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RELATIONSHIP BETWEEN FECAL CALPROTECTIN AND OTHER IMMUNOLOGICAL PARAMETERS IN DIARRHEAL CHILDREN INFECTED WITH *ENTAMOEBA HISTOLYTICA* AND PATHOGENIC BACTERIA IN THI-QAR PROVINCE, IRAQ

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Abstract

Diarrhea is one of the leading causes of illness and death in infants and children throughout the world. The present study amid to measure Fecal calprotectin (FC) and other immunological parameters as total and deferential WBC count, C-R reactive protein and ESR in diarrheal children under five years old infected with *Entamoeba histolytica* and pathogenic bacteria in Thi-Qar province. The study carried from November 2018 to April 2019, stool samples collected from 614 diarrheal children attending into Bint Al-Huda teaching hospital for children and women and Al-Mousawi hospital. The stool samples of patients infected with *E. histolytica* were cultured and tested by Fecal calprotectin (FC), Blood samples were tested for some hematological tests (CRP, ESR, WBC, RBC, PLT, Hb, HCT, MCV, MCH, MCHC). The result showed 100 out of 614 (16.4%) of stool samples from patients under five years suffer from diarrhea were infected with *E. histolytica*, 27 samples of patients infected with *E. histolytica* only. While *E. histolytica* and pathogenic bacteria were detected on 73 samples including: *E. histolytica* and *Salmonella* on 24 samples followed by *E.histolytica* and *Shigella* on 18 samples. No significant differences recorded between Fecal Calprotectin levels for male and female patients who infected with *E. histolytica* only or with *E. histolytica* and pathogenic bacteria. Most male and female patients with diarrhea in current study had positive CRP result with prevalence 76%. A significant increase recorded in (ESR, WBC, RBC, PLT, Hb, HCT) for male and female patients with diarrhea in current study when compared with control.

Key words: Diarrhea, *E. histolytica*, Pathogenic bacteria, FC Calprotectin.

Introduction

Diarrhea is one of the leading causes of illness and death in infants and children throughout the world, especially in developing countries. An estimated 2.5 million gastroenteritis deaths occur each year in children less than 5 years of age (Kosek *et al*., 2000).

Entamoeba histolytica, the etiological agent of amebiasis, the amoebic infection is the third most common cause of death among parasitic diseases, after malaria and schistosomiasis (Tanyuksel and Petri, 2003). The dysentery can mainly spread among people through contaminated food and water as well as poor sanitation, there are several numbers of bacteria that can cause acute dysentery, including *Shigella*, *Salmonella, Campylobacter* and *Escherichia coli* and (Guerin *et al*., 2004, Nath *et al*., 2013).

This community of bacteria may have a significant influence on the virulence of the amoeba itself, its ability to colonize the gut and the host's immune response at baseline and during amebiasis. The bacterial microbiota is therefore a significant environmental factor that may influence the clinical presentation and outcome of *E. histolytica* infections (Stacey *et al*., 2016).

Dysentery characterized by inflammation of the intestines, mainly the colon. Fecal calprotectin (FC) antibody produced by the neutrophils when they were in challenges with the pathogens and this antibody degranulation inside the intestinal lumen occurs as a response to local inflammation, (Foell *et al*., 2009).

The present study amid to measure Fecal calprotectin (FC) and other immunological parameters as total and deferential WBC count, C-R reactive protein and ESR

in diarrheal children under five years old infected with *Entamoeba histolytica* and pathogenic bacteria in Thi-Qar province.

Materials and Methods

The study carried from November 2018 to April 2019, stool samples collected from 614 diarrheal children under five years old both sex attending into Bint Al-Huda teaching hospital for children and women and Al-Mousawi hospital in Thi-Qar province. Stool consistency, color, odor and presence of blood or mucous were examined then the microscopic examination were involved direct wet mount smear and Iodine-stained smear were performed according to Salman (2015) while concentration technique (Formal-ether) was performed according to WHO (2003).

Small amount of stool samples for patients infected with *E. histolytica* added directly to the wells of a micro-titer plate that coated with antibodies to FC. Results for FC more than 50 ng/ml considered positive, while results for FC bellow 50 ng/ml considered negative.

