba* species by microscopic examination in a record from Egyptian (16.6% versus reported 15.4%), but apparently higher by the recorded PCR (14.6% versus reported 9.6%[9]. The latter was identified as 1.7% *E. histolytica*, 4.6% *E. dispar* and 3.3% *E. moshkovskii*. Similarly, in Malaysia, *Entamoeba* species were detected in 17.6% samples by microscopic examination and only 12.2% were proved positive by nPCR with high detection rate of *E. histolytica* (7.7%), followed by *E. dispar* (2.3%) and *E. moshkovskii* (0.7%), and mixed infection was detected in 1.4%[8]. Being from a different location the results of the Malaysian study disagreed with our results in the distribution of *Entamoeba* species recording a higher detection rate of *E. histolytica* over *E. dispar*. Results of our study showed no statistical association between abdominal pain, bloating or vomiting and amoebiasis asserting the results of Duc *et al*.[29] from Vietnam. Additionally, 4.8% of molecularly detected *Entamoeba* spp. showed positive FC levels which agrees with Rady *et al.*[30] who reported high FC levels in 15% of patients with *E. histolytica*/*dispar*.

It is worth mentioning that IBD is a disease of unknown cause with a group of conditions that cause pathological inflammation of the intestinal wall and is characterized by the presence of diarrhea and colonic lesions that can be diagnosed by endoscopy[31]. The etiology of IBD varies widely and may include some microbial agents such as *E. histolytica* that produces ulceration of the mucosa of the large intestine[32]. Accordingly, amoebiasis can exacerbate clinical manifestations of IBD and impose unfavorable effects on the disease course and treatment[33]. We reported presence of IBD in 54.4% of molecularly detected *E. histolytica* patients, and this percentage surpasses the 10% reported in Egypt[30], and 47.57% in Iraq[34].

Being of high incidence with constant rise in the number of new cases, IBD needs crucial improvement of its diagnostic methods. Diagnosis of IBD can depend on laboratory tests such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and complete blood count (CBC) but they are of low specificity. The most effective method for diagnosis of IBD is endoscopy but it has the disadvantage of being invasive and costly[35]. On the other hand, Van de Vijver *et al*.[36] demonstrated that endoscopy was not able to confirm the diagnosis of IBD in about 70% of patients and the accuracy of diagnosis was increased by detection of FC which is also noninvasive and a major inflammatory marker. Hence, FC is used as an indicator for IBD and serves as a diagnostic marker due to its resistance to degradation by pancreatic or intestinal secretions[37]. Any intestinal inflammatory process causes an influx of neutrophils into the intestinal lumen[38]. The soluble protein of neutrophils' cytosol is comprised of 60% calprotectin so, it can serve as a noninvasive quantitative marker for intestinal inflammation level[39]. An additional argument in favor of FC is that its level is usually normal in patients with irritable bowel syndrome (IBS)[40].

In conclusion, our study highlighted the crucial need for molecular diagnosis of *E. histolytica* and its differentiation from other non-pathogenic species and that in turn helps to give more reliable data on the epidemiological prevalence of *Entamoeba* species and prevents drug abuse and unnecessary treatment. Fecal calprotectin can be a good marker for the detection of the injury caused by the parasites to their host. So, PCR is recommended for diagnosis of amoebiasis, as well as FC, which although nonspecific, is also recommended as a good marker for intestinal injury. The finding of positive FC levels in *E. histolytica* patients reflects a high grade of injury caused by this protozoan on intestinal mucosa.

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Conflicts of interest: The authors declare that there is no conflict of interests.

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