Real-Time PCR For Detection CP1 & CP5 Virulence Factors Of Entamoeba Histolytica In Patients Stool Samples In Al - Najaf Al- Ashraf Province

التحري عن عوامل الضراوة CP5,CP1 للاميبا الحالة للنسيج في محافظة النجف الاشرف بتقنية التفاعل التسلسلي لإنزيم البلمرة.

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الخلاصة:
الهدف: اجريت هذه الدراسة للكشف عن عوامل الفوعة الرئيسية CP5 , CP1 لطفيلي الاميبا الحالة للنسيج المعوي Entamoeba histolytica وتشخيصها باستخدام تقنية التفاعل السلسلي لانزيم البلمرة (Real-Time- PCR).
المنهجية: اجريت هذه الدراسة على 678 من المرضى الوافدين وثلاثين من الافراد الاصحاء كمجموعة سيطرة الذين زاروا مستشفى مدينة الصدر الطبية ،مستشفى الزهراء التعليمي ،مستشفى السجاد، مستشفى الفرات ،مستشفى الحكيم ومستشفى المناذرة وكذلك المختبرات الاهلية في محافظة النجف الاشرف خلال الفترة من كانون الاول 2012 ولغاية نهاية شهر حزيران 2013.
النتائج: أظهرت نتائج الكشف عن العوامل الممرضة الرئيسية وهي السيستين بروتينيز1 (CP1) السيستين بروتينيز5 (CP5) لطفيلي الاميبا الحالة للنسيج بأجراء تقنية التفاعل السلسلي لانزيم البلمرة (Real-Time- PCR) وباستخدام بادئات محددة لطفيلي الاميبا الحالة للنسيج واظهرت النتائج ان 40 عينة من اصل 162 عينة كانت ايجابية الفحص ومن هذه العينات الايجابية الفحص كانت 16 عينة ايجابية الفحص للسيستين بروتينيز1 (CP1) و 24 عينة كانت ايجابية الفحص للسيستين بروتينيز5 (CP5) .
الاستنتاجات : من اهم عوامل الفوعة الرئيسية لطفيلي الاميبا الحالة للنسيج المعوي هي السيستين بروتينيز1 (CP1) السيستين بروتينيز5 (CP5).
التوصيات: اجراء دراسات اضافية للتشخيص الجزيئي بواسطة تقنية التفاعل السلسلي لانزيم البلمرة (Real-Time- PCR) لانواع الاميبا الغير ممرضة في الانسان .

Abstract
Objective: It aims to determine the genes characterization of CP1 and CP5 pathogenic factors of E.histolytica parasitic in stool samples of patients **in AL- Najaf Al Ashraf Province**
Methodology: This study was carried out for 6 months from the first of December 2012 till the end of June 2013The samples collected from Al-Sadar teaching hospital, AL-Zahra maternity and children hospital, AL-abasia hospital, AL-Forat hospital ,AL-Hakime hospital ,AL-Manathera hospital and private clinic in Al- Najaf governorate.
Results: To detect the major virulence factors (V.F.) cysteine proteinase1(Cp1), cysteine proteinase5(CP5) of E.histolytica, Real -Time PCR technique was conducted, by using specific primers for E.histolytica, the results showed that 40 samples out of 162 were positive, out of these positive samples, there were 16 samples positive for C P1, 24 samples were positive for CP5.
Conclusion: CP1 & CP5 is one of the most important virulence factors in E.histolytica such as toxin like activity.
Recommendation: further study development of molecular diagnosis such as real time PCR for other non-pathogenic 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.
Keyword: Entamoeba histolytica Real-Time PCR, cysteine proteinase.

INTRODUCTION:

Entamoeba histolytica is a unicellular protozoan parasite that infects about 45-50 million people each year, causing 40 thousand to 100 thousand deaths annually and may cause potentially life-threatening diseases such as hemorrhagic colitis and/or extraintestinal abscesses (1).

The genus *Entamoeba* consists of several species. Among them, six (*E. histolytica, E. dispar, E. moshkovskii, E. polecki, E. coli* and *E.hartmanni*) reside in

the human intestinal lumen. Another species, *E. gingivalis* was identified in the human oral cavity (2).

