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**Use PCR Conventional for detecting AP and PLA virulence
Factors of *Entamoeba histolytica* in patients stool samples
in Al-Qadisiyah Province**

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Abstract

The present study designed to diagnose the *Entamoeba histolytica* parasite from patients with diarrhea attended to Maternity and Childhood Teaching Hospital and General Education Hospital in Al-Qadisiya province, Theirs ages less than one year to 14 years at the period from the beginning of May to the end of November 2014. The number of samples that have been collected (100) stool samples, and the number of samples infected is (36) sample; After samples were examined in direct wet smear method using normal saline, included the study to investigate and to emphasize the presence of virulence factors amoebapore and phospholipase in parasite using Conventional PCR Technique.

The result showed that the infection rate was 36% (100). The age group of 1-5 years showed the highest rate of infection (41.6%), while patients aged 11-14 years showed the lowest rate of infection (11.1%). The infection rate in male was higher (58.3%) than in female (41.6%). It was found that the majority of cases (61.1%) were from Urban areas.

DNA was extracted from positive from stool samples and then after amplified using special designed for primers *E. histolytica* virulence factors genes that called amoebapore & phospholipase and the amplified DNA passed in Electrophoresis apparatus for DNA. The results showed that the mentioned factors is present in all positive samples of *E. histolytica*.

Introduction

Amebiasis caused by *Entamoeba histolytica*. it is still mentioned as one of the major health problems in tropical and subtropical areas (1).

It is the third leading cause of death due to parasites, after malaria and schistosomiasis. Amoebiasis presents a high index of morbidity and mortality, mainly in developing countries. According to the World Health Organization (WHO), 500 million people are infected with amoebae; 10% of infected individuals have virulent *E. histolytica* resulting in 40,000–100,000 deaths annually (2).

This infection is usually predominant in low socioeconomic status and poor hygienic situations that favor the indirect fecal-oral transmission of the infection (3).

Virulence is a complex phenomenon that depends on two general properties; the invasiveness, or ability of microorganism to multiply and to cause localized tissue destruction, and toxigenicity, or the ability to produce and secrete substances that can cause distant lesions. However, the virulence of *E. histolytica* related strains likely depends mainly on the tissue-damaging potential of individual trophozoites and the number of invasive amoebae in the infected host(4).

The major pathogenic function and the most prominent property of *Entamoeba histolytica* is its remarkable cytolytic capacity. A number of *E. histolytica* molecular components have been thoroughly established as contributors to its pathogenesis. During initial intestinal colonization, a protein Twenty years ago termed amoebapore(AP) which is capable of forming ion channels ,or pores in lipid membranes, and depolarizing target cells, was discovered in *E. histolytica* (5,6,7).

Trophozoites of *E. histolytica* secrete pore-forming peptides known as ‘amebapores’ that assemble within host cell membranes to trigger cell death (8). Amoebapores insert into the membranes of bacteria or eukaryotic cells and form pores that result in lysis of the target cells. The addition of purified Amoebapores to eukaryotic cells results in cell necrosis and possibly apoptosis (11).

Another important virulence factor called phospholipase A (PLA), which is facilitates host cell penetration by the two protozoan species *Toxoplasma gondii* and *Entamoeba histolytic* (10) . The term “phospholipases” refers to a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids . Although, all phospholipases target phospholipids as substrates, each enzyme has the ability to cleave a specific ester bond (9).

Materials and Methods:

Samples collections

One hundred human feces samples were provided from Microbiology laboratory of Al-Qadisiyah Hospital, then transported to laboratory and stored in freeze.

Microscopic examination

The direct method is the method used to mix the amount of stool with salt solution on a glass slide using a metal wire ,or a wooden skewer .aoda, then slide the cover and examine under the microscope on the two powers 25X,40X.

Genomic DNA Extraction

Genomic DNA was extracted from stool samples by using (Stool DNA extraction Kit, Bioneer. Korea). The extraction was done according to company instructions by using stool lysis protocol method with Proteinase K. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20C at freeze until used in PCR amplification.