Stool samples of patients infected with *E. histolytica* were cultured by using some media cultures (MacConkey agar, Blood agar, *Salmonella Shigella* Agar, Xylose Lysine Deoxy-cholate agar XLD, Chromatic Detection agar) which prepared according to the instructions of the company and sterilized by autoclaving at 121°C for 15 minutes.

Five ml of venous blood samples were collected by a well-trained nurse from volunteer patients in tube and divided to two parts, the first part of blood allowed to clot at room temperature for 30 minutes, then it was centrifuged for 5 minutes at 4000 RPM, serum was collected and frozen at -20°C to estimate CRP. The second part of blood was added to ETDA containing tubes for assessment the hematological examination and ESR, Twenty five healthy children under five year were selected as control.

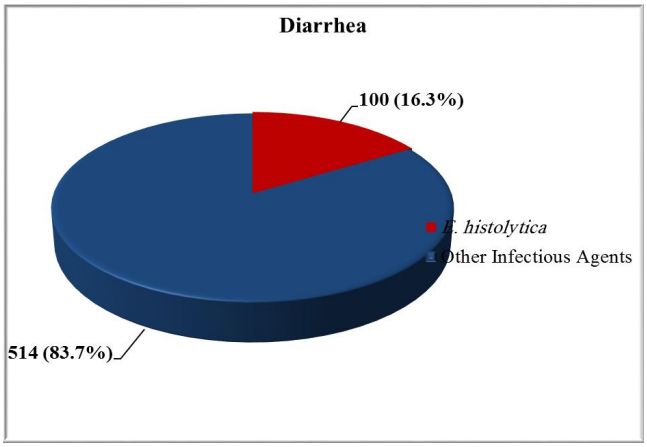

Fig. 1: Prevalence of *E. histolytica* Among Diarrheal Patients by Using Direct Examination.

The statistical package for social science (SPSS) version used in the current study to analyze data and the results expressed as (Mean ± S.E.). Independent sample Chi-square-test used to determine the statistical differences by consideration P-value $\leq$ 0.05 was statistically significant.

Results

The result showed 100 out of 614 (16.4%) of stool samples from patients under five years suffer from diarrhea were infected with *E. histolytica*. Fig. 1.

Distribution of Stool Sample According to Type of Infectious

A total of 100 stool samples of patients infected with *E. histolytica* were cultured to diagnose the pathogenic bacteria associated with *E. histolytica*. The result showed, there are 27 samples of patients were infected with *E. histolytica* only with prevalence 27%. While pathogenic bacteria were detected on 73 sample with prevalence 73%.

The results of the present study recorded that the *E. histolytica* and *Salmonella* were found in 24 samples (24%) followed by *E. histolytica* and *Shigella* were found in 18 samples (18%). While the lowest percentage was recorded with *E. histolytica, Salmonella, Shigella* and *Pseudomonas* which found in three samples only (3%). Table 1.

Prevalence of *E. histolytica* Infection and Pathogenic Bacteria According to Gender

No significant differences (P $\leq$ 0.05) were recorded between males 59% and females 41% in current study, the results showed 43% of males were infected with *E. histolytica* and pathogenic bacteria, while 16% infected with *E. histolytica* only and 30% of females infected with *E. histolytica* and pathogenic bacteria, while 11%

Table 1: Distribution of stool sample according to type of infectious agents.

S. No.	Infectious agents	No.	%
1	*E. histolytica* only	27	27
2	*E. histolytica* and *Salmonella*	24	24
3	*E. histolytica* and *Shigella*	18	18
4	*E. histolytica* and *Proteus*	8	8
5	*E. histolytica* and *Pseudomonas*	5	5
6	*E. histolytica* and *Salmonella and Proteus*	8	8
7	*E. histolytica* and *Salmonella and Shigella*	7	7
8	*E. histolytica* and *Salmonella and Shigella and Proteus*	3	3
	Total	100	100

Table 2: Distribution of infections according to gender.