E. histolytica is the only species definitely associated with pathological manifestations in humans (3,4) . The others are considered nonpathogenic .

As *E. moshkovskii*, *E. histolytica* and *E. dispar* are morphologically indistinguishable; it is not possible to differentiate the three species on the basis of traditional microscopic examination. In the identification of *E. histolytica*, new approaches are used, based on detection of *E. histolytica* specific antigen and DNA in stool and other clinical samples.

Molecular diagnostic tests, including conventional and real-time PCR, have been developed for the detection and differentiation of *E. histolytica*, *E.dispar*, and *E. moshkovskii* in clinical samples. (5,6) .

The PCR method is a powerful tool for the genetic typing of different amoebic strains. Together these two methods contributed in both improved clinical diagnosis and treatment of amoebiasis, and a greater understanding of the epidemiology of *E. histolytica*. 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*. **(**7).

In the present study, we have examined a molecular method with a Real Time-PCR for amplification of a part of CP1 gene and a part of CP5 gene enabling us to study the features of this pathogenic factors and to differentiate the pathogenic species, *E. histolytica*, from the non-pathogenic species, *E. dispar* .

MATERIALS AND METHODS

This study was carried out for 6 months from the first of December 2012 till the end of June 2013 The samples collected from (Al-Sadar teaching hospital, AL-Zahra maternity and children hospital, AL-abasia hospital, AL-Forat hospital,AL-Hakime hospital,AL-Manathera hospita and private clinic) in Al- Najaf governorate. (678) samples of stool were included in this study, 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, and asking about clinical symptoms like abdominal pain, presence of blood in stool, appetite, dryness and fever)) of unknown etiology, no patient was treated prior to specimen collection.

Stool samples were taken from each patient, fresh unpreserved stool samples were collected in sterile containers for microscopic examination (wet mount), the specimen containers were labeled with name and age of the patients, the specimens were examined with in 30 minute for three times.

(162) stool samples positive for *E. histolytica/E. dispar* (one year-70years old) using microscopic examination (Wet mount,) were tested at the laboratories.

Positive Samples were quickly frozen for DNA detection by Real time PCR, and kept at _20°C prior to analysis.

Extraction of DNA from stool samples:

Procedure of DNA Extraction from Stool According to (Bioneer) Kit (Korea).

Specific Primer Sequences used for PCR Amplification

The *E. histolytica* detection primers used in this study previously evaluated by (Bioneer-Korea) and are listed in table (1) .

Table (1): The specific primers and their sequences

Primer	DNA Sequence		References
Cysteine-proteinase 1(CP1)	**F**	**5'-ATT GAT TTC AAT ACA TGG GTT-3'**	**(Melendez–Lopez,2007)**
	R	**5'-GAG ATA TTC AAC ACC AGT TGG-3'**	
Cysteine-proteinase 5(CP5)	**F**	**5' GTT CACTGTCTCGTTATTAG 3'**	**(Rostamighalehjaghi *et al* ., 2010).**
	R	**5' CATCAGCAACCCCAACTG 3'**	

Molecular technique

Protocol

1. Lyophlized, master mix with Sybr Green

PCR F- Primer(10 pmole)	2 µ l
PCR R- Primer(10 pmole)	2 µ l
DNA Template	10 µ l
DEPC-distlled water.	6µ
Total sumation	Adjust to 20 µ l

2. Seal the Optical adhesive film for real – time PCR on tube
3. Completeiy mix by vortexing for resuspension of premix pellets.
4. Centrifuge at 3,000 rpm, for 2 min
5. Start Real-Time PCR instrument and load it
6. Program the PCR setting

Step	Condition	Cycle
Pre-Denaturation	**95 ^{0}C, 5 min**	**1**
Denaturation	**95 ^{0}c 20 sec**	**40**
Annealing/Extension	**52 ^{0}c 40sec for CP1**	
Detection (Scan)	**59 ^{0} c 40sec for CP5**	
Melting		**1**

