Diagnostic study of some species of Genus Entamoeba by PCR technique in City Tikrit

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Abstract :

A total of 317 stool samples were collected from patients attended Tikrit Teaching hospital and some clinics laboratories in the Tikrit city center and its environs during the period extend from 25 /10 / 2009 to 1/4 /2010. Microscopic examination of direct wet smear , formalin – ether concentration methods and polymerase chain reaction (PCR) were used for diagnosis and differentiation of *Entamoeba histolytica , E.dispar , E.moshkovskii.* The results revealed that out of 317 stool samples examined by wet mount and formalin – ether concentration 54 (17.03%) were infected with *E.histolytica* / *E.dispar* trophozoite and / or cyst as found in stool sample varying from solid , semi – solid , diarrhea (watery or bloody and mucus together).whereas the PCR carried out on the stool samples recorded positive for *E.histolytica* / *E.dispar* in microscopic examination showed that 45 out of 54 (83.33%) were *E.dispar* and 1 out 54 (1.85%) was mixed infection of *E.dispar* and *E.moshkovskii* , and 14.18% (8 of 54) were negative, no infection with *E.histolytica*.The diagnosis of *E.moshkovskii* is first considered in the present study from Tikrit city.

Introduction:

The genus *Entamoeba* contains many species, six of which *E.histolytica, E.dispar, E.moshkovskii, E.poleks , E.hartmani and E.coli* reside in the human intestinal lumen . *E.histolytica* only can invade the tissue. The first three species are identical morphologically but are biochemically and genetically different (1). Although *E.histolytica* is capable to cause disease (invasive amoebiasis) the ability of *E.dispar* and *E.moshkovskii* are uncertain (2).

The diagnosis of *E.histolytica* may be carried out by microscopic examination and finding trophozoites or cyst in the feces but this method is incapable of distinguishing between the cysts and trophozoites of *E.histolytica* and *E.dispar,E.moshkovskii* in addition the presence of other amoebae cyst like *Endolimax* , *Iodamoeba* makes the diagnosis difficult (3,4).These factors pose a problem an the accuracy of the data of the epidemiological studies carried out in the last decade in different parts of the world and makes the quoted prevalence of *E.histolytica* 500 million worldwide annually is very misleading(5).

The diagnosis of intestinal amoebiasis that depend on in-vitro cultures, the detection of antigens in the feces and other molecular techniques are more sensitive and specific in detection of *E.histolytica* in comparison to microscopical examination (6). Recently there are kits of ELISA that differentiation between *E.histolytica* , *E.dispar* , but a recent comparative study between using ELISA and PCR already shown that PCR is more advantageous from the other tests in epidemiological studies and impose a new evaluation about the prevalence of each species of amoeba and correct the epidemiological information (7).

The aim of the following study is to a comparison between microscope examination of direct wet smear , formalin-ether concentration and PCR technique in the diagnosis and differentiation of the 3 species of amoeba *E.histolytica* , *E.dispar* , *E.moshkovskii* in stool samples.

Material and methods :

A total of 317 stool samples were collected patients with diarrhea or abdominal pain attended Tikrit Teaching hospital and some clinics laboratories in the city center and its environs during the period extend from 25 / 10 / 2009 to 1 / 4 / 2010.

The stool samples were examined microscopically within 30 minutes using direct wet preparation of 1% lugal iodine , 1% eosine (8,9), and formalin – ether concentration method (10).

Extraction of DNA from stool samples :

The solutions were prepared according to (11) and DNA extraction was carried out directly from the stool samples by two methods , the first one used by (11) included the distraction of the membranes of the parasites mechanically by using small metal balls , the second was fast CTAB DNA isolation method according to (12) which included the distraction of the parasites membrane enzymatically by proteinase K.

Multiplex – PCR was used to detect the presence of DNA specific for *E.histolytica* and *E.dispar* at the same sample and at the same time (13). The primers used were (Eh2R- Ehp1F) for *E.histolytica* which generate 132 bp amplicon (Edp2R- Edp1F) for *E.dispar* which generate 96 amplicon.single round PCR according to (11) was carried out to detect the specific DNA for *E.moshkovskii* using the primer (EntaF – EmR) which generate 580 bp amplicon .

