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 ٢٠١١ to the end of May ٢٠١٢. 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 ٥ (EhCP٥) 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 ١٠٠٠٠٠ people die yearly due to this parasite infection world-wide [١].The percentage of the world population infected by *Entamoeba histolytica* is calculated to be ١٠٪ and ٩٠٪ of these individuals are not

symptomatic [٢]. 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* [٣,٤,٥,١٧]. In Iraq, there have been several studies on the prevalence of *E.histolytica*/*E.dispar* complex which used morphological or ElISA Technique [٦, ٧] 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 ١٦٥ 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 ٢٠١١ to the end May ٢٠١٢. Stool was collected with a clean and labeled container and patients were asked and instructed on how to bring approximately ٣ g of stool, which was enough for direct saline [١٨].

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 ٥ (EhCP٥) gene, present only in *E. histolytica* and Actin gene which is present in both *E. Histolytica* and *E. dispar* is shown in Table ١. These primers synthesized by Alpha DNA Company, Canada.

PCR reaction was conducted in ٥٠µl of a reaction mixture containing ٢٥ µl Go Taq® Green Master (Promega, USA), ١µlof ١٠٠pmol of each of primer (Ehcp٥+Act) ,٣µl of DNA template, ١µl $MgCl_2$ and ١٧µl 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 ١ cycle at ٩٥°C for ٥min; ٣٠ cycles of ٩٣°C for ١min, ٥٩°C for ١min, ٧٢°C for ٤٥sec; ٧٢°C for ١٠min. The amplified products were subjected to ٢٪ Agaros gel electrophoresis, and visualized under UV (Image master VDS, Pharmacia Biotech, USA) after Ethedium bromide staining.

Table١: Primers used for the amplification of cystein proteinase ٥ (CP٥) & actin genes.

Gene	Primers	Primer sequence (٥'–'٣)	Amplicons size bp	GenBank accession number	Ref.
CP٥	Forward Ehcp٥	٥GTTGCTGCTGAAGAAAC TTG ٣	٢٤٢	١٨٣٢٣٣٢٠١ ١٨٣٢٣٣٢٠١/ ٦٤٥٨٤٥,٢/ *E.histolytic*	[٩,١٢]
	Reverse Ehcp٥	٥GTACCATAACCAACTAC TGC٣			
Actin	Forward Act	٥GGGACGATATGGAAAA GATC ٣	٣٠٠	٦٤٥٨٤٥,٢ ١٦٧٣٧٦٧٥٣/ ٠٠١٧٣٤٠٨٠,١/ *E.dispar*	[١١,١٢,١٣]
	Reverse Act	٥CAAGTCTAAGAATAGCA TGG ٣			

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, ٢٠٠٤) as in the following equation: $x^2 = \sum \frac{(o-e)^2}{e}$

Result

Microscopic examination

According to microscopic examination, the results showed that ٢٧(٥٤٪) of patient with symptoms and ٣٣(٣٤,٧٣٪) 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<٠,٠١), Table٢.

Table ٢. 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.١٤٥	Microscopic examination(Wet mount) for identical *E.histolytica/E.dispar*	Chi-square (χ^2)
Symptom	٥٠	(٥٤,٠٠٪)٢٧	**(p<o.o١) ٠,٠
A symptom	٩٥	(٣٤,٧٣٪)٣٣	

DNA Isolation:

One hundred and seven isolates, ٤٧ symptom and ٦٠ 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 figure١.

