Detection of major virulence factor of *Entamoeba histolytica* by using PCR technique

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الخلاصـــة

اجريت هذه الدراسة لتحديد عوامل الضراوة الرئيسية لطفيلي الاميبا الحالة للنسيج في الامعاء وتشخيصها باستخدام تقنية التفاعل السلسلي لانزيم البلمرة PCR ((كما تمت دراسة نسبة انتشار هذا الطفيلي في محافظة الديوانية .تم جمع **186** عينة براز من الاشخاص المصابين المشتبه باصابتهم الذين تراوحت اعمارهم بين سنة واحدة و76سنة للفترة بين كانون الاول **2011**وحزيران **2012** تم تشخيص جنس طفيلي الاميبا الحالة للنسيج كمسبب للإسهال بفحص عينات البراز بطريقة المسحة الرطبة وطريقة التركيز كفحص عام للبراز وأظهرت النتائج ان **126**عينة من اصل **186**كانت ايجابية للفحص المجهري, وقد حفظت عينات البراز بدرجة حرارة -11°م .وللكشف عن مستضد الطفيلي في عينات البراز تم اجراء فحص الادمصاص المناعي المرتبط بالأنزيم الايلايزا (ELISA) حيث كانت النتائج **89**عينة موجبة من اصل **186**عينة .تضمنت الدراسة الحالية التحري عن بعض عوامل الضراوة الرئيسية (Lectin, Amoebapore cysteine proteinase)لطفيلي الاميبا الحالة للنسيج بالاعتماد على تقنية التفاعل السلسلي لإنزيم البلمرة(PCR) وباستخدام البادئات الخاصة لهذه العوامل (primers)حيث اظهرت النتائج ان **61**عينة من اصل**186**عينة كانت موجبة حيث كانت **55**عينة موجبة لإنزيم اللكتين و51عينة موجبة لإنزيمالاميبا بور, جميع العينات**(61)** كانت موجبة للسستين بروتنيز .بالمقارنة بين العينات الدموية والغير دموية حسب نتائج تقنية التفاعل السلسلي لأنزيم البلمرة لتشخيص طفيلي الاميبا الحالة للنسيج , اظهرت النتائج ان **58** (41%) من أصل 140 عينة دموية كانت موجبة و **4**(9%) من اصل **46** عينة غير دموية كانت موجبة, مع وجود فروق معنوية بمستوى(P value < 0.01) . وبالمقارنة بين طريقة الفحص المجهري وفحص الايلايزا وتقنية التفاعل السلسلي لأنزيم البلمرة اظهرت النتائج انه من مجموع **186** عينة كانت **126**عينة موجبة بطريقة الفحص المجهري (68%) و أظهرت **89** عينة موجبة بفحص الادمصاص المناعي المرتبط بالأنزيم الايلايزا (48%) و **61**عينة موجبة بتقنية التفاعلالسلسلي لألنزيم البلمرة (33%).

اظهرت نتائج هذه الدراسة ان نسبة اصابة هذا الطفيلي كانت عالية في المناطق الريفية في محافظة الديوانية حيث كان عدد الاشخاص المصابين باستخدام تقنية التفاعل السلسلي لأنزيم البلمرة 22.58% بينما كانت نسبة الاصابة للساكنين في مركز المدينة بنسبة 10.7% واظهرت الدراسة الحالية ان نسبة الاصابة كانت عالية لدى الاشخاص بالأعمار اليافعة مع عدم وجود فرق معتمد احصائيا عن باقيالمجاميع العمرية الاخرى.

ABSTRACT **Aim of the study**: This study was undertaken to detect the major virulence factors of the intestinal parasite E.histolytica by using PCR technique, and to study the prevalence of this parasite in Al-Diwanyia Governorate.

Patients and method: Stool samples were collected from one hundred eighty- six patients suffering from acute diarrhea, 146 of them had bloody diarrhea. Their ages vary from one to seventy six years old at the period between (December 2011 and June 2012). Microscopic examination with wet mount and concentration method as general stool examination (GSE) were done to all samples, ELISA assay and PCR were done to all samples.