The detection of the products of DNA extraction and PCR were carried out in agarose gel electrophoresis according to (11).

The solutions , buffers used in the extraction and electrophoresis were provided by promega company , The solutions and materials used in PCR were provided by Alpha DNA , promea , sigma compantes.

Results :

1: **The percentage of infection with *E.histolytica*/*E.dispar* by microscopic examination of direct wet smear and formalin - ether concentration method.**

The positive samples were 54 out of 317 (17.03%) , wheras the negative samples were 263 out of 317(82.73%).

2: Extraction of DNA from stool sample:
It appeared the fast CTAB DNA isolation method used was more efficient than the other method used in extraction more concentration and purity for use as a template in PCR as shown in fig (1).

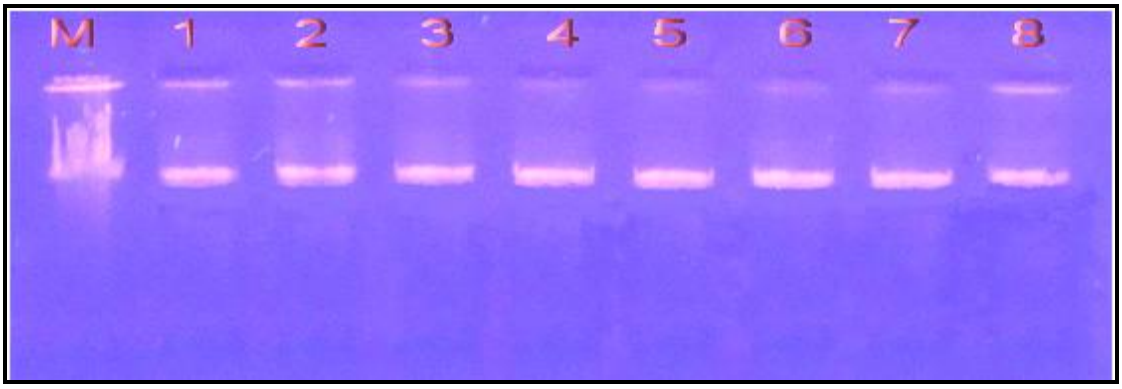

Fig (1): Electrophoresis of DNA extracted from stool samples in 1% agarose. M:represent the DNA size marker and the samples (1-8) the DNA samples extracted.

3: The percentage of infection with *E.histolytica* , *E.dispar* , *E.moshkovskii* by PCR.
The specific primers used showed the lances / bands required .The Edp1R- Edp2F generated 96 bp amplicon showed that 83.33% (45 out of 54) samples were positive for *E.dispar* as shown in fig (2) , whereas the primer (Ehp1F- Ehp2R) Showed no band corresponding to 132 bp indicating negative *E.histolytica* . The primer (EntaF- EmR) generated 580 bp showed that 1.58%(1 out of 54) as shown in fig (3) was positive for *E.dispar* and *E.moshkovskii* .The negative samples comprises 14.81 % (8 out of 54).

Table (1) summaries the percentage of the infection with the 3 species of *Entamoeba* by PCR technique .

No.of sample examined by PCR	The+ve samples	The parasites	% infection	-ve sample	%
54	45	*E.dispar*	83.33	8	14.81
	1	*E.dispar* +*E.moshkovskii*	1.85		
The total	46		85.18	8	14.81

