Prevalence and species distribution of *E.histolytica* and *E.dispar* in symptomatic and asymptomatic patients

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Abstract

The objectives of this study were shed light to determine the prevalence and differential detection of two species *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (non pathogenic) that were morphologically identical as both cysts and trophozoite in two different groups , the first groups includes stools of fifty patient have diarrhea and abdominal pain (symptomatic) and ninety five patient have no diarrhea and abdominal pain (asymptomatic), who attending the AL-alwya childhood Teaching Hospital and AL-zafaranyia General Hospital in Baghdad were collected during the period from the beginning of Jully 2011 to the end of May 2012. The polymerase chain Reaction (PCR) was used to identify the *Entamoeba* species, *E.histolytica* (pathogenic) and *E.dispar* (non-pathogenic) by amplification DNA sequences of two genes , cystein proteinase 5 (EhCP5) gene, present only in *E. histolytica* and Actin gene(Act) which is present in both *E. histolytica* and *E. dispar*. The Results showed that both parasites were presents in both groups of patients and the percentage of *E. dispar* was higher than *E.histolytica* in two groups symptomatic and asymptomatic. In conclusion, it should not depend on direct wet mount technique identification of *Entamoeba* and it should be used PCR for exact identification of both species *E. histolytic*a and *E. dispar* in the diagnosis of amoebic dysentery.

Key word: Prevalence, distribution, *E.histolytica, E.dispar,* symptomatic, asymptomatic.

Introduction:

Amoebiasis is an important public health problem in developing countries and it's the third cause of death among parasite disease. Approximately 100000 people die yearly due to this parasite infection world-wide [1].The percentage of the world population infected by *Entamoeba histolytica* is calculated to be 10% and 90% of these individuals are not

symptomatic [2]. It has been known that many people who are apparently infected with *E. histolytica* never develop symptoms and spontaneously clear their infection. Differential diagnosis of *E. histolytica* and *E.dispar* in stool samples is not easy on the basis of microscopy alone. Currently some expensive methods such as amoebic antigen and DNA detection, isoenzyme electerophoretic pattern, PCR-basis methods, are available to differentiate both non pathogenic *E. dispar* from pathogenic *E. histolytica* [3,4,5,17]. In Iraq, there have been several studies on the prevalence of *E.histolytica/E.dispar* complex which used morphological or ElISA Technique [6, 7] the aim of this study, however, was to address the prevalence of *E.histolytica* and *E.dispar* in stool samples of patients with symptomatic and asymptomatic diarrhea using PCR.

Materials and Methods

Collection sample& Microscopy

A cross-sectional study was conducted on 165 stool samples collected from fifty patients with symptoms diarrheas, Ninety five patients with a symptom diarrheas and twenty healthy individuals as a control. The samples collected from the childhood Teaching Hospital and AL-zafaranyia General Hospital in Baghdad Governorate, during July 2011 to the end May 2012. Stool was collected with a clean and labeled container and patients were asked and instructed on how to bring approximately 3 g of stool, which was enough for direct saline [18].

DNA Isolation

All DNA isolation procedures were carried out in a biological safety cabinet in a room physically separated from that used to set up nucleic acid amplification and also from the "post-PCR" room, in order to minimize contamination and hence the possibility of false positive results. Parasites genomic DNA was extracted from stool samples by using Genomic DNA Purification kit Accuprep® stool DNA Extraction kit (Bioneer, Korea) according manufacture instructions.

PCR assays

Specific primers were used for PCR analysis of the two genes sequences, the internal segment of the cystein proteinase 5 (EhCP5) gene, present only in *E. histolytica* and Actin gene which is present in both *E. Histolytica* and *E. dispar* is shown in Table 1. These primers synthesized by Alpha DNA Company, Canada.

PCR reaction was conducted in 50µl of a reaction mixture containing 25 µl Go Taq® Green Master (Promega, USA), 1µlof 100pmol of each of primer (Ehcp5+Act) ,3µl of DNA template, 1µl $MgCl_2$ and 17µl of Deionized water. Negative control (containing Deionized water instead of DNA template)

amplification was included in every set of PCR reactions. Amplification was conducted using a Master cycler (Eppendrof) programmed with 1 cycle at 95°C for 5min; 30 cycles of 93°C for 1min, 59°C for 1min, 72°C for 45sec; 72°C for 10min. The amplified products were subjected to 2% Agaros gel electrophoresis, and visualized under UV (Image master VDS, Pharmacia Biotech, USA) after Ethedium bromide staining.