Result: the results showed that 126 samples out of 186 were microscopically positive for E.histolytica. To detect the antigens of E.histolytica from stool, ELISA assay was done for all samples, 89 samples out of 186 were positive. To detect the major virulence factors (V.F.) (lectin, cysteine proteinase, and amoebapore) of E.histolytica, PCR technique was conducted, by using specific primers for E.histolytica, the results showed that 62 samples out of 186 were positive, out of these positive samples, there were 55 samples positive for lectin, 58 samples were positive for amoebapore, and all samples (62) were positive for cysteine proteinase.

Conclusion: the study show that PCR technique and ELISA assay are much specific and sensitive more than microscopic examination for detecting E.histolytica and distinct from non pathogen species.

Introduction

Amoebiasis is defined as invasive intestinal or extra intestinal infection with the protozoan parasite *E.histolytica*. It is still mentioned as one of the major health problems in tropical and subtropical areas

that is more than 50 million people worldwide are infected, and up to 100,000 of these die every year. [1] *E.histolytica* is a pseudopod-forming, non-flagellated protozoan parasite that causes proteolysis and tissue lysis (hence its name). It considered the most aggressive protozoan disease that affects the human bowel and is considered as the second or third most common cause of death among the parasitic diseases [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]. Previously two morphologically identical species of *Entamoeba* had been found, and shown that only one of them is able to cause infection in kittens or human volunteers. However, *E.histolytica* has recently been re-described as two distinct species; the pathogenic species *E.histolytica* and the nonpathogenic species *E.dispar*[4]. As these two species are morphologically similar, developments of new methods for their rapid differentiation is currently under investigation. Humans and primates are only the natural hosts; ingestion of E.histolytica mature cysts from the environment is followed by excystation in the terminal ileum or colon to form highly motile trophozoites. Upon colonization of the colonic mucosa, the trophozoites may encyst and thenexcreted in the feces or may invade the intestinal mucosal barrier and gain access to the blood stream and disseminate to the liver, lungs, and other sites, excreted cysts reach the environment to complete the cycle[5] . It is the cause of various infectious diseases ranging from dysentery to abscess of liver or other organs. Infection with E.histolytica results from invasion of the intestine by the parasite followed by tissue damage and inflammation, the severity of the disease may depend on multiple virulence factors with poorly understood mechanism [5,6]. One of the proposal mechanisms is the attachment of Entameba trophozoite to the enterocyte which is essential for colonization of the large intestine and is considered a prerequisite for parasite-induced enterocyte dysfunction and clinical disease. The adherence of trophozoites to colonic epithelial cells seems to be mediated by galactose/N–acetylgalactos amine (GAL/GalNAc)– specific lectin[7]; several other proteins that might participate in adherence have been also identified, Other virulence factors are the amoebapores, that can lysis the target cell and can also induce apoptosis, amoebapores are found within cytoplasmic granules of E.histolytica and may exocytose to the target cell membrane on contact[8]**.** Another important mechanism is secretion proteins with toxin-like activities, these proteins are present on the surface of E.histolytica known as cysteine proteinases that are the key of the virulence factors of E.histolytica, which play a central role in tissue invasion and disruption of host defenses.

Cysteine proteinases are among the most important enzymes in many microorganisms and are known to play essential roles in pathogenesis of such organisms. From known cysteine proteinases, CP1 and CP5 exist in *E. histolytica* and not in *E. dispar*. [9] In the present study, examination of molecular method with a PCR was done for amplification of a part of CP5 gene enabling us to differentiate the pathogenic species, *E.histolytica* from the non-pathogenic species, *E.dispar*.

Patients and Methods

This cross section study was carried out from the first of December 2011 till June 2012. The samples were collected from al-Diwaniya teaching hospital, maternity & children hospital, Afak general hospital and privet clinics in Diwaniya province.

One hundred eighty six patients of both genders were included in this study. All included patients had acute onset of diarrhea with inflammatory features. All those patients undergo full history (a questioner and full information were obtained from the patient like age, address, type of drinking water, and asking about clinical symptoms like abdominal pain, presence of blood in stool, and fever) and physical examination including vital signs and abdominal

examination were done by specific physician. The patients excluded from the study are those with features of irritable bowel syndrome, patients with chronic diarrhea, those with a known case of inflammatory bowel disease and patients with food poisoning are excluded from this study. The stool samples were taken from each patient, fresh unpreserved stool samples were collected in sterile containers for microscopic examination.