7. After reaction is completed, perform data analysis.

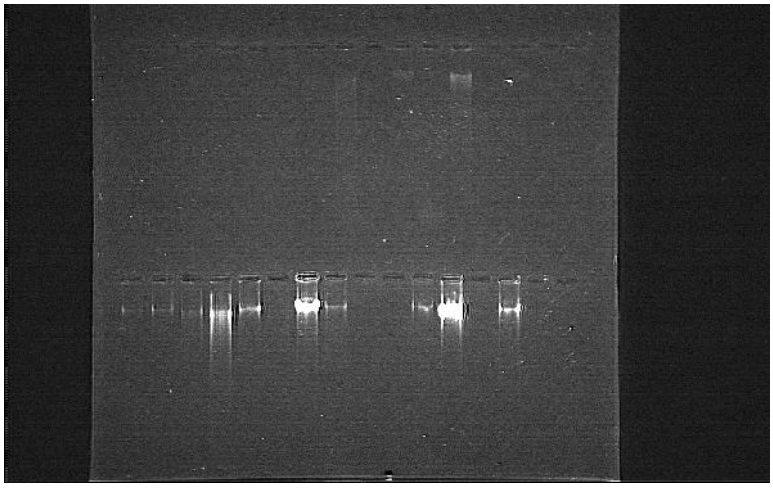
Melting Curve Analysis

After completion of (45) cycles PCR amplification , the PCR products were melted by raising the temperature from (50 Cº) to (95 Cº) at rate (1Cº/min.) .The Exicycler thermal block soft ware displayed the data collection during melting curve analysis as result melting temperature were derived from melting peaks by melting curve analysis of the amplified DNA specimens.

RESULTS

Molecular Test (PCR) FOR Gel electrophoresis of DNA extraction from stool samples.

The molecular results were detected by electrophoresis on 1% agarose gel and exposed to UV light in which the DNA appear as compact band (Pic.1).



Picture (1): Gel electrophoresis of DNA extraction from stool samples. Lanes 1-16 show positive DNA results.

Amplification of CP1 gene of *E. histolytica* with Sybr Green Using primers CP1

Amplification of the gene CP1 can be determined by a serving the fluorescence emission curves , the fluorescence curve which exceed the threshold line represent the amplified CP1 gene of *E. histolytica*.The negative samples did not amplified and the fluorescence curves remained below the threshold line.

Different types of melting peak of *E. histolytica* for gene and with Sybr Green.This graph represent the amplified DNA products as distinct melting peaks with specified melting temperature.(Fig.1)

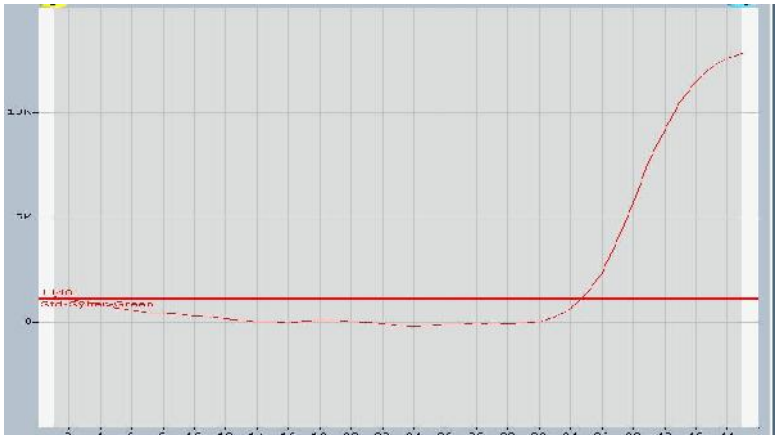


Fig.(1) .Amplification of CP1 gene of *E. histolytica* with Sybr Green Using Real-Time PCR

Amplification of CP5 gene of *E. histolytica* with Sybr Green Using primers(CP5)

Amplification of the genes CP5 can be determined by a serving the fluorescence emission curves , the fluorescence curve which exceed the threshold line represent the amplified CP5 gene of *E. histolytica*.The negative samples did not amplified and the fluorescence curves remained below the threshold line.