Table1: Primers used for the amplification of cystein proteinase 5 (CP5) & actin genes.

Gene	Primers	Primer sequence (5'–'3)	Amplicons size bp	GenBank accession number	Ref.
CP5	Forward Ehcp5	5GTTGCTGCTGAAGAAAC TTG 3	242	183233201 183233201/ 645845.2/ *E.histolytic*	[9,12]
	Reverse Ehcp5	5GTACCATAACCAACTAC TGC3			
Actin	Forward Act	5GGGACGATATGGAAAA GATC 3	300	645845.2 167376753/ 001734080.1/ *E.dispar*	[11,12,13]
	Reverse Act	5CAAGTCTAAGAATAGCA TGG 3			

Statistical Analysis

Only **C**hi-squire was used for the statistical analysis to analyze the results and comparing between the rates of parasites isolation from stool as well as evaluating the variances between wet mount method and PCR method in identification of *E.histolytica and E.dispar* (SAS, 2004) as in the following equation: $x^2 = \sum \frac{(o-e)^2}{e}$

Result

Microscopic examination

According to microscopic examination, the results showed that 27(%54) of patient with symptoms and 33(%34.73) patients with a symptom were infected with *E.histolytica/E.dispar*. The presence of both amoeba species in both groups (symptom &a symptom) were statically significant ($p<0.01$), Table2.

Table 2. Distribution of *E.histolytica /E.dispar* in the two groups of patients (symptom and a symptom) of selected positive stool samples, according to microscopic examination.

Patients groups	NO.145	Microscopic examination(Wet mount) for identical *E.histolytica/E.dispar*	Chi-square (χ^2)
Symptom	50	(%54.00)27	**(p<o.o1) **0.0**
A symptom	95	(%34.73)33	

DNA Isolation:

One hundred and seven isolates, 47 symptom and 60 a symptom patients and twenty fecal samples were collected from healthy individuals as a control, were subjected to DNA isolation. Sharp bands for genomic DNA were obtained for each preparation, the band intensity varied according to concentration of each preparation as shown figure1.

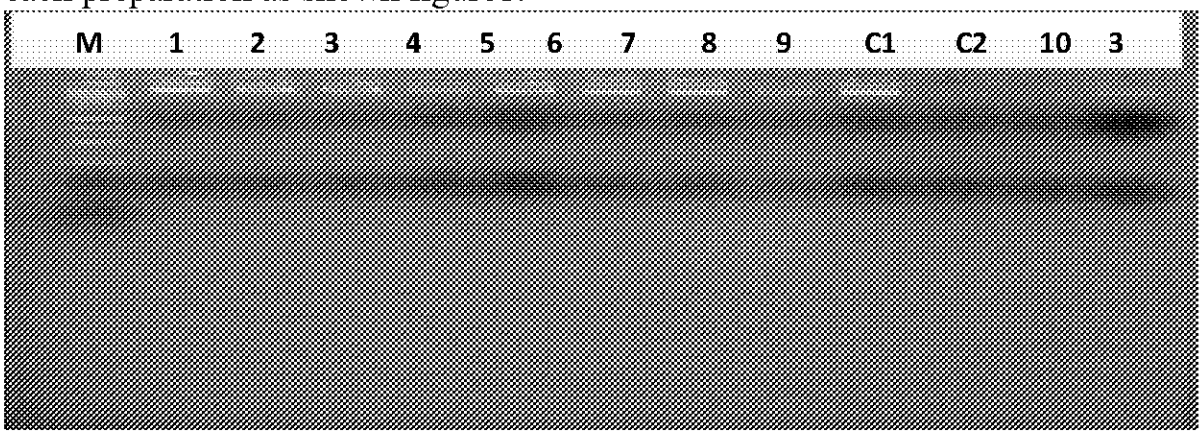

Figure 1: An Ethedium bromide stained Agaros gel (2%) electrophoresis (70 volt for 2 hour) of genomic DNA isolated from stool samples (Lanes:1-10)collected from symptom and a symptom patients .M: 100bp ladder.(Lanes:C1-C2) negative fecal sample by (containing distilled water instead of DNA template)

Duplex PCR (dPCR)

Two DNA sequences were amplified, cystein proteinase5 gene (242bp) present only in
E. histolytica and Actin gene (300bp) present both in *E. histolytica* and *E. dispar*, Figure2.