The specimens were examined within 30 minute for three times. According to the results of macroscopic and microscopic examination, we classified these samples into bloody and non bloody diarrhea.

After microscopic examination, the stool samples were stored at –20ºC until used for PCR and ELISA.

Microscopic examination (wet mount and concentration method) was used to identify the trophozoite and cyst stages of E.histolytica. Two types of wet mount were used for each of fecal sample (saline and iodine) and some samples stained with methylene blue. The wet mount was used for the initial microscopic examination of stool and to demonstrated amoebic trophoziotes, cysts and can also reveal the presence of RBC and pus cell. Then ELISA was used to detect E.histolytica's antigen from stool samples by using usual principles of ELISA assay, finally PCR was used to detect three proteins (Gal/GAL Nac lectin, amoebapore C, and cysteine proteinase 5) as a major virulence factors of E.histolytica.

Three steps were done for molecular technique (PCR): Extraction of DNA from fecal samples, Gel electrophoresis and thermocycling

Specific primer sequences used for PCR amplification, these primers were prepared according to information of company by dissolve primer in 60 ul of deionized distilled water to obtain in concentration 100 p.mol the primer. The primer used in this study was used according to the method of Bhattacharya et al[10].

The primer sequence was:

F: TCAGATTTTGCTTGACGAGTG

R: TTTCAATACTTGGGTTGCAAAT.

Results

A total of 186 samples were investigated in this study. The ages of patients were varied between (l – 67) years.

Table (1) will show the demographic distribution of patients. According to our study E.histolytica infection is more prevalent in younger age groups. The study also shows that the male was higher prevalence of infection with E.histolytica than female (101 male and 85female) with P value < 0.05).

The results showed that the infection with this parasite was high prevalence in rural than urban areas (97 patients from rural areas and 89 from urban) with P value < 0.05).

Table (1): Demographic distribution of the patients.

		number	percentage
Genders	Male	101	54%
	female	85	46%
Geographic distrebutiobn	Rural	97	52%
	urban	89	48%
Age/ years	< 9	47	25%
	10-19	27	15%
	20-29	20	11%
	30-39	23	12%
	40-49	30	16%
	>50	39	21%

Macroscopically and microscopically, 140 out of 186 stool samples are bloody samples. By microscopic examination revealed that 126 samples were infected with *E.histolytica* (110 (78.5%) out of 140 bloody samples while only 16 (35%) out of 46 non-bloody samples as shown by table 2.

Although the microscopic examination remains the main method for the diagnosis of amoebiasis and is used in most countries, however, it cannot differentiate between E.histolytica and other non-pathogenic species like E.dispar. The present study showed that accuracy of this method in detecting E.histolytica had sensitivity and specificity (91%, 44% respectively) which compared with polymerase chain reaction (PCR).

Table (2): Distribution of E.histolytica according to the type of diarrhea by using macroscopic and microscopic results.

Type of diarrhea	No. of samples	No. of infected samples	%
Bloody diarrhea	140	110	78.5%
Non-bloody diarrhea	46	16	35%
total	186	126	

Table three showed that Eighty nine (48%) out of 186 stool samples were positive for *E.histolytica* by ELISA assay (80 out of 140 bloody samples and 9 out of 46 non-bloody samples) with a significant statistically (P value < 0.01) between type of diarrhea and number of infected patients, this indicates that the bloody diarrhea is highly correlating with E.histolytica. This study also showed that the sensitivity and specificity of ELISA test were (93%), (75%) respectively.

Table (3): Distribution of E.histolytica according to the type of diarrhea by using ELISA results.

Type of diarrhea	**No. of examined samples**	**No. of infected samples**	**%**	**Statistical value**
Bloody dia.	**140**	**80**	**57%**	**P value =0.001**
Non bloody dia.	**46**	**9**	**11%**	
total	**186**	**89**		

The isolated DNA tested by three primers provided by bioneer (korea) which were: GAL/GalNAc–specific lectin, amoebapores C and cysteine proteinase 5, by this analysis, these three proteins were identified successfully in 62 samples out of 186 samples are positive in PCR, out of these positive samples, there are 55 samples positive for lectin (49 of the m are bloody), 58 samples are positive for amoebapore(55 of them are bloody), and all samples (62) are positive cysteine proteinase(58 of them are bloody).(table 4)