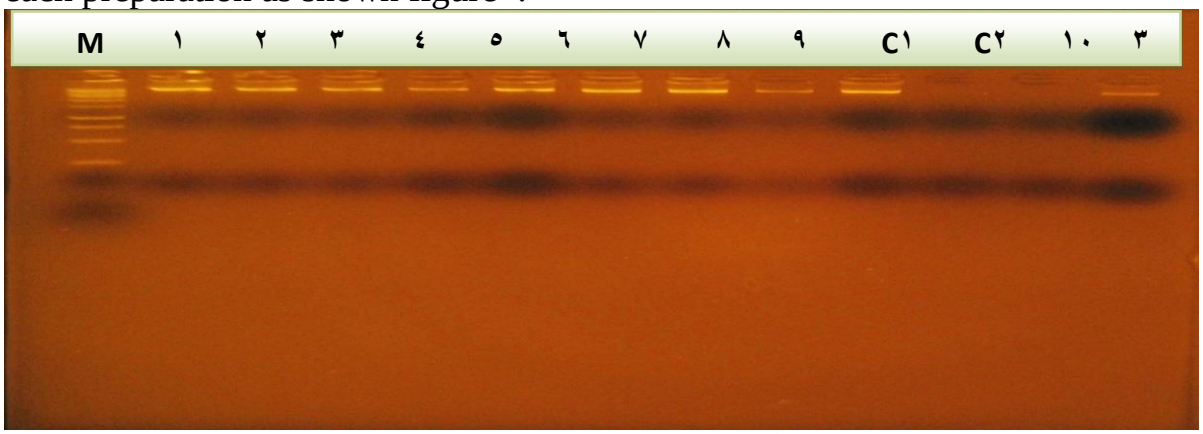

Figure ١: An Ethedium bromide stained Agaros gel (٢٪) electrophoresis (٧٠ volt for ٢ hour) of genomic DNA isolated from stool samples (Lanes:١-١٠)collected from symptom and a symptom patients .M: ١٠٠bp ladder.(Lanes:C١-C٢) negative fecal sample by (containing distilled water instead of DNA template)

Duplex PCR (dPCR)

Two DNA sequences were amplified, cystein proteinase٥ gene (٢٤٢bp) present only in

E. histolytica and Actin gene (٣٠٠bp) present both in *E. histolytica* and *E. dispar*, Figure٢.

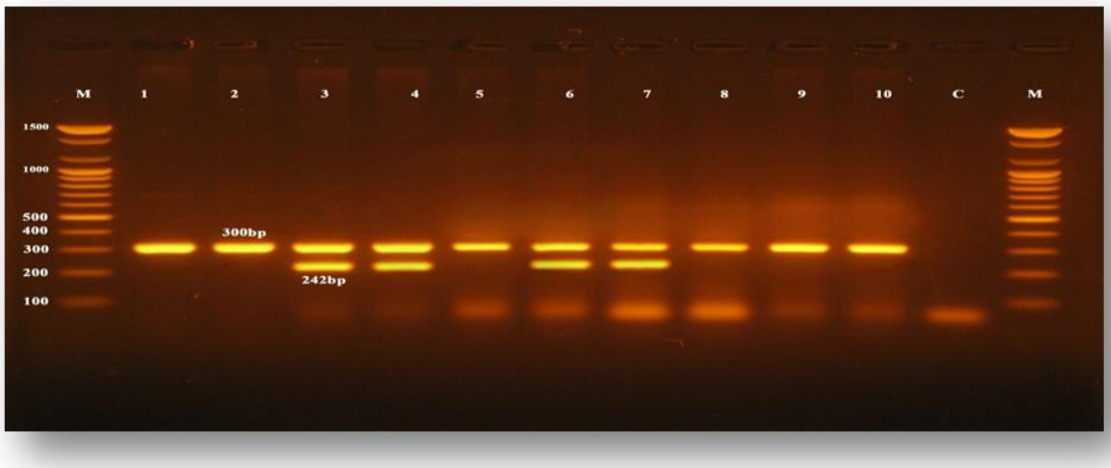

Figure٢: An Ethedium bromide stained Agaros gel (٢٪) electrophoresis (٧٠V, ١٢٠min) duplex PCR results showing diagnostic differentiation of *Entamoeba histolytica* from *E. dispar*. The amplicons from the Actin gene, common to both amoebae, and the Ehcp٥ specific to *E. histolytica*, are identified as ٣٠٠ bp and ٢٤٢ bp, respectively. Lane M is

١٠٠-bp DNA ladder marker. Other lanes are: (١,٢,٥,٨-١٠) samples for *E.dispar* ; (C) negative fecal sample by (containing distilled water instead of DNA template).*E. histolytica* was identified in direct samples (٣,٤,٦,٨).