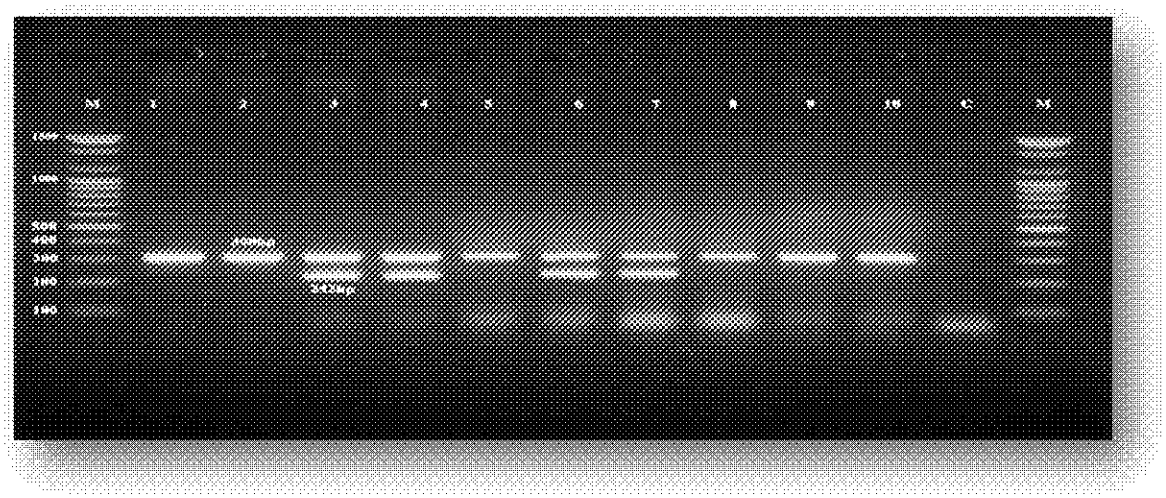

Figure2: An Ethedium bromide stained Agaros gel (2%) electrophoresis (70V, 120min) duplex PCR results showing diagnostic differentiation of *Entamoeba histolytica* from *E. dispar*. The amplicons from the Actin gene, common to both amoebae, and the Ehcp5 specific to *E. histolytica*, are identified as 300 bp and 242 bp, respectively. Lane M is

100-bp DNA ladder marker. Other lanes are: (1,2,5,8-10) samples for *E.dispar* ; (C) negative fecal sample by (containing distilled water instead of DNA template).*E. histolytica* was identified in direct samples (3,4,6,8).

The dPCR analysis showed that 39/165 of stool samples were infected with *E.histolytica/E.dispar*, 31 (%79.48) infected with *E.dispar* (only Actin gene amplified) and 8 (%20.15) were infected with *E.histolytica* (both genes Ehcp5 and Actin amplified), Table 3. Which showed a statistically significant?

Table 3: Distribution of *E. histolytica/ E. dispar* in the two groups of patients (symptom and a symptom) according to dPCR assay which amplified both genes (Ehcp5 and Act).

Patients groups	NO. Samples	Actin gene only	Both genes (Cystein proteinase5 gene & Actin gene)	Chi-square(χ^2)
Symptom	19	14 (%73.68)	5 (%26.31)	** **8.74**
A symptom	20	17 (%85.00)	3 (%15.00)	** **9.61**
Total	39	31 (%79.48)	8 (%20.51)	****8.89**
Chi-square)(χ^2)		*3.95	* 3.86	

significant (P<0.05)* ،Higher significant=(P<0.01) **

Microscopic results comparison to the dPCR technique.

Microscopically result was classified according to the wet preparation. This result was compared with the positive sample result of dPCR. There was statistically significant(P<0.01) and negative sample result of dPCR was statistically significant(P<0.05) respectively symptom and a symptom group, and control positive result negative for microscopic and dPCR with infected *Entamoeba* sp .and was no statistically significant between present *E.histolytica/E.dispar* showed Table 4.

Table 4: The number and percent of positive sample for dPCR for positive and negative of wet mount microscopic examination.