The present study detected CP5 successfully, from known cysteine proteinases CP1 and CP5 exist in E.histolytica and not in other nonpathogenic species like E.dispar

Table (4): E.histolytica enzymes distributed among sample by using PCR, N.186.

enzyme	Positive result	%	Bloody samples	%
Lectin (hg13)	55	29.5%	49	89%
Amoebapore-C	58	31.1%	55	95%
Cysteine proteinase 5	62	33.3%	58	93.5%

Table five show the comparison between the results of microscope with the results of the PCR, showed that 126 out of 189(68%) samples positive with microscopically while only 62(33%) of these samples will be positive with PCR, and out of this total numbers there were 58 samples positive with both microscopic examination and PCR, and

there were only 4 samples negative with microscopic but positive with PCR with statistically was highly association (P value < 0.01). Tab.(5,6)

Table (5): A comparison between bloody and non-bloody stool samples according to PCR results.

Type of diarrhea	**No. of examined samples**	**No. of infected samples**	**%**	**Statistical value**
Bloody diarrhea	**140**	**58**	**41%**	**P value =0.001**
Non bloody diarrhea	**46**	**4**	**9%**	
total	**186**	**62**		

Table (6): comparison results of microscopic examination with PCR in detection of E.histolytica.

PCR / microscopic	No. of posative samples	No. of negative samples	total	Statistical value
Positive samples	58	68	126	P value =0.001
Negative samples	4	56	60	
total	62	124	186	

The results of ELISA were compared with the results of the PCR, and showed that the positive cases from the total number of samples; 89(48%) samples positive with ELISA, 62(33%) samples positive with PCR, and out of this total numbers, there were 55 samples positive with both ELISA and PCR, and there were 7 samples negative with ELISA and positive with PCR, with statistically highly significant (P value < 0.01)**.**

In the present study, the primers which used to detect the three virulent factors (Lectin, amoebapores and cysteine proteinase) were calculated specifically for E.histolytica, this makes the PCR sensitivity and specificity (100%) therefore cross reactivity should not occur.

Table (7): ELISA in comparison with PCR in detection of E.histolytica.

PCR / ELISA	positive samples	Negative samples	total	Statistical value
Positive samples	55	34	89	P value =0.001
Negative samples	7	90	97	
total	62	124	186	

Table (8): The validity of different tests in detection of E.histolytica infection in comparison with the validity of PCR (100%).

test	Sensitivity	Specificity	PpV	NpV	Accuracy
Microscope	91 %	44%	45%	91%	60%
ELISA	93%	75%	65%	95%	81%

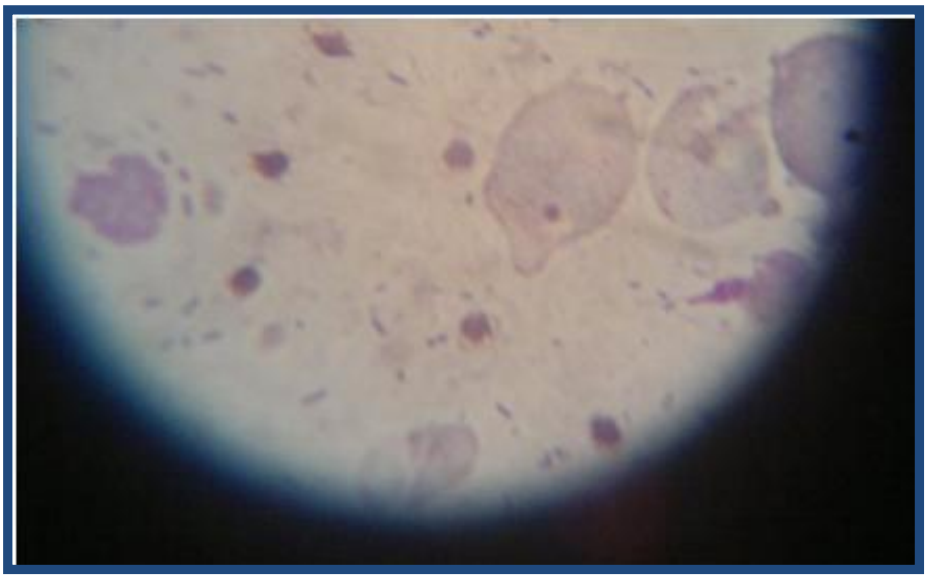

Figure 1: Results of microscopic examination, *E.histolytica*.

