IRAQI Academic Scientific Journals
Samarra Journal of Pure and Applied Science
SJPAS
www.sjpas.com
p ISSN: 2663-7405
e ISSN: 2789-6838

Role of Fecal calprotectin and P-Selectin in intestinal Amoebiasis

Saif Lokman Mohammed*, Ali Mohammed Abed
Department of Biology, College of Science, Tikrit University,Tikrit,Iraq

https://doi.org/10.54153/sjpas.2025.v7i1.875

Article Information
Received: 05/04/2024
Revised: 11/05/2024
Accepted: 20/06/2024
Published: 30/03/2025

Keywords:
Entamoeba histolytica1, *ELISA 2*, *Fecal calprotectin 3*,*P-selectin4*, *PCR5*.

Corresponding Author
Gmail (Saiflokman.1985@gmail.com)
Mobile: 07740939902
E-mail: am.abdnasir@tu.edu.iq
Mobile: 07703754666

Abstract

Entamoeba histolytica a gastrointestinal protozoa causes amoebiasis, which is spread by contaminated water and food. This study aims to diagnose amoebiasis by using the direct wet smear method and PCR assay to detect E.histolytica and other species, as well as investigate levels of some inflammation markers such as fecal calprotectin and P-selectin in sera patients.**:** A total of 563 stool samples were collected from patients who attended Ibn Al-Atheer Hospital, Al-Khansaa Hospital, and Al-Salam Hospital in the governorate of Nineveh suffered diarrhoea (infants and children) in the environment during the period extending from June 2023 to November 2023. The result reversed that out of 563 stool specimens examined by direct smear 100 (18%) the infected with amoebiasis cyst or/and Trophzoite found in fecal samples varying from semi-liquid diarrhea (watery or bloody, mucous together). The PCR technique recorded a rate of 74 (74%) on faecal samples. Infection with the parasite *E.histolytica*. Out of a total of 100 samples that were positive by microscopic examination, the remaining 26 (26%) samples that were negative by PCR technology may belong to other species of the *Entamoeba* genus. There was a considerable increase in mean concentrations fecal calprotectin and P-selectin in serum patients .fecal calprotectin levels concentration 200.8±96.6ng/ml, compared to the control group, whose average concentration 39.1±15.3ng/ml. Similarly, P-selectin levels in patients were significantly higher compared to the control group, with a mean of 241.8±102.9ng/mL, and 51.2±16.2ng/ml, respectively. The study showed that Amoeba histolytica is a common pathogen that causes diarrhea among children and infants, and high levels of some intestinal inflammatory indicators in the serum of patients.

Introduction:

The protozoan parasite *Entamoeba histolytica* is the causative agent of invasive amoebiasis, a disease that represents a major health problem in subtropical and tropical areas as well as in returnees from amebiasis endemic region[1] A greater proportion of those infected do not exhibit any symptoms, and this condition can last up to a year. The illness can be fatal even before symptoms show up because it can cause diarrhea, which can result in severe dehydration[2,3] and to improve breakdown, amoeba pore protein and cysteine proteinases are discharged into the phagosome[4] To thrive, *E. histolytica* needs much iron, which it can get from host proteins such transferrin, ferritin, haemoglobin, and lactoferrin.

ferritin is endocytosed by clathrin-coated pits in *E. histolytica* trophozoites, and specific cysteine proteases subsequently degrade ferritin in the endosome/lysosome pathway[5] is regarded as the third most common cause of death from protozoan parasites[6] .

The World Health Organization (WHO) estimates that 500 million people may have *Entamoeba* infections globally; only 10% of those cases are expected to involve the species *E. histolytica*, with the remaining cases presumably involving nonpathogenic species. Each year, amoebiasis results in 40,000–100,000 deaths[7] The trophozoites, particularly those of *E. histolytica*, have the ability to become highly pathogenic and invasive. They initiate the destruction of the protective mucous-epithelial barrier, leading to an excessive production of mucus, the death of host cells, and the onset of inflammation. This sequence of events ultimately results in the development of amoebic colitis[8].

The diagnosis of parasites can be conducted through microscopic examination, specifically by identifying trophozoites or cysts in the fecal. However, this method cannot differentiate between the cysts and trophozoites of *E.histolytica* and other similar species such as *E.dispar* and *E.moshkovskii*. Additionally, the presence of other amoebae cysts, such as *Endolimax and Iodamoeba,* further complicates the diagnosis.[9,10].