Group patient	Number and Percentage Wet-mount identical *E.histolytica/E.dispar*		NO. Positive dPCR	Number and percentage Detection Entamoeba.sp using technique dPCR		Chi-square
				E.histolytica	*E.dispar*	
Symptom No.50	Positive sample	(%54.00) 27	(%55.55) 15	(%20.00)3	(%80.00) 12	**7.84** **
	Negative	(%46.00) 23	(% 17.39)4	(%50.00)2	(%50.00) 2	**3.62** *

	sample					
A symptom	Positive sample	(%34.00) 33	(%48.48) 16	(%6.25) 1	(%93.75) 15	**7.96 ****
No.95	Negative sample	(%65.00) 62	(%6.45)4	(%50.00) 2	(%50.00) 2	**NS**
Control No.20		0	0	0		**NS**

non-significant = NS, significant =(P<0.05)* ،Higher significant=(P<0.01) **

Discussion:

In this report, we used duplex PCR assay for differential diagnosis &prevalence of two species of *Entamoeba* ,*E.histolytica* and *E.dispar* which share identical morphology .Recently differences between *E.histolytica and E.dispar* in expression patterns of protein thought to be involved in the virulent behavior of *E.histolytica* [8] .So two primer used for amplification of two gen cystein proteinase (Ehcp5) found only in *E.histolytica* and Actin gene found in both species *E.histolytica* and *E.dispar*[9,11,12,13,16,17] .

The results of this study showed the result by microscopy indicates that 27(54%) and 33 (34%) of the 165 symptomatic and asymptomatic to be infected with *E.histolytica/E.dispar* .but the species –specific diagnostic with *E.histolytica* found only in 8(20.51%) and *E.dispar* found in 31(79.48%) sample of the 145 symptomatic & asymptomatic. so that results indicated that the assay successfully amplified the positive sample with *E.histolytica* & *E.dispar* from each group (symptom &a symptom) .also the result showed that the percentage of *E.dispar* were more than the *E.histolytica* and close to the results of international studies, however, percentages may differ slightly due to time of study, location, sex, age and epidemiology [10,14,15].

Presence of this parasite in asymptomatic patients refers to the need for performing the diagnostic tests in symptomatic in addition to asymptomatic patients.

References

1-Haque, R.; Huston, C. D. ; Hughes, M. ; Houpt, E. and Petri, W. A. (2003). Amoebiasis. ***N. Engl. J. Med. 348:1565–1573.***

2-Ngui, R.; Siti, L. A.;Fakhrurrazi, A.; Lian, Y. L.; Ling, L. Y.; Ibrahim J. and Mahmud, R.(2012).Differentiating *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovkii* using nested polymerase chain reaction (PCR) in rural communities in Malaysia. ***Parasites & Vectors, 5:187.***

3-Gonin, P. and Trudel, L. (2003). Detection and Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* Isolates in Clinical Samples by PCR and

Enzyme-Linked Immuno sorbent Assay. ***J. Clil. Microb. Jan. 2003, p. 237–241.***

4- Sanchez-Guillen, M. C.; Merino-Guzman, G.; Perez-Fuentes, R.; Rosales-Encina , J.; L. and Talamas-Rohana, P.(1997). Serologic Characterization of *Entamoeba histolytica* Asymptomatic Carriers from a Community of Puebla State, ***Mexico. Suppl., 28:322-324.***

5- Redondo, R. P.; Mendez, L. G and Baer, G. (2006).*Entamoeba histolytica and Entamoeba dispar differentiation by Enzyme-Linked Immuno sorbet Assay (ELISA) and its clinical correlation in pediatric patients.* ***Parasito. Latin. 61: 37 – 42.***

6-Jarallah, H. M. (2012). Intestinal parasitic infections among rural villages in Basrah marshes regions .***J. Basrah Res. (Sciences) 38. 2 .A (2012).***

7-Raza H. H and Sami R.A. (2009).Epidemiological study on gastrointestinal parasites among different sexes, occupations, and age group sinsulaimani district. ***J. Duhok Univ. (12).1: 317-323.***

8- Bruchhaus, I.; Roeder, T.; Lotter, H.; Schwerdtfeger, M.and Tannich, E. (2002). Differential gene expression in *Entamoeba histolytica* isolated from amoebic liver abscess. ***Mol. Microb. 44: 1063–1072.***

9-Bruchhaus, I.; Jacobs, T.; Leippe, M. and Tannich, E. (1996). *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cystein proteinase genes. ***Mol. Microbiol.(22): 255-263.***

10-Hooshyar, H.;Rezaian, M.; Mahmoodi, M.;Farnia, S.;Mohammadi, S. (2004). A Field Study of the Distribution of *Entamoeba histolytica dispar* Cyst Passers in Northern, Central, and Southern Iran. ***Iranian .J Publ .Health, 33(2) :28-32.***