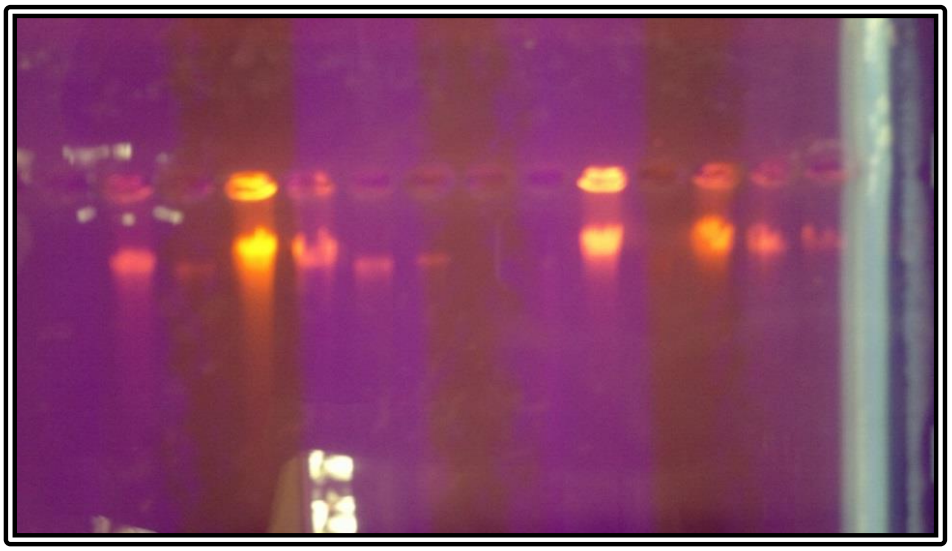

Figure 2: Gel electrophoresis of DNA extraction**.**

Lane 1: shows positive *E.histolytica* DNA result. Lane 2: shows positive *E.histolytica* DNA result. Lane 3: shows positive *E.histolytica* DNA result. Lane 4: shows positive *E histolytica* DNAresult. Lane 5: shows positive *E.histolytica* DNA result.Lane 6: shows positive *E.histolytica* DNA result. Lane 7: shows negative *E.histolytica* DNA result. Lane 8: shows negative *E.histolytica* DNA result.Lane 9: shows positive *E.histolytica* DNA result.Lane 10: shows negative *E.histolytica* DNA result.Lane 11: shows positive *E.histolytica* DNA result. Lane 12: shows positive *E.histolytica* DNA result.
Lane 13: shows positive *E.histolytica* DNA result.

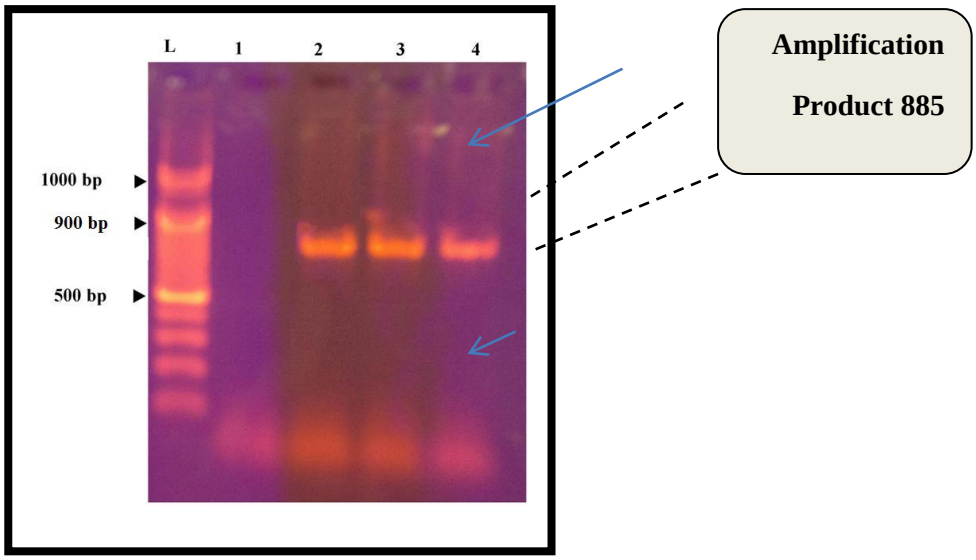

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Figure 3: PCR products of the CP5 gene by using the specific primer.
M: 100 bp standard size reference marker.
Lane 1: *E.histolytica* shows negative result with CP 5 gene.
Lane 2: *E.histolytica* shows positive result with CP 5 gene.
Lane 3: *E.histolytica* shows positive result with C P 5 gene.
Lane 4: *E.histolytica* shows positive result with C P 5 gene.