Different types of melting peak of *E. histolytica* for gene and with Sybr Green.This graph represent the amplified DNA products as distinct melting peaks with specified melting temperature. Fig.(2)

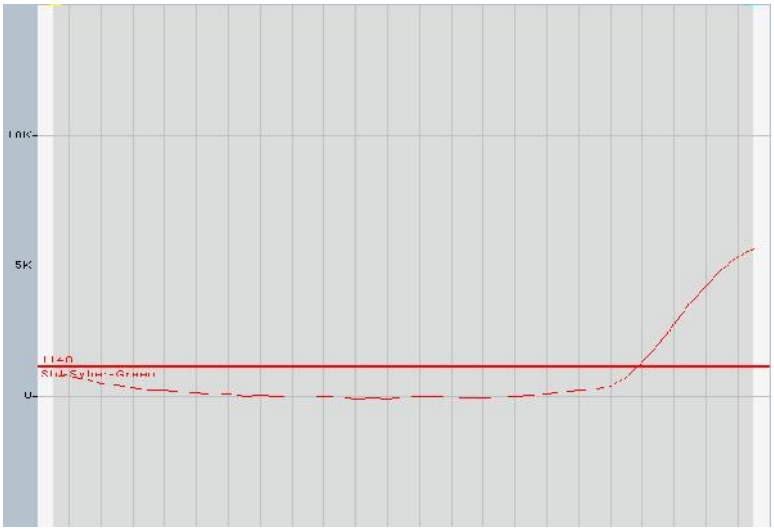


Fig.(2) .Amplification of CP5 gene of *E. histolytica* with Sybr Green Using Real – Time PCR

The curves were produced due to binding Sybr Green to the PCR Sybr green is a double strand binding dye and when it bind to double strand DNA , the Fluorescence emission occurs which is used for the visualization of amplified product during real-time PCR of CP1,CP5 Gene with Sybr Green the amplification of each DNA specimen was determined by observation the Fluorescence emission curve (Fig.4-6), these curves were produced due to binding Sybr Green to the PCR product, and the Fluorescence reading were taken after each extension step of real-time PCR.The Fluorescence emission increases due to the increase cycles of PCR. Fig.(3)

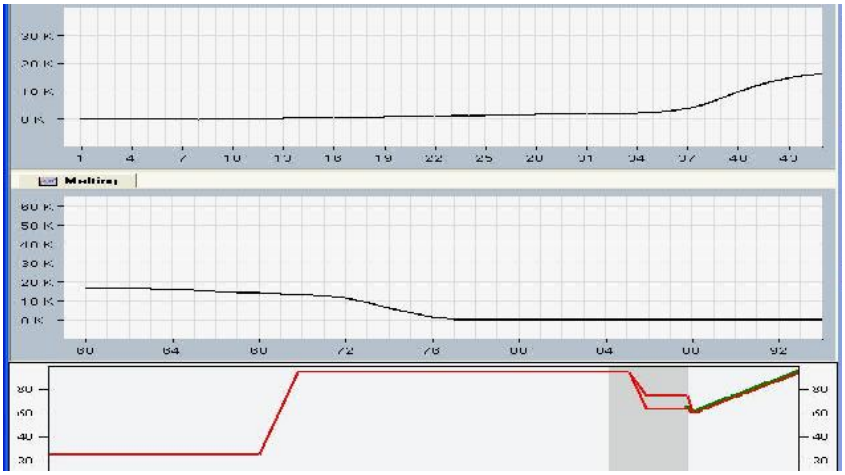


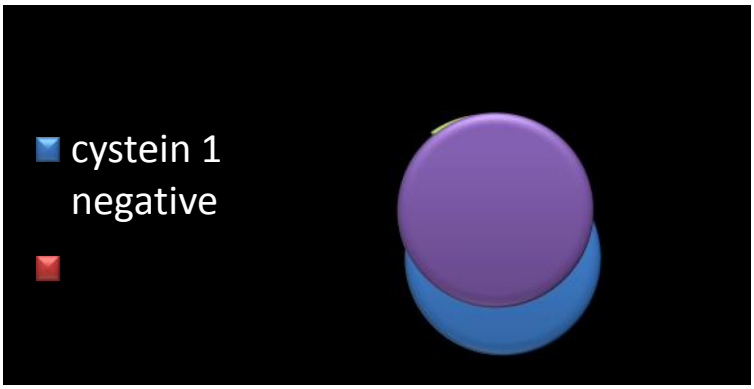
Fig.(3) the Fluorescence emission curve product