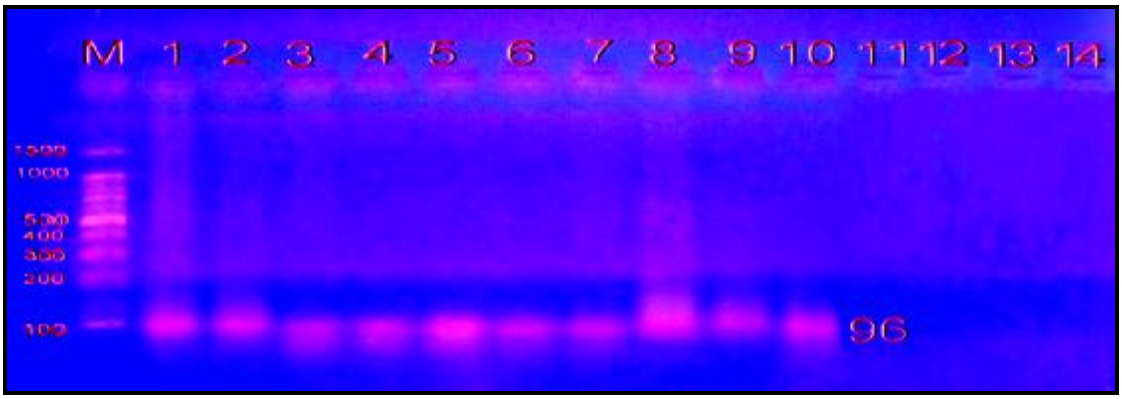

Fig 2: Electrophoresis of PCR product in 2% agarose .M: represent DNA size marker (100 bp ladder) Bands / lanes 1-10 positive for *E.dispar* , (11-13) negative , (14) without DNA(control).

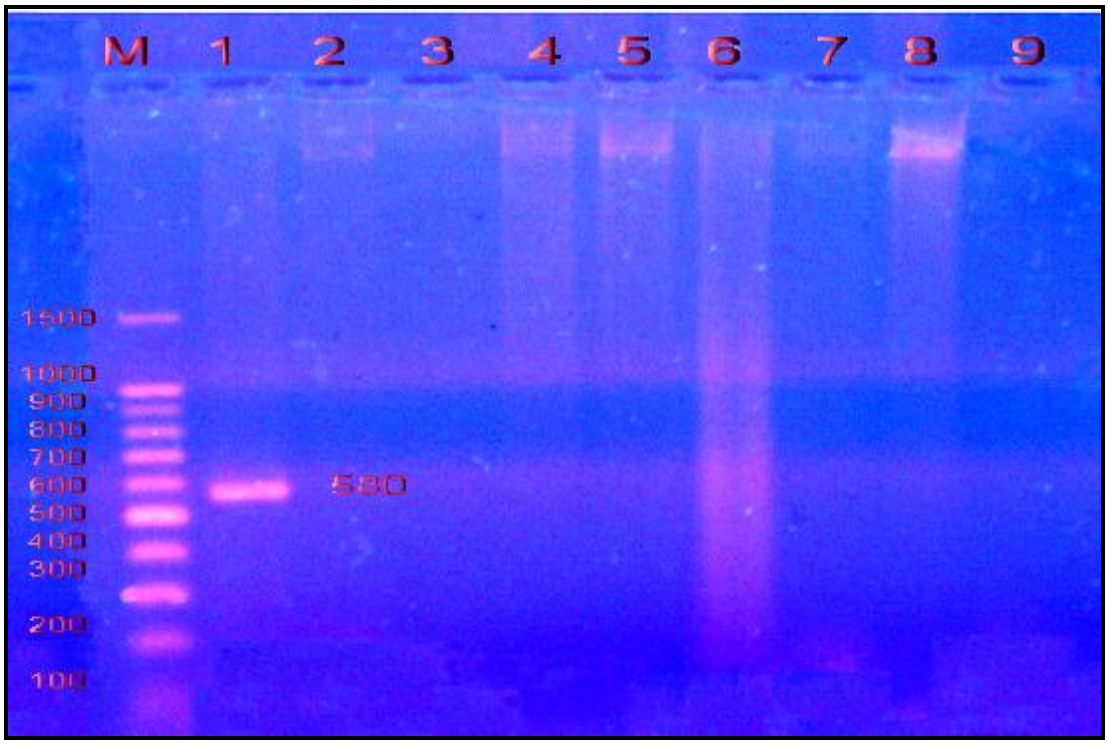

Fig 3 : Electrophoresis of PCR product in 2% agarose .M: represent DNA size marker (100 bp ladder) Bands / lanes (1) positive for *E.moshkovskii* , (2-8) negative and (9) without DNA (control).