Discussion

The present study shows that the prevalence of E.histolytica (according to PCR results) is high among children and young adult, this agrees with another study in Erbil done by Hamad and Ramzy et al.[11]. The prevalence of E.histolytica is high among families who eat together from the same plate, among those who eat with their hands, among those who eat away from home, sanitary workers. The results also showed that the infection with this parasite was high prevalence in rural than urban areas which is often influences to peoples with low socioeconomic status (fecal-oral transmission) and may be due to contaminated water in rural areas, this agrees with another study done by Lebbad, SG and Svard[12].

Microscopic examination remains the main method for the diagnosis of amoebiasis and is used in most countries; however, it cannot differentiate between E.histolytica and other non-pathogenic species like E.dispar. The results of this estimation revealed that the amplified DNA has (885 bp) for cysteine proteinase 5. *E.histolytica* secretes proteinases (which are the most important virulence factor.of *E. histolytica*) that dissolve host tissues, kills host cells on contact, and engulfs red blood cells. *E. histolytica* trophozoites also invade the intestinal mucosa, lysing host cells and causing ulcers (amoebic colitis) [6].

The present study showed that the sensitivity and specificity of microscopic examination were 91%, 44% respectively and the accuracy of wet mount (60%) Tab. (4-8), this agrees with study of Al-Oumashi, Haque et al., and Al-Yaquob who proved that microscopy was apparently less sensitive and specific for identifying Entamoeba species than antigen detection test by ELISA and PCR technique[13,14]. These studies revealed that the sensitivity and specificity of wet mount (81.3%), (40%) respectively. The

present study showed that Eighty nine (48%) out of 186 stool samples were positive with ELISA assay; 80 out of 89 samples were bloody, with statistically highly association between type of diarrhea and number of infected patients (P value < 0.01), this indicates that the bloody diarrhea is highly correlating with E.histolytica. The present study showed that the sensitivity and specificity of ELISA test were (93%), (75%) respectively.The use of ELISA has been shown to be useful in routine work. This agrees with study of Hague et al. who proved that this test has demonstrated a good sensitivity and specificity for detection of E.histolytica antigen in stool specimens of people suffering from amebic colitis and asymptomatic intestinal infection [15].

The molecular results were detected by electrophoresis on 1% agarose gel and exposed to UV light in which the DNA appear as compact band. The isolated DNA tested by three primers provided by bioneer (korea) which were: GAL/GalNAc–specific lectin, amoebapores C and cysteine proteinase 5, by this analysis, these three proteins were identified successfully in 55, 58 and 62 samples respectively. The results of this technique revealed that the amplified DNA has (900 bp) for lectin in 55 samples (49 of them were bloody). This study searched for GAL/GAL Nac lectin and the importance of the identification of this protein rests in the probable central role in pathogenesis of amoebiasis, This was agrees with Petri et al.; Haque et al ., whose proved that the adherence of trophozoite to the intestinal mucosa occurs by lectin as the first step in pathogensis[15,16]. Also in this study the amplified DNA showed that the amoebapore C has (928 bp) in 58 samples, 55 of them were bloody, this result due to that amoeabapores produced by E.histolytica trophozoite play critical role in formation of bloody diarrhea because it cause tissue damage and amebic colitis. This would agrees with Andra, J.; Herbst, et al., ;Bracha et al., who improved that E.histolytica trophozoites lacking or decreased amoebapore level shows less virulence[8,9].