Fecal Calprotectin is a highly abundant protein that binds to calcium. It is a member of the S100 family and is mostly produced by neutrophils. It is also produced to a lesser amount by monocytes and reactive macrophages[11] Calprotectin makes up 5% of the total protein and 60% of the cytosolic protein in human neutrophils[12] This substance possesses qualities that inhibit the growth of bacteria and fungus. Additionally, research has demonstrated that levels of plasma calprotectin significantly rise of 5 to 40 in the presence of infectious and inflammatory situations.[13] Significantly increased concentrations of calprotectin have been identified in the stool of individuals suffering from Inflammation occurring within the large intestines [14].

Produced P-selectin is stored in alpha granules of platelets and endothelium-specific storage granules. When activated, it expression and show to the platelet and endothelial cell surfaces,[15] It is thought to play a major role in adhesive interactions during inflammation, particularly between granulocytes, platelets, and vascular endothelial cells (specifically, those in post-capillary venules). It is increased on the cell surface during cell activation[16] The study's findings showed that, compared to the control group, The concentration of serum p-selectin is notably elevated in individuals afflicted with intestinal parasitic infection. which calls for higher expression of cell adhesion molecules to fulfil their function in attracting effector cells to the infection site. Several studies have highlighted the significance of P-selectin in leukocyte homing to the inflamed tissues[17]

Materials and working methods:

Sample collection

During the period from July 2023 to 2023 November, a study was conducted involving the collection of fecal and serum samples from 563 children with diarrhea, aged between 1 day and 10 years, of both genders. The questionnaire form included in-kind observations including sample size, sample color, and presence of blood or mucus. The samples were obtained from Ibn al-Ather hospital, El Khansa Educational Hospital for Births and Children, and Al salam Educational Hospital in Nineveh Governorate.

Microscopic Examination:

The microscopic analysis where take a quantity of feces using a wooden stick, then place it on a glass slide with a glass cover over it and examine at10X then 40x magnification used wet mount treated with normal saline or a Lugol's iodine stain 1%, revealing the presence of a cyst or trophzoite *E.histolytica*.[18] .**Fig .1 and. 2**

Molecular Assay:

DNA Extraction

DNA extractions were performed with genomic DNA mini kits(Genaid). The first technique used was physically using 2 small glass beds to distract the parasite membranes. The second method followed the steps of the kit, which included distracting the parasite membrane enzymatically by proteinase K.[19] The precipitate is stored at -20 ℃.

Assessment of Concentration and Purity of DNA extracted

Modern molecular laboratories place a premium on both efficient DNA extraction methods and the precise measurement of nucleic acid concentration and purity. To conduct the DNA quantification and purity tests, a fraction was taken from the final amount of 100 µl of extracted and processed DNA. Spectrophotometric analysis was performed on the isolated genomic DNA to evaluate its DNA yield (ng) and purity (absorbance ratio at 260/280; according to the guidelines laid forth[20] . The highest concentration was taken along with the lowest concentration and also for purity.**Table:1**

Table:1 Data Concentration and Purity of DNA extracted

Nanophotometer Analysis		
Number sample	High	Low
Concentration (ng/µL)	584.8	3.9
Purity (A260/A280)	1.9	1.4

PCR Assy:

Primers for polymerase chain reaction (PCR) assays targeting certain *Entamoeba* genera were designed using sequences from the small-subunit rRNA gene. The *Entamoeba* genus-specific primers were prepared from (Microgen Company, Korea) The primers used were gen target 18S ribosomal RNA gene Entamoeba histolytica, primers names(EntaF,EhR) Sequence (5'-3') size for EntaF:5´-GTT GAT CCT GCC AGT ATT ATA TG- 3´and) Sequence (5'-3') size for EhR:5-´CAC TAT TGG AGC TGG AAT TAC-3´ which generate 550 bp [21].PCR amplifications unique to each genera were carried out in a final volume of 25 includes 12.5 µl of PCR buffer, 0.5 µl of each forward and reverse primer Entam1, 0.5 µl of DNA material, and 8.5 µl of free water nuclease. A thermocycler (Px2 Thermal Cycler, thermoHybaid, UK) PCR System was used to conduct the reactions. Samples were first denatured for five minutes at 94˚C. They were then heated in 35 cycles for one minute at 94˚C, one minute at 55˚C, and one minute at 72˚C. Finally, they were extended for seven minutes at 72˚C. amplicons derived from genus-specific and single-round PCR methodologies were subjected to electrophoresis in a 1.5% agarose matrix and subsequently observed under a gel documentation system after staining with 0.5 µg/ml of ethidium bromide(22).