Polymerase Chain Reaction (PCR)

PCR was performed for detection virulence factors genes in *Entamoeba histolytica* using specific primer which was designed in this study for amoebapore C and Phospholipase by using Genbank NCBI database and primer3plus. These were provided by (Bioneer company . Korea), as the following table:

Primer		Sequence	amplicon	Genbank
amoebapore C	F	TCCAGTTCTTTGTCCTGTTTGT	229bp	AY956434.2
	R	ACATGCATGAATCAACCCACA		
Phospholipase	F	TGCTGATTTGGCTCTTGGGA	420bp	DS571171.1
	R	CCAAGCCCTCTTTCCCAAA		

After that, PCR master mix was prepared by using (AccuPower[®] PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250 μ M, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl₂ 1.5mM, stabilizer, and tracking dye) and the PCR reaction prepared according to kit instructions in 20ul total volume by added 5ul of purified genomic DNA and 1.5ul of 10pmole of forward primer and 1.5ul of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20ul and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene Bioneer. Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 60 °C for 30sec, and extension 72 °C for 1 min and then final extension at 72 °C for 5 min. PCR products(420bp) were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under U.V transilluminator.

Results:**1. Prevalence of *E. Histolytica* According to Microscopical Examination .**

Prevalence of *E. Histolytica* in Human according to direct wet smear, 36 out of 100 stool samples were positive distributed over the months as the study is shown in the figure (1).

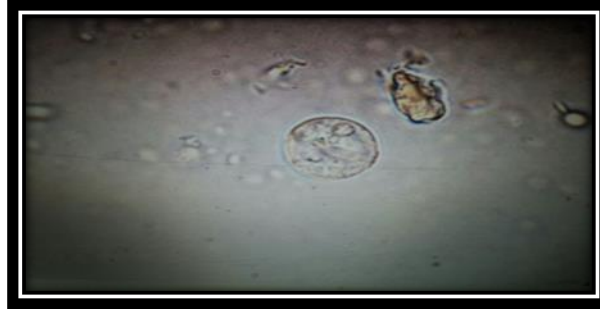


fig (1) *E. histolytica*

2. Prevalence of *E. Histolytica* According to the age groups.

The results of the present study showed the high percentage (41.6%), which was recorded in age group (1-5 years), while the lowest percentage infect (11.1%) in the age group (11-14 years) with significant difference at $p < 0.05$. Table(1)

Table (1) The prevalence of *E. Histolytica* parasite according to the age

Age groups	The number of infected Patients	%
Less than one year	10	27.7 ab
1-5	15	41.6 a
6-10	7	19.4 bc
11-14	4	11.1 c

3. Prevalence of *E. histolytica* According to the sex .

The results showed that 21 (58.3%) out of 36 and 15 (41.6%) out of 15 of male and female were positive respectively with significant differences at $p < 0.05$. Table (2)

Table (2) The prevalence of *E. histolytica* parasite according to sex

Sex	The number of Patients	%
male	21	58.3 a
female	15	41.6 a

3. Prevalence of *E. histolytica* According to the nature of residence.

The results showed that the incidence of *E. histolytica* in rural areas amounted to 61.1%, which is higher than the urban 38.8% . Results of statistical analysis showed that there were significant differences in the incidence between infect rates and the nature of residence at the level of probability ($p < 0.05$) as shown in Table (3).

Table (3) The prevalence of *E. histolytica* parasite according to residence

nature of residence	The number of Patients	%
Urban area	22	61.1 a
rural areas	14	38.8 b

A note

* Similar letters indicate no significant difference at the level of probability of 0.05 using test X2.

* Different letters indicate the existence of a significant difference at the level of probability of 0.05 using test X2.

4. Molecular study

Results of positive samples *E. histolytica* by PCR showed that all these samples 100% contain pathogenic virulence factors under study which are : amoebapore and phospholipase as the pictures show (2 and 3) the molecular weight of the PLA factor at PCR product size 420 bp, and the molecular weight factor of the AP factor at PCR product size is 229 bp, and they were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and examined under UV transilluminator .



fig (2): Agarose gel electrophoresis image that show PCR product analysis of *E. histolytica* parasite gene Phospholipase . Where, Lane (M) DNA marker (2000-100bp), Lane (1,2,3,4,5,6,7&8) positive as gene PLA at PCR product size 420bp.