Groups	Male		Female		Total	
	No.	%	No.	%	No.	%
E. histolytica	16	16%	11	11%	27	27%
E. histolytica and Pathogenic bacteria	43	43%	30	30%	73	73%
Total	59	59%	53	41%	100	100%

Calx² = 0.01 Tabx² = 3.84 Df= 1 P. Value= 0.974

with *E. histolytica* only. Table 2.

Prevalence of *E. histolytica* Infection and Pathogenic Bacteria According to Habitat

The most infected patients were found in rural habitat 54 (54%), while the patients in urban habitat were 46 (46%) with non-significant statistical difference ($P \leq 0.05$). Table 3.

Prevalence of *E. histolytica* and Pathogenic Bacteria According to Age Groups

The high prevalence of infection with *E. histolytica* and pathogenic bacteria were found in the first age group (≤ 1 year) 23% and the lowest prevalence of infection in the second age group 1-2 years 10% with no significant difference ($P \leq 0.05$). The high prevalence of infection with *E. histolytica* only were found on the second age group 9%, while the lowest prevalence in the fifth age group (4 - ≤ 5 years) 3% with no significant difference ($P \leq 0.05$). Table 4.

The Relationship between Diarrheal Patients Infected with *E. histolytica* and Pathogenic Bacteria and Some Immunological Parameters

The Relationship between Fecal Calprotectin Result and The Infection with *E. histolytica* and Pathogenic Bacteria: Fecal Calprotectin levels were positive (>50> ng /ml) for most patient with diarrhea caused by *E. histolytica* only or *E. histolytica* and pathogenic bacteria.

No significant differences were recorded between Fecal Calprotectin levels for male and female patients who infected with *E. histolytica* only or with *E. histolytica* and pathogenic bacteria. Table 5.

The Relationship between CRP and infection with *E. histolytica* Associated with Pathogenic Bacteria: Most

Table 3: Distribution of infections according to habitat.

Groups	Male		Female		Total	
	No.	%	No.	%	No.	%
E. histolytica	14	14%	13	13%	27	27%
E. histolytica and Pathogenic bacteria	40	40%	33	33%	73	73%
Total	54	54%	56	46%	100	100%

Calx² = 0.096 Tabx² = 3.84 Df= 1 P. Value= 0.793

Table 4: Distribution of infections according to age groups.

Age Groups (months)	*E. histolytica*		*E. histolytica* and Pathogenic bacteria		Total	
	No.	%	No.	%	No.	%
0 - 11	7	7%	23	23%	30	30%
12 - 23	9	9%	10	10%	19	19%
24 - 35	4	4%	14	14%	18	18%
36 - 47	4	4%	11	11%	15	15%
48 - 59	3	3%	15	15%	18	18%
Total	27	27%	73	73%	100	100%

Calx² = 5.388 Tabx² = 9.49 Df= 4 P. Value= 0.25

male and female patients with diarrhea in current study had positive CRP result with prevalence 76%. Table 6.

Erythrocyte Sedimentation Rate: A significant increase $P \leq 0.05$ were recorded in ESR for male and female patients with diarrhea when compared with control. No significant increased $P \leq 0.05$ in ESR for male and female patients with diarrhea caused by *E. histolytica* and pathogenic bacteria when compared with patients with diarrhea caused by *E. histolytica* only. Table 7.

White Blood cell Count and Differential: The result of current study showed a significant increase $P \leq 0.05$ in total count of WBCs for male and female patients with diarrhea when compared with control. No significant increased $P \leq 0.05$ in total count of WBCs for male and female patients with diarrhea caused by *E. histolytica* and pathogenic bacteria when compared with patients with diarrhea caused by *E. histolytica* only.

The results recorded a significant increase $P \leq 0.05$ in the differential count of WBCs for male and female patients with diarrhea when compared with control except the Lymphocyte. No significant increased $P \leq 0.05$ in differential count of WBCs except the Lymphocyte for male and female patients with diarrhea caused by *E. histolytica* and pathogenic bacteria when compared with patients with diarrhea caused by *E. histolytica* only. Table 6.

Other Hematological Parameters: A significant increase $P \leq 0.05$ were reported in the blood parameters

Table 5: Distribution of infections according to gender.

