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Entamoeba Typing using Multiplex-PCR and Clinical Features Among Patients with Inflammatory Diarrhea in Khuzestan, Southwest Iran

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Abstract: Amoebiasis is one of the most important causes of inflammatory diarrhea. *Entamoeba histolytica, Entamoeba dispar and Entamoeba moshkovskii* are similar morphologically but biochemically and genetically are different. Therefore, differentiation in *Entamoeba* species is one of the most important challenges in parasitology. In this cross sectional study carried out from May, 2014 to October, 2015, 242 stool samples that showed colitis by microscopic examination and then all specimens evaluated by multiplex-PCR method for the presence of amoeba and to differentiate the *Entamoeba* species. Statistical analysis was performed using SPSS version 17 and p-<0.05 was considered statistically significant. Using PCR analysis, of the 242 stool samples, 10 (4.1%) samples were identified as *E. histolytica* 13 (5.4%) were *E. dispar* and 2 (0.8%) were *E. moshkovskii*. Abdominal pain in 96% of patients (p<0.001) and nausea and vomiting in 72% of patients (p<0.001) were the most prevalent clinical manifestations in patients with amoebiasis. In this study *E.dispar* was more prevalent like in other studies. PCR is a gold standard and highly sensitive and specific molecular method for differentiated Entamoeba typing to avoid unnecessary treatment and over diagnosis but since, PCR is technically expensive and not available everywhere, we suggest that PCR method use in patients with abdominal pain and vomiting (The odds ratio of abdominal pain and vomiting in the amoebiasis group were 22.7 and 6.8 times more than in the parasite free group, respectively).

INTRODUCTION

Diarrhea is defined as the passage of loose or watery stool, typically at least three times in a day[1]. Inflammatory diarrhea is generally, associated with pain, fever and bleeding. Entamoeba is an enteric protozoan parasite and one of the most important causes of infective diarrhea. There are different types of Entamoeba: *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii*[2] *E. histolytica* is an invasive enteric parasite that should be treated whereas *E. dispar* and *E. moshkovskii* are nonpathogenic and do not require treatment[3]. Diagnosis of different types of Entamoeba is very difficult by traditional diagnostic methods however, it is important that they are differentiated clinically. Therefore, more specific methods like Polymerase Chain Reaction (PCR) or stool antigen detection are needed[2]. WHO estimates that about 50 million people are infected in developing countries annually resulting in over 1 00,000 deaths every year[4]. Entamoeba histolytica, dispar and moshkovskii are similar morphologically but biochemically and genetically different[5]. Therefore, species differentiation in Entamoeba is one of the most important challenges in parasitology[6].

To increase the sensitivity of diagnoses, PCR-based methods have been utilized, since, the early 1990's[7]. Antigen detection in stool by ELISA technique may be useful as an additional test concomitant with stool examination. However, a comparative study on the use of ELISA and PCR for the detection of *E. histolytica* shows that PCR was more sensitive[8]. With serological methods, it is not possible to differentiate remote from recent infections but PCR-based methods on amplification of *small subunit* rRNA gene (SSU-rRNA) was reported to be 100 times more sensitive than ELISA for *E. histolytica* detection[4].

The aim of this study included the following: detection of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* by molecular methods and comparison of results of PCR with microscopic assay on stool sample. Comparison of isolated Entamoeba with clinical presentation. To determine the relative frequency of Entamoeba species in human fecal samples using molecular techniques. In order to avoid unnecessary treatment of individuals with non-pathogenic *Entamoeba* species and also to prevent microbial resistance, it is important to discriminate these species from the pathogenic *E. histolytica*.

MATERIALS AND METHODS

In this cross sectional study which was conducted from May, 2014 to October, 2015, patients with diarrhea from Ahvaz and it's surrounding towns and villages referred to Razi's Governmental Hospital of Ahvaz Jundi Shapur University Medical Science were studied. The patients underwent physical examination and also completed a checklist including: age, gender, site of residence, type of drinking water, signs and symptoms. Then, fecal samples were sent to Razi's Hospital Laboratory. If there were evidence of colitis in fecal sample with or without cyst and trophozoite of *Entamoeba histolytica\dispar*, then a fresh fecal sample was sent to Parasitology Department of school of medicine for evaluation by PCR. Finally, PCR was performed on the 242 fecal samples. Exclusion criteria included: non inflammatory diarrhea, the use of nitroimidazoles for >48 h.

Multiplex-PCR: Extraction of DNA was performed on the 242 fecal samples according to the protocol of QIAamp DNA extraction stool kit (QIAGEN, Hilden, Germany). The extracted DNA was stored at -20°C until use in PCR analysis.

The primers were prepared and lyophilized. One primer (EntaF) was forwarded for all three Entamoeba species whereas every Entamoeba species had a reverse primer including EhR, EdR and EmR[9]. All primer sequences were compared to sequences in Genbank. sequences used were as follows for EntaF,5'-ATGCACGAGAGCGAAAGCAT-3'; For EhR, 5'-GATCTAGAAACAATG CTTCTCT- 3'; for EdR, 5'-CACCACTTACTATCCCTACC-3'. and for EmR 5´TGACCG GAGCCAGAGACAT-3'. The PCR product for *E.histolytica* generated 166-bp++ for *E.dispar*, a 752 bp PCR product and a 580-bp product for *E.moshkovskii* DNA.