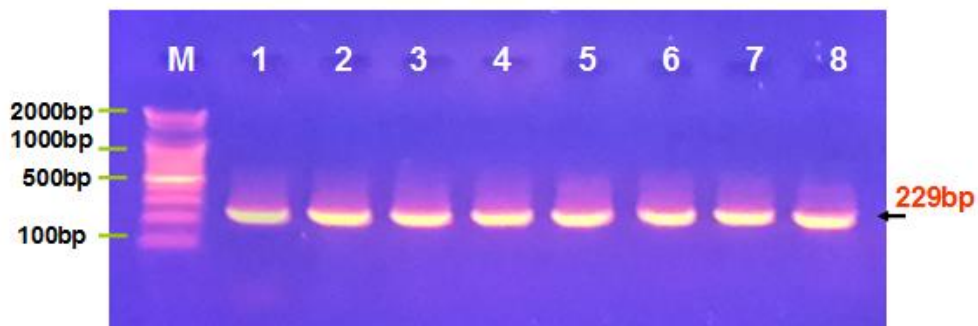


fig (3): Agarose gel electrophoresis image that show PCR product analysis of *E. histolytica* parasite gene amoebapore . Where as Lane (M) DNA marker (2000-100bp), Lane (1,2,3,4,5,6,7&8) positive as gene AP at PCR product size 229bp.

Discussion:

Entamoeba histolytica is a disease of the intestinal parasites. The regional prevalence of amoebic infection worldwide varies from (4 – 81%); because of widespread in the world and all regions the study was found to prove the high infection, use PCR conventional in detection AP and PLA virulence factors. The results founded the prevalence of cases in the age groups between (less than 1-14 years) the number of infection ownership amounted to (36) of (100) cases of infection were highest (1-5) years, reaching 41%. Hedda and agreed with the study in Thi-Qar Governorate (12). The study recorded the prevalence of infection stood at (42%). The study recorded the number of infection in male of 21 infection and the prevalence is (58.3%) and females number of 15 cases and the percentage of (41.6). The result of our study seems similar to the results of other studies done in Iraq and the world. Interpretation of this engagement on the basis of the behavior of females with their surroundings than males, This finding is in agreement with the result of a study done in Diala Governorate by (13). Recorded the prevalence in males (56.7%), and females (43.3%), while (14) in Baghdad, record rate of 41% for the year 2012 with regard to Candidiasis study reported infection in female 15% ratio while at male 21%. The study showed prevalence in rural areas is (38.8%) and in Urban area is (61%). The result of our study seems similar to the results of other studies done in *Entamoeba histolytica* Epidemiological. The reason for return prevalence in rural areas more than in Urban area, because of Water sources, and differences in educational level, and the presence of insects, and spread rodents in the countryside. Results proved the existence of the first factor of 100% of samples positive for injury and this is in line with (15). Results proved the existence of the second factor of 100% of samples positive for injury and this is in line with (16).

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استخدام تقنية PCR لتشخيص AP and PLA عوامل الضراوة لل

Entamoeba histolytica من المرضى المصابين بالإسهال في

محافظة الديوانية

صفاء رسن عبدالله /جامعة القادسية -كلية التمريض

رنا صالح صاحب الدفاعي /جامعة القادسية -كلية الطب البيطري

الخلاصة

صممت الدراسة الحالية من اجل تشخيص *Entamoeba histolytica* المزولة من المرضى المصابين بالإسهال في مستشفى الديوانية التعليمي ومستشفى الولادة والاطفال في محافظة الديوانية .

تراوحت اعمار المصابين ما بين (اقل من سنة واحدة - 14 سنة) للفترة الواقعة ما بين الاول من ايار ولغاية نهاية تشرين الثاني للعام 2014 . عدد العينات التي تم جمعها (100) عينة براز ، وكان عدد العينات المصابة (36) عينة ، بعد فحص العينات بطريقة الفحص المباشر للعينة الرطبة باستخدام المحلول الملحي الفسلجي (النورمال سلاين) .

تضمنت الدراسة التحقق من عوامل الضراوة , amoebapore و phosphlipase في الطفيلي باستخدام طريقة conventional PCR .
اظهرت الدراسة نسبة الاصابة في حالة 36 من 100 . ففي اعمار (1-5) سنة كانت اعلى نسبة اصابة حيث بلغت (41%) بينما الفئة العمرية من (11-14) سنة اظهرت اقل نسبة وهي (11,1%) . وكانت نسبة اصابة الذكور (3,58%) اعلى من نسبة اصابة الاناث (6,412%) وايضا المقارنة بين نسب الاصابة في المناطق الريفية كانت الاعلى (1,61%) .

تم مضاعفة الحامض النووي المستخلص من العينات الموجبة باستخدام عوامل الضراوة للطفيلي , amoebapore و phosphlipase واظهرت النتائج وجود هذه العوامل في جميع العينات الموجبة لل *Entamoeba histolytica* .