Fecal Calprotectin levels	Male			Female			Total		
	Examined	50 ng /ml	<50 ng / ml	Examined	>50 ng /ml	<50 ng / ml	Examined	>50 ng /ml	<50 ng / ml
E. histolytica	16	5	3	11	7	5	27	12	8
E. histolytica and Pathogenic bacteria	43	19	20	30	13	14	73	32	34
Total	59	24	23	41	20	19	100	44	42

Calx² = 7.51 Tabx² = 11.07 Df= 5 P. Value= 0.185

Table 6: CRP for patients with diarrhea compared with control.

Fecal Calprotectin levels	Male			Female			Total		
	Examined	Positive	%	Examined	Positive	%	Examined	Positive	%
E. histolytica	16	10	62.5	11	5	45.4	27	15	55.5
E. histolytica and Pathogenic bacteria	43	36	83.7	30	25	83.3	73	61	83.5
Total	59	46	77.9	41	30	73.1	100	76	76

Calx² = 8.925 Tabx² = 7.81 Df= 3 P. Value= 0.03

of male and female patients with diarrhea ($RBC_{s,}$ PLT, Hb, HCT) when compared with control. No significant increased $P \leq 0.05$ were showed in male and female patients with diarrhea caused by *E. histolytica* and pathogenic bacteria when compared with patients with diarrhea caused by *E. histolytica* only.

Other blood parameters (MCV, MCH, MCHC) with no significant differences $P \leq 0.05$ for male and female patients with diarrhea when compared with control. Table 7.

Discussion

The direct wet preparation method and Lugol's iodine were used in the current study for examination of diarrheal stool samples, the microscopy was the gold standard for parasitology examination and it is remain the backbone particularly in developing countries (Saeed *et al* ., 2015).

The result of current study showed 100 out of 614 (16.4%) of stool samples from patients under five years suffer from diarrhea were infected with *E. histolytica* and these results agreed with previous studies.

In Genet, Southern Ethiopia, Nyantekyi *et al*., (2010) examined 288 diarrheal stool and found the percentage of infection with *E. histolytica* is 13.2%.

In Spain Goñi *et al*., (2012) examined 160 diarrhea stool by using light microscope for detect *E. histolytica* and they found 12.8% which positive for *E. histolytica* and 75.0% negative for *E. histolytica.*

Saeed and Sandstrom (2015) in Khartoum, Sudan examined 437 diarrheal samples by microscope for detect *E. histolytica* and found 5% positive for *E. histolytica.*

The results of present study disagree with Tellevik *et al*., (2015), in Dares Salaam, Tanzania, they examined 701 diarrheal stool by light microscope and they found no infection with *E. histolytica* (0.00%) and with Mbae *et al*., (2013), in Nairobi, Kenya, they examined 541 stool sample with diarrhea and found 225 (36.7%) sample infected with *E. histolytica.*

The immunological effect of *E. histolytica* was showed by estimate some immunological parameters as calprotectin, C-reactive protein and erythrocyte sedimentation rate. The results showed there are (86%) positive sample positive for calprotectin, included 47% males 24% of which at concentration <50 and 23 samples at concentration 50-200 and 39% females positive sample for calprotectin 20% at <50 and 19% sample positive for calprotectin at 50-200, the results also showed the positive result in co-infection was 66%, while in patients infected with *E. histolytica* only was 20%. This current result agree with previous study obtained by Ali and Mohammad, (2018), in Kirkuk province-north of Iraq, they examined 419 patient with diarrhea and found 18 male and 32 female positive for calprotectin at <50 and 12 male and 25 female at 50-200, the result also agree with study obtained by Paul Chubb, (2018), in Australia, he examined 136 sample positive for *E. histolytica* and found 79.2 positive sample for fecal calprotectin, the result disagree with previous study obtained by (Hestvik *et al*., 2011), in urban Kampala, the examined 302 stool sample and found 152 (50.3) positive sample for calprotectin, the result also disagree with study obtained by Holtman *et al*., (2016), the examined 114 stool sample and found no sample positive for calprotectin at concentration <50 and 30 (26.3) positive for calprotectin at concentration 50-200. In fact, it would seem that false-positive results are very rare in children, probably because of the lower frequency of associated disease and the less-frequent use of drugs that could damage the intestinal mucosa. As in adult patients, the sensitivity of the calprotectin assay in children was limited by false-negative results in patients with celiac disease (Holtman *et al*., 2016). In the current study the patients infected with *Entamoeba histolytica* or with co-infection and have positive results for fecal calprotectin was refers to acute infections of protozoan infection among this patients. The probability in computable and different. This interpretative ambiguity, together with the fact that there is not yet a standardized