Discussion :

The prescent study recorder 17.03% infection with *E.histolytica* / *E.dispar* from a total of 317 stool samples examined by direct wet smear and concentration methods. This is similar to the finding of (14,15, 16) who recorded 17.8% , 15.9% and 18.7%, respectively .

The results of using PCR technique shows that *E.dispar* represent 83.33% (45 out of 45 samples) this is similar to (17,18) who recorded 83.78% (31 out of 57) and 81.4%(22 out of 27), respectively.

The high percentage of infection by *E.dispar* may be due to the fact that this species is the more common 11.7 and 14.4 time than other species of amoeba (19, 20) respectively. The presence of *E.dispar* in patient with diarrhea , gastric and intestinal symptoms may be due to the presence of other organisms causing diarrhea (*Rota virus* , *Shigella species* , *Campylobacter* species , enterohemorrhagic or enteroinvasive *Esherichia coli* , and *Salmonella* species) although there are some clues that infection with *E.dispar* may result in blood changes in some person (21) and recently the infection with *E.dispar* was recorded in patient infected with liver abscess .

The 1.85 % of mixed infection with *E.dispar* and *E.moshkovskii* is similar to (19 ,23) and others . The low percentage of *E.moshkovskii* may be explained that the parasite is considered free living and seldom infect the human and has a similar distribution to to *E.histolytica* (24).

The negative results from shown by PCR for some stool samples recorded positive by microscopic examination may be due 1: the limited advantage of microscope examination in differentiating trophozoites or cyst of *E.histolytica* / *E.dispar* from polynucleated white blood cells or the cyst stages of other species of amoebae like *Endolimax* , and *E.hartmani* (13, 25) , 2: The presence of inhibitory materials in some stool samples which may interfere with DNA polymerase and inhibit its action and prevent DNA amplification of the parasite (26), 3: The stool samples are considered very complicated material it contain several inhibitors as heme , bilirubin , bile salts complex carbohydrates which are frequently extracted with the DNA of the infecting parasite(27).

In conclusion : we recommend the use of PCR as complementary test for the routine examination and epidemiological studies . we expect to collect better information and understanding of the pathogenesis of the (3)species of amoeba in human and offer the necessary treatment .

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دراسة تشخيصية لبعض أنواع الجنس انتاميبا *Entamoeba* بتقنية الـPCR في مدينة تكريت

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الملخص:

تضمنت الدراسة الحالية فحص 317 عينة غائط للمرضى المراجعين لمستشفى تكريت التعليمي وبعض المختبرات الأهلية في مركز مدينة تكريت وضواحيها خلال الفترة مابين 2009/10/25 الى 2010/4/1.

أظهرت الدراسة الحالية نسبة خمج كلي بلغت 17.03% (54 من 317) بطفيلي*Entamoeba histolytica / E.dispar* فحصت بالمسحة المباشرة الرطبة وطريقة التركيز بالأيثر- فورمالين بأستخدام المجهر الضوئي؛ وذلك للكشف عن الأطوار المتغذية والمتكيسة للطفيلي، إذ وجدت في عينات غائط مختلفة القوام من الصلبة الى شبه الصلبة الى أسهال (تراوح من مائي الى دموي ومخاط معاً).

أستعملت تقنية تفاعل السلسلة المتبلمرة (PCR) في كشف وتفريق الخمج بطفيليات (*E.histolytica* , *E.dispar* , *E.moshkovskii*) في العينات الموجبة الفحص المجهري ، أظهرت نسبة خمج كلية بلغت 85.18%(46 من 54) ونسبة خمج منفرد بطفيلي *E.dispar* بلغت 83.33% (45 من 54) ونسبة خمج مختلط بطفيليات (*E.dispar* +*E.moshkovskii*) بلغت 1.85% (1 من 54). سجلت 8 عينات سالبة بنسبة 14.81 % (8 من 54)، ولم يسجل الخمج بطفيلي *E.histolytica*، و يعد تشخيص الخمج بطفيلي*E.moshkovskii* أول تسجيل في مدينة تكريت.