The PCR mixture were carried out using 200 μM of deoxynucleoside triphosphate, 0.1 μM of each forward and reverse primer, 0.5 U of Taq polymerase, 1×Taq buffer, 6 mM $Mgcl_2$ and 10 μL of extracted DNA samples. Then micro tubes were placed in the thermocycler. The program of thermocycler used is as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min with a final extraction at 72°C for 7 min. Then, agarose gel 2% was prepared using agarose powder and TBE buffer. After electrophoresis performed on 2% agarose gels, amplified products were visualized with ethidium bromide staining.

Statistical analysis: Finally, statistical analysis was performed using SPSS Version 17 (SPSS Inc., Chicago, IL, USA). Categorical variables were analyzed using Chi-square test or Fishers exact test. For comparison of multiple means, one way ANOVA was used. For each statistically significant signs and symptoms an Odds Ratio (OR) and 95% Confidence Interval (CI) were calculated by multivariate logistic regression analyses $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In this study, 242 stool samples showed colitis by microscopic examination and multiplex PCR. About 10

Table 1: Baseline characteristic between groups of patients

	PCR subgroup				
Characteristics	Neg	*E. histolytica*	*E. dispar*	*E. moshkovskii*	p-values
Age(mean±SD)	38.1±16	37±18.4	38.8±19.4	35±42	0.972
Sex {No. (%)}					
Male	101(41.7)	4(1.6)	8(3.3)	2(0.8)	0.242
Female	116(47.9)	6(2.4)	5(2)	-	
Living site					
Urban	161(66.5)	5(2)	2(0.8)	-	0.008
Rural	56(23.1)	5(2)	11(4.5)	2(0.8)	
Habitancy site					
Home	212(87.6)	10(4.13)	11(4.5)	1(0.4)	<0.001
Care facility	5(2)			1(0.4)	
Day care	-	-	2(0.8)	-	
Underlying condition					
Diabetes	15(6.19)	2(0.8)	1(0.4)	-	0.407
Hypertention	9(3.7)	1(0.4)	- -		
Pregnancy	6(2.47)	-	- -		
Immunodeficiency	2(0.8)	3(1.23)	1(0.4)	-	
Healthy	185(76.4)	4(1.6)	12(4.95)	2(0.8)	

Table 2: Clinical signs and symptoms of patients

	PCR subgroups			
Clinical features	Negative	*E. histolytica*	*E. dispar* and *E. moshkovskii*	p-values
Fever				
Yes	120(55.3)	4(40)	4(26)	<0.001[a]
No.	97(44.7)	6(60)	11(73.3)	
Diarrhea				
Bloody	127(58.5)	7(70)	2(13.3)	0.886[a]
Watery	90(41.5)	3(30)	13(86.7)	
Abdominal pain				
Yes	116(53.5)	9(90)	3(20)	<0.001[a]
No	101(46.5)	1(10)	12(80)	
Tenesmus				
Yes	130(60)	6(60)	2(13.3)	0.016[a]
No	87(40)	4(40)	13(86.7)	
Nausea and vomiting				
Yes	39(18)	8(80)	6(40)	<0.001[a]
No	178(82)	2(20)	9(60)	
Dehydration				
Yes	14(6.5)	4(40)	2(13.3)	0.046[b]
No	203(93.5)	6(60)	13(86.7)	

[a]Chi-square test [b]fisher's exact test p<0.05 was considered statistically significant; In Table 3, crude and adjusted odds ratio for signs and symptoms of patients were compared in the two groups with and without amoebiasis *p<0.05 was considered statistically significant

Table 3: Crude and adjusted odds ratio in sign and symptom in patients

Sign/symptom	Crude OR (CI)	Adjusted OR (CI)	p-values
Vomiting	11.6 (4.38-28.49)	6.87 (2.40-19.69)	<0.001*
Abdominal pain	21.31 (2.83-160)	22.79 (2.87-187)	0.004*
Fever	0.46 (0.2-1.10)	0.25 (0.09-0.72)	0.01*
Dehydration	3.66 (1.2-11.22)	1.52 (0.38-6.1)	0.55

ORs are adjusted for living site, gender and water

(4.1%) samples were positive for *E. histolytica*, 13 (5.4%) samples for *E. dispar*, 2 (0.8%) samples for *E. moshkovskii* and 217 (89.6%) samples were negative (Fig. 1).

Of the total patients, 127 (52.4%) cases were female. In total, 168 (69.4%) cases were living in urban areas while 74 (30.6%) cases were in rural areas. The baseline characteristics of every group are shown in Table 1.