3-Fecal calprotectin and P-Selectin ELISA test:

The concentrations of fecal calprotectin and P-selectin in the serum were determined using the enzyme-linked immunosorbent assay.. Sixty serum patients and thirty serum controls were included in the study using 96-well plates for the Enzyme-Linked

Immunosorbent Assay (ELISA) obtained from BT LAB China. The ELISA plates were pre-coated with antibodies against fecal calprotectin and P-selectin. The samples were added to the plates, and the color development in the substrate solution correlated with the level of Fcal and P- selectin. After applying a stop solution, the process was halted, and the absorbance was measured at 450 nm. both serum p-selectin and fecal calprotectin antibodies.

Statistical analysis

To perform statistical analysis, IBM SPSS software program, version 26.0 was used. Frequencies and percentages were used to present categorical information. The format for continuous variables was mean ± SD. Values of $P \leq 0.05$ were regarded as significant by using the t test, The program Graph Pad Prism 10.a was used to create the graphs.

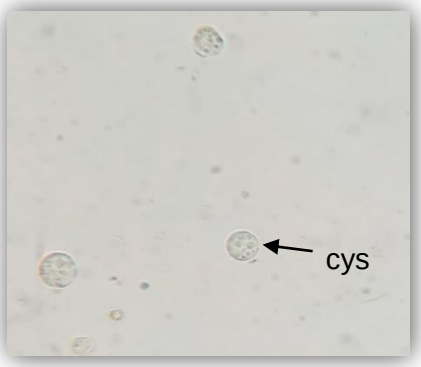
Results and Discussion

Diagnosis of Entamoeba by Microscopic Examination

The total number of this study was 563, 100 sample (18%) test result was positive for microscopic light infection of *Entamoeba*, Uninfected samples (82%) **Table:2**

Table:2 Diagnosis of *Entamoeba* by Microscopic Examination

Total number examined	positive		negative	
	NO	%	NO	%
563	100	12%	463	82%



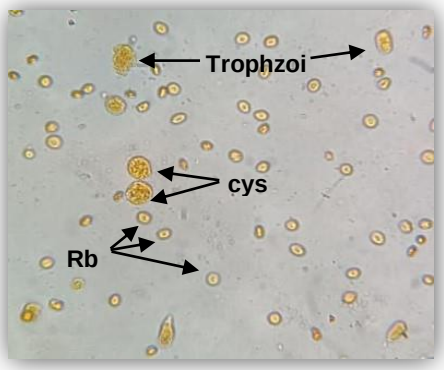


Fig.1 *show cyst of by wet smear normal saline(0.9%)*

Fig.2 *cyst&Trophzoite by Lugol's iodine (1%)*

Diagnosis of *Entamoeba histolytica* by PCR assay:

the PCR technique on 100 samples diagnosed with an optical microscope, only 74 samples were found to contain DNA of the specific genus *Entamoeba*.**Table:3, Fig.3**

Table .3 Diagnosis of *Entamoeba histolytica* by PCR assay**:**

Total number examined	positive		negative	
	NO	%	NO	%
100	74	74%	26	26%

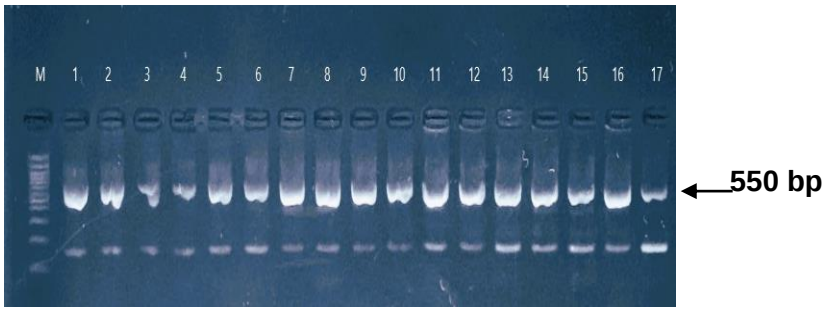


Fig .3 *Diagnosis of Entamoeba histolytica by PCR assay DNA electrophoresis on 2% agarose gel*

Serum fecal calprotectin level in patients and comparison groups:

The study observed a significant advancement in the average concentration of Fecal calprotectin in infected children (Mean ± SD200.8±.96.6ng/mL) compared to the control group (39.1±15.3ng/mL) **Table:4 and Fig.4**