The dPCR analysis showed that ٣٩/١٦٥ of stool samples were infected with *E.histolytica/E.dispar*, ٣١ (٪٧٩,٤٨) infected with *E.dispar* (only Actin gene amplified) and ٨ (٪٢٠,١٥) were infected with *E.histolytica* (both genes Ehcp٥ and Actin amplified), Table ٣. Which showed a statistically significant?

Table ٣: Distribution of *E. histolytica/ E. dispar* in the two groups of patients (symptom and a symptom) according to dPCR assay which amplified both genes (Ehcp٥ and Act).

Patients groups	NO. Samples	Actin gene only	Both genes (Cystein proteinase٥ gene & Actin gene)	Chi-square(χ^2)
Symptom	١٩	١٤ (٪٧٣,٦٨)	٥ (٪٢٦,٣١)	** ٨,٧٤
A symptom	٢٠	١٧ (٪٨٥,٠٠)	٣ (٪١٥,٠٠)	** ٩,٦١
Total	٣٩	٣١ (٪٧٩,٤٨)	٨ (٪٢٠,٥١)	**٨,٨٩
Chi-square)(χ^2)		*٣,٩٥	* ٣,٨٦	

significant (P<٠,٠٥)* ,Higher significant=(P<٠,٠١) **

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<٠,٠١) and negative sample result of dPCR was statistically significant(P<٠,٠٥) 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 ٤.

Table ٤: 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.٥٠	Positive sample	(٪٥٤,٠٠) ٢٧	(٪٥٥,٥٥) ١٥	(٪٢٠,٠٠)٣	(٪٨٠,٠٠) ١٢	٧,٨٤ **
	Negative	(٪٤٦,٠٠) ٢٣	(% ١٧,٣٩)٤	(٪٥٠,٠٠)٢	(٪٥٠,٠٠) ٢	٣,٦٢ *

	sample					
A symptom No.٩٥	Positive sample	(٪٣٤,٠٠) ٣٣	(٪٤٨,٤٨) ١٦	(٪٦,٢٥) ١	(٪٩٣,٧٥) ١٥	٧,٩٦ **
	Negative sample	(٪٦٥,٠٠) ٦٢	(٪٦,٤٥)٤	(٪٥٠,٠٠) ٢	(٪٥٠,٠٠) ٢	NS
Control No.٢٠	٠		٠	٠		NS

non-significant = NS, significant =(P<٠,٠٥)* ,Higher significant=(P<٠,٠١) **

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* [٨] .So two primer used for amplification of two gen cystein proteinase (Ehcp٥) found only in *E.histolytica* and Actin gene found in both species *E.histolytica* and *E.dispar*[٩,١١,١٢,١٣,١٦,١٧] .

The results of this study showed the result by microscopy indicates that ٢٧(٥٤٪) and ٣٣ (٣٤٪) of the ١٦٥ symptomatic and asymptomatic to be infected with *E.histolytica/E.dispar* .but the species –specific diagnostic with *E.histolytica* found only in ٨(٢٠,٥١٪) and *E.dispar* found in ٣١(٧٩,٤٨٪) sample of the ١٤٥ 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 [١٠,١٤,١٥].

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

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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 ٥٠عينة براز لمرضى يعانون من اسهال والم في البطن (المجموعة العرضية) اما المجموعة الثانية فتضمنت ٩٥ 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 ٢٠١١ الى نهاية شهر ايار ٢٠١٢ . 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 gene(Ehcp٥) ٥cystein 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* .