The amplified DNA (885 bp) for cysteine proteinasewas estimated in 62 samples 58 of them were bloody. The characterization of the cysteine proteinase (which is the most important virulence factor secreted by E.histolytica) can dissolves host tissues, kills host cells on contact, induce of apoptosis in host target cells[14]. By this mechanism the pathogenic trophozoite are distinct from the harmless nonpathogenic species, this agree with Irvine et al., who proved that the cysteine proteinases(CPs) of E.histolytica play crucial roles in the interactions between parasite and host[17]. The present study detected CP5 successfully, from known cysteine proteinases CP1 and CP5 exist in E.histolytica and not in other nonpathogenic species like E.dispar, this agrees with another study done in Iran by Rostamighalehjaghi et al., who used cysteine proteinase 5 to differentiate between of E.histolytica and E.dispar by using PCR technique, they successfully detect CP5 in E.histolytica but not in E.dispar, and they observed that this method (PCR) have showed high specificity and sensitivity[18].

Conclusion:

Amoebiasis still mentioned as one of the major health problems in tropical and subtropical areas which caused by *E.histolytica* which had recently been re-described as two distinct species; the pathogenic species *E.histolytica* and the nonpathogenic species *E.dispar*. These two species are morphologically similar[4].

E.histolytica can breach the mucosal barrier and travel through the portal circulation to the liver, where they cause abscesses that are 100% fatal if untreated. A variety of virulence factor are currently being investigated to better define pathogenic mechanisms. *E. histolytica* (pathogen) is a distinct species from *E. dispar* (a harmless commensal).This agree with Irvine., *et al*, who proved that the cysteine proteinases of *E.histolytica* play crucial roles in the interactions between parasite and host, including acquisition of nutrients, facilitation of tissue invasion, and defense against immune attack, therefore, the amebic

cysteine proteinases are important targets for novel chemotherapeutic strategies, these cysteine proteinases degrade the host extracellular matrix and muco-proteins, dislodge epithelial cells, and degrade epithelial basement membrane[16]. Similarly with study done by Reed *et al*., who proved that the observation of *E.histolytica* cysteine proteinase gene is presented only in pathogenic isolates of *E. histolytica* suggests that this aspect of virulence in amebiasis is genetically predetermined[19]. The characterization of the CP5 cysteine protease and the induction of apoptosis in host target cells have led to a better comprehension of the mechanisms by which trophozoites can lyse target cells.

From known cysteine proteinases, CP1 and CP5 exist in *E.histolytica* and not in *E.dispar*. This agrees with other study in Iran on the use cysteine proteinase 5 to differentiate between of *E.histolytica* and *E. dispar* by using PCR. They successfully detect CP5 in *E.histolytica* but not in *E.dispar*, and they observed that this method have showed high specificity and sensitivity [17]. Bruchhaus *et al*., have reported that the EhCP1, EhCP2, and EhCP5 enzymes contribute to approximately 90% of the total CP activity from the parasite [9]. They have compared cysteine proteinase genes and their expression in *E.histolytica* and in non-pathogenic twin *E.dispar*. Recently a study has shown that two of CPS genes including ehcp1 and ehcp5 are unique to *E. histolytica*, as the former (ehcp1) is absent and the latter (ehcp5) is nonfunctional in *E.dispar*, a morphologically identical but noninvasive *Entamoeba* species [20]. Identification and differentiation of *E.histolytica* from *E. dispar* has been the most important advancement in intestinal protozoalogy with clinical and epidemiological application. These methods have showed high specificity and sensitivity, although some cannot generally be used in developing countries due to their cost and complicated methodology, recently nucleic acid-based approaches have successfully[15].

Recommendations:

1. this speculation should be proven by the further development of molecular diagnosis such as real time PCR (RT-PCR) for other nonpathogenic Entamoeba species which found in human, such as E.moskoviskii in comparison with E.histolytica, because the RT-PCR quantitative and qualitative improved method for the specific diagnosis of E.histolytica infection.
2. Study the other virulence factors produced by E.histolytica like serine rich protein, actin, caspase, and study the parasite ability to circumvention of the host factors.
3. Proper and effective diagnostic techniques, such as, the use of sensitive and specific methods like ELISA other than microscopic examination and treatment of infected individuals should be encouraged and made available in hospitals and rural health centers.
5. Fundamental studies on the immunity against amoebiasis are essential for evaluating the feasibility of developing an E.histolytica vaccine, and the vaccine will develop with the studies of inhibitions the effect of V.F. by using update technique such as cloning.

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