Table:4 Serum Fecal calprotectin level in patients and control groups:

fecal ng/mL	Patients N= 60	Control N= 30	P value
Mean± SD	200.8±96.6	39.1±15.3	0.001*
P value is high significant at P≤ 0.05*			

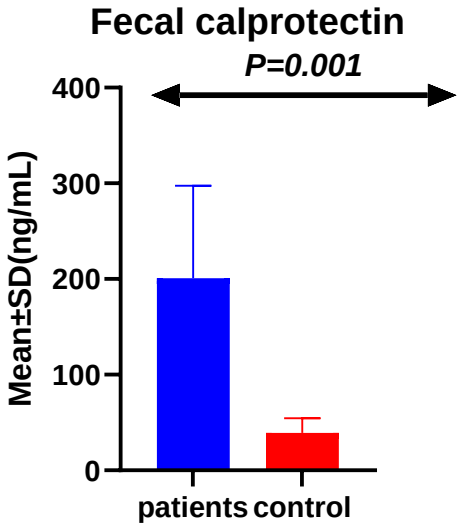


Fig. 4 *concentration serum Fecal calprotectin in patients and control groups*

Serum P-selectin level in patients and comparison groups:

The study observed a significant increase in the average concentration of P-selectin in infected children (Mean ± SD 241.8±102.9 ng/mL) compared to the control group (51.2± 16.2 ng/mL)**. Table:5 and Fig.5**

Table.5 Serum P-selectin level in patients and control groups:

P-selectin ng/mL	Patients N= 60	Control N= 30	P value
Mean± SD	241.8±102.9	51.2±16.2	0.001*
P value is high significant at P≤ 0.05*			

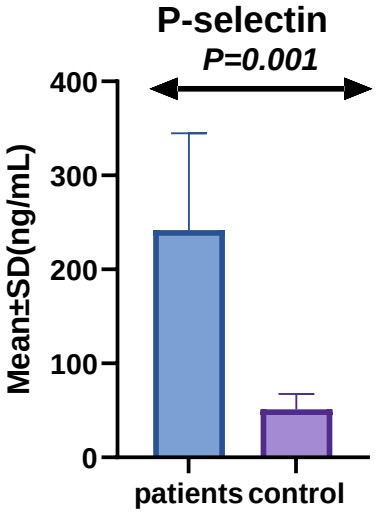


Fig .5 *concentration serum P-selectin in patients and control groups*

Discussion

The current study recorded an *E. dispar* / *E. histolytica* infection rate of 12% out of a total of 563 stool samples examined microscopically by direct wet smear, as shown in Table 1. This percentage is almost in agreement with was recorded by [22][23][24]and did not agree with all of [25][26]The difference in the ratios of the values recorded in the current study compared to previous studies may be due to the difference in several factors, including the level of sanitation, personal hygiene, population density, geographical location, climatic conditions, the total number of samples examined, the extent of accuracy and skill of the sample examiner, and diagnostic methods.

In this study, the infection rate of *E. histolytica* was 74% of the total of 100 positive samples by microscopic examination using PCR, as in Table No. 2, which is a percentage that agrees with was recorded [27][28]and did not agree with was recorded[29][30] The reason may be due to the difference in methods for extracting DNA from stool samples, the type of PCR assay, and the type of targeted genes, the difference in the amount of the parasite present in the stool samples, or the parasite may be spreading irregularly in countries of the world due to differences in climatic conditions and customs and traditions. The negative samples may be other non-pathogenic species such as *E.dispar* or *E.moshkivishi*, or there are contaminants and materials that inhibit PCR technique in the fecal samples.

The current study shed light on the level of Fecal calprotectin in the serum of patients with amoebic dysentery compared to the control group, as a highly significant increase in the average concentration was observed compared to the control group. The results of this study

agree with a previous study in Kirkuk - northern Iraq conducted by Ali and Muhammad 2018 [23], and also the study of Zainab Al-Salhi and Zainab Muhammad (2020) in Thi- Qar, southern Iraq [31]. While it does not agree with Hustvik et al. 2011 in Kampala [32]. As well as With Holtmann *et al.,* 2016. FC elevated in the serum of patients due to the immune response, and thus neutrophil blood cells are attracted to the site of infection, and thus its level increases in serum and feces [33]. As for the reason for the decrease in FC production, it may be due to some genetic diseases (leukemia, hyponemia, or Crohns disease) or taking some inhibitory medications. [34]