11-Edman , U.; Meza, I. and Agabian, N. (1987). Genomic and cDNA actin sequences from a virulent strain *of Entamoeba histolytica*. Proceedings of the National Academy of Sciences, ***USA., 84: 3024–3028.***

12-Freitas, M. A.; Vianna, E. N. ; Martins, A. S.; Silva, E. F. ; Pesquero, J. L. and Gomes. M. A. (2004). A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*. ***Parasitolo., 128:625–628.***

13-Huber, M.; Garfinkel, L.; Gitler, C.; Mirelman, D.; Revel,M. and Rozemblatt, S. (1987). *Entamoeba histolytica* cloning and characterization of actin cDNA. ***Mol. Bioch. Parasit.,24, 227–235.***

14-Ehsan,.N.;Mojarad,Z.N.;Navid,S.;Mohammad,R.N.;Hossein,D.;Ali,H.(2010) characterization of *Entamoeba histolytica* and *Entamoeba dispar* in fresh stool by pCR., ***Gastr . Hep. Bed .Bench . 3(1):37-41.***

15- Solaymani-MOhammadi, S.; Rezaian, M.; Babaei, Z.; Rajabpour, A.;

Meamar, A.R. andPourbabai, A.A. (2006) . Comparison of a stool antigen detection kit and PCR for diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* infections in asymptomatic cyst passers in lran **. *J .Clin. Microbiol .44: 2258-61.***

16- Tengku, S.A. and Norhayati, (2011) Public health and clinical importance of amoebiasis in Malaysia. ***Tro. Bio.med.,Rev 28(2): 194–222***

17- Fotedar, R., D. Stark, N. Beebe, D. Marriott, J. Ellis, and J. Harkness. (2007). Laboratory Diagnostic Techniques for *Entamoeba* Species., ***J. Clin. Microbiol. Rev.,45:511-532.***

18- Tanyuksel, M.; Yilmaz, H.; Ulukanligil, M.; Araz, E.; Cicek, M.; Koru, O.; Tas, Z. and Petri, W.A. (2005). Comparison of two methods (microscopy and enzyme-linked immune sorbent assay) for the diagnosis of Amoebiasis. ***Exp. Parasitol., 110:322-326.***

انتشار وتوزيع الاميبا الحالة للنسج والاميبا المتغايرة في المرضى العرضيين واللاعرضيين

الخلاصة

تهدف الدراسة الحالية لتسليط الضوء على تحديد وتشخيص نسبة الأصابة بنوعين من الاميبا ،الاميبا الحالة للنسج (المرضية) *Entamoeba histolytica* والاميبا المتغايرة (الغير مرضية) *Entamoeba dispar* واللذان يمتازان بشكلهما المتماثل في كلا الطوريين الكيسي والخضري في مجموعتين مختلفين المجموعة الاولى تضمنت 50عينة براز لمرضى يعانون من اسهال والم في البطن (المجموعة العرضية) اما المجموعة الثانية فتضمنت 95 عينة لمرضى لايعانون من اسهال اوالم في البطن (المجوعة اللاعرضية)تم جمع هذه العينات من المراجعين لمستشفى اطفال العلوية التعليمي والزعفرانية العام في بغداد للفترة من الشهر تموز 2011 الى نهاية شهر ايار 2012 . تم تشخيص طفيلي الاميبا بطريقة الفحص الرطب و تقنية التفاعل التسلسل التضخمي المزدوج dPCR assay في تشخيص طفيلي الاميبا المرضية *E.histolytica* والغير مرضية *E.dispar* من خلال تضخيم زوج من الجينات احدهما 5gene(Ehcp5) cystein proteinase الذي يدل وجوده على الاميبا الحالة للنسج اما الجين الاخر فيمثل Actin gene(Act) والذي يتواجد في كلا من الاميبا الحالة للنسج والاميبا المتغايرة اشارت النتائج PCR الى تواجد كلا النوعين وفي كلا المجوعتين العرضية واللاعرضية ولكن نسبة تواجد الاميبا المتغايرة تفوق نسبة تواجد الاميبا الحالة للنسج . نستنتج من هذه الدراسة الى انه لايمكن الاعتماد على الفحص الرطب في تشخيص الاميبا ولغرض تميز كلا النوعين يجب استخدام تقنية PCR في تميز *E.histolytica E.dispar* .