Table 7: ESR for patients with diarrhea compared with control.

Sex		ESR
Males	Control	5.71 ± 1.20^{a}
	E. histolytica only	13.60 ± 7.65^{b}
	E. histolytica and pathogenic bacteria	15.73 ± 8.08^{b}
	L.S.D.	7.88
Females	Control	4.81 ± 1.83^{a}
	E. histolytica only	14.91 ± 7.31^{b}
	E. histolytica and pathogenic bacteria	17.59 ± 5.73^{b}
	L.S.D.	10.09

Table 6: ESR for patients with diarrhea compared with control.

Sex	Parameters	Total WBC *10³ M+SD	MON% M + SD	LYM% M + SD	NEU% M + SD	BASO% M + SD	EOS% M + SD
Males	Control	6.64 ± 1.21[a]	0.21 ± 0.09[a]	1.45 ± 0.26[a]	2.57 ± 0.60[a]	0.33 ± 0.17[a]	0.15 ± 0.56[a]
	E. histolytica only	10.89 ± 2.55[b]	0.88 ± 0.44[b]	3.59 ± 1.53[a]	6.41 ± 3.46[b]	0.90 ± 0.51[b]	1.21 ± 0.50[b]
	E. histolytica and pathogenic bacteria	11.8 ± 5.49[b]	0.74 ± 0.34[b]	3.83 ± 1.45[a]	7.19 ± 2.85[b]	0.79 ± 0.52[b]	0.88 ± 4.33[c]
	L.S.D.	4.73	0.71	5.49	3.84	0.56	1.06
Females	Control	6.39 ± 1.42[a]	0.33 ± 0.19[a]	1.41 ± 0.38[a]	2.61 ± 0.67[a]	0.29 ± 0.1[a]	0.34 ± 0.55[a]
	E. histolytica only	11.96 ± 3.96[b]	0.98 ± 0.47[b]	4.09 ± 1.71[a]	5.86 ± 2.84[b]	0.82 ± 0.61[b]	1.13 ± 0.63[b]
	E. histolytica and pathogenic bacteria	12.59 ± 4.03[b]	1.15 ± 0.68[b]	4.9 ± 6.67[a]	7.16 ± 3.76[b]	0.86 ± 0.54[b]	0.69 ± 0.35[c]
	L.S.D.	5.57	0.64	2.6	3.25	0.52	0.83

method for fecal calprotectin measurement, indicates that recommended cut-off values would have to be determined depending on the clinical setting in which they are used.

The result of CPR and ESR showed there are (76%) sample positive for CRP 61% of which in patients with co-infection and 15% in patients infected with *E. histolytica* only and there are significant difference within patients and between patients and control, also the rate of ESR is high in the female infected with co-infection but normal value, but the ESR recorded significant difference computable with control. The current study agree with study obtained by Hegazi and Patel, (2013), in Kingdom of Saudi Arabia, south Jeddah, the examined 120 stool sample infected with *E. histolytica*, they found 60 (50%) samples have a positive result for CRP, but the current disagree with same study according to ESR being found there are no significant differences between ESR patients and control, the result also agree with study Zainab, (2018), in Karbala, Iraq, they examined 75 stool sample infected with *E. histolytica* and they found (74.4), patients positive for CRP and showed the level ESR slightly higher than control. Studies established that *E. histolytica* caused the removal of the protective mucus coat during the first two hours of incubation, detached the enterocytes and then penetrated into the lamina propria. The mechanism of invasion by *E. histolytica* lead to inflammatory responses and activate alternative pathway *via* CRP (Selsted and Ouellette, 2005, Karimi and Saffar, 2013).