The results showed a significant increase in the average concentration of P-selectin in the serum of amoebic dysentery patients compared to the healthy control group, as it agrees with Al-Hadrawi *et al*. 2022 [35]and also with Issekutz.AC and Issekutz.TB 2002 [17].It is possible that P-selectin is the main endothelial selector that attracts and recruits leukocytes to the site of chronic inflammatory lesions. According to the results of a previous study, patients with intestinal parasitic infections had significantly higher serum concentrations of p-selectin than those in the control group. It may be due to the host immune response to infection and inflammation, which necessitates increased expression of cell adhesion molecules to fulfill their role in recruiting effector cells to the site of infection. A study was also conducted on amebiasis and giardiasis, which showed a decrease in its level in the blood serum of people with diarrhea [36]. Many studies have indicated that p-selectin facilitates the migration of T cells to the site of infection and inflammation.

Conclusion:

The study showed that Amoeba histolytica is a common pathogen that causes diarrhea among children and infants, and high levels of some intestinal inflammatory indicators in the serum of patients.

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IRAQI Academic Scientific Journals

Samarra Journal of Pure and Applied Science

SJPAS

www.sjpas.com

p ISSN: 2663-7405
e ISSN: 2789-6838

دراسة كالبروتكتين البراز وبي- سيليكتين في داء الأميبات المعوي

سيف لقمان محمد*، علي محمد عبد

قسم علوم الحياة، كلية العلوم ، جامعة تكريت ، تكريت ، العراق

معلومات البحث:

تأريخ الاستلام: 2024/04/05
تأريخ التعديل: 2024/05/11
تأريخ القبــول: 2024/06/20
تأريخ الـنشر: 2025/03/30

الكلمات المفتاحية:

الكلمة المفتاحية 1 المتحولة الحالة للنسج، ELISA 2، 3كالبروتكتين البرازية ، P-selectin 4، PCR5.

معلومات المؤلف

الايميل
(Saiflokman.1985@gmail.com)
رقم الهاتف:07740939902
الايميل
am.abdnasir@tu.edu.iq
رقم الهاتف:07703754666

الخلاصة:

المتحولة الحالة للنسيج هي ابتدائيات معوية معدية تسبب داء الأميبات الذي ينتقل عن طريق الماء والغذاء الملوث، الأهداف: هدفت هذه الدراسة إلى تشخيص داء الأميبات باستخدام طريقة المسحة الرطبة المباشرة وتقنية ال PCR لأجل كشف وتمييز طفيلي الاميبا الحالة للنسيج وأنواع اخرى، وكذلك تقييم مستويات بعض موشرات الالتهاب مثل الكالبروتكتين البرازى والـ P-selectin في عينات مصل المرضى. المواد والطرق: جمعت 563 عينة غائط من الاطفال والرضع الذين يعانون من الاسهال المراجعين الى مستشفيات ابن الأثير والخنساء و السلام في محافظة نينوى خلال الفترة الممتدة من حزيران 2023 إلى تشرين الثاني 2023.النتائج: اظهرت النتيائج من مجموع 563 عينة غائط فحصت بالمسحة المباشرة (18%) نسبة الإصابة بالطور الناشط و/او المتكيس وجدت في عينات غائط متفاوتة من إسهال شبه سائل (مائي أو دموي ومخاطي معاً). سجلت تقنية ال PCR على عينات غائط نسبة 74 (74%). إصابة بطفيلي .E.histolytica من مجموع 100 عينه موجبة الفحص المجهري، أمّا باقي 26 (26%) عينه سالبة بتقنية ال pcr قد تعود لانواع أخرى من جنس الانتيميبا. أظهرت الدراسة ارتفاع معنوي لكل من الكالبروتكتين البرازية والـ P-selectin في مصل المرضى مقارنع مع مجموعة السيطرة وكما مبين على التوالي. تركيز مستويات الكالبروتكتين البرازية 200.8 ± 96.6 نانوغرام / مل، مقارنة لمجموعة السيطرة التي بلغ متوسط تركيزها 39.1±15.3 نانوغرام/مل. وبالمثل، كانت مستويات P-selectin في المرضى أعلى مقارنة بالمجموعة الضابطة، بمتوسط 241.8 ± 102.9 نانوغرام / مل، 51.2 ± 16.2 نانوغرام / مل، على التوالي. الاستنتاجات: أظهرت الدراسة أنّ الأميبا الحالة للنسيج ممرض شائع مسبب لاسهال بين الأطفال والرضع، وارتفاع مستوى بعض الموشرات الالتهابية المعوية في مصل المرضى.