Age, sex, living in urban or rural and underlying conditions had no significant correlations between thegroups of patients (Table 2). Signs and symptoms of patients in every group are shown in Table 3. Only nine of the 67 microscopy-positive stools confirmed by PCR. In comparison of the two tests; PCR and microscopy were both positive in 9 samples while 14 samples were positive only by PCR and 58 samples were positive only by microscopy (Table 4). The sensivity and specificity of microscopy compared to PCR was 39.1 and 73.5%, respectively (Table 5).

Table 4: Comparison of PCR and microscopy results

	PCR		
Microscopy (N, %)	Positive N (%)	Negative N (%)	Total N (%)
Positive	9 (39.1)	58 (26.5)	67 (27.7)
Negative	14 (60.9)	161 (73.5)	175 (72.3)
Total	23 (100)	219 (100)	242 (100)

Table 5: Evaluation of results microscopy methods according to PCR

Variables	Percent	CI (95%)
Sensitivity	39.1	19.7-61.5
Specificity	73.5	67.2-79.2
PPV	+13.4	6.3-24
NPV	92	86.9-95.6
LR+	1.7	0.97-3
LR-	0.79	0.56-1.11
Kapa	0.068	0.18-0.045
AUR	58.1	47.7-68.5

CI: 95% Confidence Interval, PPV: Positive Predictive Value, NPV: Negative Predictive Value, LR: Likelihood Ratio, AUR: Area Under ROC

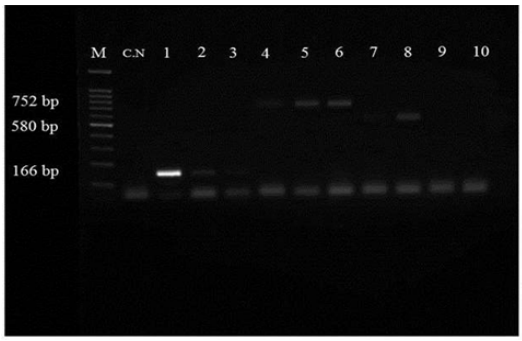

Fig. 1: Lane M, 100-bp lader DNA marker; Lane 1, 2, 3: *E. histolytico* DNA (166 bp); Lane 4, 5, 6 *E. dispar* DNA (752 bp); Lane 7, 8 *E.moshkovskii* DNA (580 bp). Lane 9, 10: negative control

Exact diagnosis of different types of Entamoeba is important clinically and epidemiologically because their treatment varies. Differentiation between pathogenic and non-pathogenic Entamoeba is not possible using microscopy, except for the instances of haematophagous trophozoites. In this study three species Entamoeba was differentiated by using multiplex-PCR which consists of multiplex primer sets within single template[10, 11]. Accordingly, the results of the present study showed that the relative frequency of Entamoeba by PCR is 10.3% in Khuzestan of which *Entamoeba histolytica* is 4.1% which is similar to findings by Hooshyar *et al*.[12] and Solaymani-Mohammadi *et al*.[6] in Tehran. However, the prevalence rate of *E. histolytica/E. dispar* in the central, Northern and Southernparts of Iran was 0.78, 3.9 and 4.6%, respectively[12]. High prevalence rate of the disease in Ahvaz is perhaps related to substandard drinking water, inadequate health systems and flow of sewage in some areas. The result of our study demonstrated that *E. dispar* is more prevalent than *E. histolytica* and *E. moshkovskii* (5.4% vs. 4.1% and 0.8%) in Khuzestan and is similar to results of other studies by Kurt *et al*.[13] in Turkey, Hooshyar *et al*.[4] in Iran and Subhan Chandra, etc. in India. However, in studies performed by Intarapuk *et al*.[14] in Thailand and Noor Azian *et al*.[15] in Kuala Lumpur *E. histolytica* was greater than *E. dispar* which is in contrast with the results obtained from other studies in Iran. Perhaps it is related to inadequate hygiene facility, poor environmental health and substandard drinking water. In this study, more prevalent clinical manifestations of amoebiasis were abdominal pain, tenesmus, nausea, vomiting and bloody diarrhea. The odds ratio of abdominal pain and vomiting in the amoebiasis group were 22.7 and 6.8 times more than in the parasite free group, respectively. Odds ratio of fever in amoebiasis group was 4 times less than in the parasite free group. The results of our study was almost similar to findings obtained by Tengku *et al*.[16] in Malaysia and Kurt *et al*.[13] in Turkey.

CONCLUSION

In this study, *E. dispar* was more prevalent like in other studies. PCR is a gold standard and highly sensitive and specific molecular method for differentiated Entamoeba typing to avoid unnecessary treatment and over diagnosis but since, PCR is technically expensive and not available everywhere, we suggest that PCR method use in patients with abdominal pain and vomiting (The odds ratio of abdominal pain and vomiting in the amoebiasis group were 22.7 times and 6.8 times more than in the parasite free group, respectively).

LIMITATIONS

The limitations of our study as following absence of a special freezer for storing stool samples in hospital. Dispose of some samples by laboratory staff) some patients fail to cooperate in giving samples) problems buying the kit.

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