***E.histolytica* enzymes distributed among sample by using Real- Time PCR**

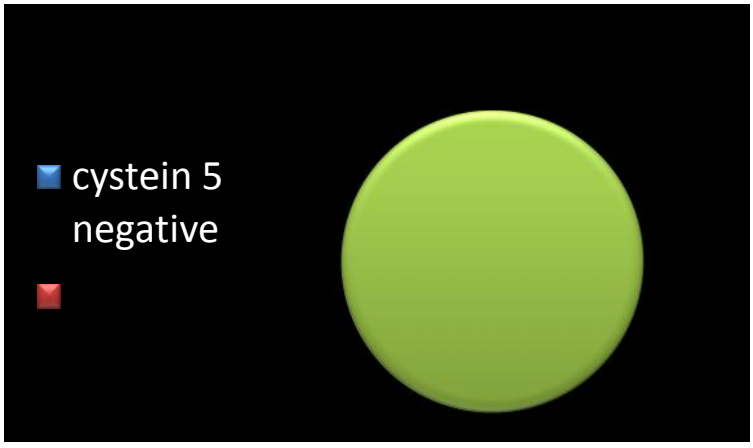
The table (2) shows that 40 samples out of 162 samples are positive in Real- Time PCR, out of these positive samples, there are 16 samples positive for Cysteine proteinase 1 , 24 samples are positive for Cysteine proteinase **5. Fig.(1)and(2)**

Table (2): E.histolytica enzymes distributed among sample by using Real- Time PCR

Enzyme	Positive **samples**	%
Cysteine proteinase 1	16	9.9
Cysteine proteinase 5	24	14.8
Total	40	24.7



fig(4):*E.histolytica* enzymes Cysteine proteinase 1 distributed among sample by using Real- Time PCR



fig(5):*E.histolytica* enzymes Cysteine proteinase 5 distributed among sample by using Real- Time PCR.

DISCUSSION

The results of this estimation revealed that the amplified DNA has for cysteine proteinase in 40 samples. The characterization of the cysteine proteinase (which is the most important V.F. secreted by *E.histolytica*) have led to a better understanding the pathogenic mechanisms of *E.histolytica* (pathogen) are distinct species from the nonpathogenic (a harmless commensal). By this mechanism the trophozoites can

dissolves host tissues, kills host cells on contact, induce of apoptosis in host target cells and engulfs red blood cell. *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. Amoebic liver abscesses grow inexorably, and at one time were almost always fatal, but now even large abscesses can be cured by one dose of antibiotic. This agree with (8) who proved that the cysteine proteinases(CPs) 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 proteinase is important targets for novel chemotherapeutic strategies, this cysteine proteinase degrade the host extracellular matrix and muco-proteins, dislodge epithelial cells, and degrade epithelial basement membrane, similarly with (9) who proved that the observation of *E.histolytica* cysteine proteinase gene is presented only in pathogenic isolates suggests that this aspect of virulence in amoebiasis is genetically predetermined

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 (10).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, and disagrees with another study done by (11) that the EhCP1 is a major released cysteine proteinase of invasive *E. histolytica*, which is important in both invasion and disruption of the host response.

CONCLUSIONS

1. Molecular tool by Using Real-Time Polymerase Chain Reaction is a perfect methods that differentiate between *E.histolytica and E.dispar*
2. Cysteine proteinase is one of the most important virulence factors in *E.histolytica* such as toxin like activity. The characterization of the CP5 cysteine protease has led to a better comprehension of the mechanisms by which trophozoites can lyse, and induce the apoptosis in host target cells.

RECOMMENDATION:

1-further studies mast be carried on the virulence strains of *E.histolytica* to determine the strain which more prevalence in Iraqi.
2. further study development of molecular diagnosis such as real time PCR (RT-PCR) for other non-pathogenic *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.
3. 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.

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