The current study showed the effect of *E. histolytica* associated with pathogenic bacteria or non on levels of hematological parameters. The result showed the count of WBC is higher in patient infected co-infection and there are non-significant difference between patients, but with control, also the percentage of MON also higher in patients infected with co-infection followed by *E. histolytica* infected patients with significant difference between two class patients and control, the result also showed non-significant difference in LYM percentage between patients and control. The current result showed the percentage of differential GRA was higher in patient with co-infection especially NUE and BASO, but the percentage of EOS was higher in *E. histolytica* infected patients with significant difference between patients and control, also the count of RBCs, is low in patients with co-infection and count of PLT was higher in co-infection patients also with significant difference between patients and control. The level of Hb and percentage of HCT, is low in patients with *E. histolytica* followed by patients with co-infection with significant difference between

Table 7: ESR for patients with diarrhea compared with control.

Sex	Parameters	RBCs *106 M+SD	PLT *10³ M + SD	Hb gm/dl M + SD	HCT% M + SD	MCF fl M + SD	MCH pg M + SD	MCHC gm /dl M + SD
Males	Control	5.14 ± 0.31[a]	240.0 ± 33.9[a]	12.8 ± 0.68[a]	39.5 ± 2.1[a]	90.35 ± 3.8[a]	32.38 ± 1.9[a]	31.70 ± 0.97[a]
	E. histolytica only	4.30 ± 0.57[b]	365.2 ± 104.9[b]	10.63 ± 1.55[b]	31.9 ± 3.65[b]	90.77 ± 5.3[a]	29.25 ± 3.7[a]	30.57 ± 3.2[a]
	E. histolytica and pathogenic bacteria	4.13 ± 0.57[b]	328.2 ± 94.4[b]	11.01 ± 1.32[b]	32.6 ± 3.8[b]	89.10 ± 5.5[a]	30.33 ± 3.1[a]	30.99 ± 4.7[a]
	L.S.D.	0.84	125.2	2.16	14.8	2.84	4.63	1.23
Females	Control	4.95 ± 0.32[a]	240.63 ± 33.8[a]	12.9 ± 1.13[a]	39.56 ± 3.6[a]	93.72 ± 4.1[a]	30.43 ± 2.8[a]	32.55 ± 2.3[a]
	E. histolytica only	4.32 ± 0.49[b]	405.33 ± 103.0[b]	10.44 ± 1.7[b]	31.32 ± 4.15[b]	90.58 ± 4.2[a]	29.72 ± 4.9[a]	30.99 ± 1.98[a]
	E. histolytica and pathogenic bacteria	4.26 ± 0.68[b]	347.66 ± 152.6[b]	10.47 ± 1.35[b]	32.27 ± 3.3[b]	89.54 ± 6.9[a]	29.18 ± 3.7[a]	30.77 ± 3.7[a]
	L.S.D.	0.62	164.69	2.47	13.83	3.14	5.71	1.56

patients and control, also the result showed there are non-significant difference in the level of MCV, MCH and MCHC between patient and control, the current study is agree with previous study obtained by Zainab, (2013), in Karbala, they found the patients infected with *E. histolytica* have a some change in the hematological parameters as high WBC, increase the level of EOS and NEU and slightly decrease level of hemoglobin, the results also agree with study of Obaid, (2014), in Al Haweeja / Kirkuk, Iraq, he examined 207 patient with co-infection of *E. histolytica* and bacteria and found the infection have a negative relation with hematological parameters as high WBC count and differential and decrease count of RBCs and low level of hemoglobin, the result disagree with study obtained by Al Laham and Ridwan, (2015), in Gaza, they found the *E. histolytica* non has effect on hematological parameters in patients. The reason for the difference may be that the researcher examined patients whose parasite was not sufficient to cause malabsorption or sufficient blood loss to affect iron levels. Some researchers have found that the high prevalence of intestinal pathogens, especially parasites, is associated with anemia and a marked rise in leukocyte rates, because the bleeding cause by invading *E. histolytica* (Akram, 2018).

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