Original Article

Investigating Specific Calprotectin and Immunological Markers Associated With Intestinal Infections Caused by *Entamoeba histolytica*

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ABSTRACT

Background: *Entamoeba histolytica* is a parasitic organism that infects the intestines and causes dysentery, characterized by intestine inflammation.

Objectives: We aimed to estimate calprotectin levels and their relationship to the severity and progression of intestinal inflammation.

Methods: We conducted a cross-sectional study in Al-Habbobi Teaching Hospital Thi-Qar, Al-Nasseriah, Iraq, from March 10 to October 1, 2023. The sample included 50 men and 50 women aged in three groups: 25–29, 30-34, and >34 years. We employed microscopy, direct wet method, and formal-ether concentration approach to detect parasites. Also, we employed nzyme-linked immunosorbent assay (ELISA) for measuring fecal calprotectin (FC) and C-reactive protein (CRP), and XP-Sysmex for detecting white blood cells.

Results: The calprotectin levels below 50 ng/mL were more common in the second age group. The third age group had calprotectin values of 50 ng/mL. In the first age group (25–29 years old), calprotectin frequency was similar. CRP levels were equal in the first age group. In the second age group (30–34 years), negative CRP outnumbered positive CRP. Positive CRP was more common than negative in the third age group of >34 years. The group above 35 had the highest general stool examination (GSE) and antigen Ag detection rates, while those aged 30–34 years in the control group had the highest.

Conclusion: Calprotectin levels less than 50 ng/mL were more frequent in the age group of 30-34 years, while calprotectin levels greater than 50 ng/mL were more frequent in the age group <35 years. CRP levels were equal in frequency in the first age group. Negative CRP was more common than positive CRP in the second age group. Positive CRP was more common than negative CRP in the third age group.

Keywords: *Entamoeba histolytica*, C-reactive protein (CRP), Calprotectin, Gastroenteritis, Intestine inflammation

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Introduction

Entamoeba histolytica is a type of single-cell organism that is responsible for causing intestinal amebiasis. It can also lead to other health problems outside of the intestines. Most cases of *E. histolytica* infection do not show symptoms (Stanley, 2003; Kareem Kadhim et al., 2023). There are 50 million people who suffer from obvious clinical symptoms, which leads to about 100,000 deaths annually (Mewara et al., 2023; Dhubyan, 2022). The infection occurs in countries with poor sanitation and low economic status (Stanley, 2003; Saleh Mohammed Al-Samarrai et al., 2022). The prevalence of *Entamoeba* varies from one city to another. As results have shown in Thi-Qar Governorate, of the 341554 cases of intestinal parasitic infections, 38,004 individuals (11.1%) were recorded as having amoebiasis, which accounted for the highest proportion of infections in 2015 (26.1%) and the lowest in 2020 (8.1%). Amoebiasis is distributed among all age groups (Flaih et al., 2021). Research in Baghdad Governorate has also shown that the prevalence of *E. histolytica* was 15.89% among 497 patients. The two stages of parasitism are found in the same patient as 50.63%, while one stage was found in 49.36%. In relation to sex, the incidence was higher in males (51.89%) than in females (48.10%) (Nayyef et al., 2022).

E. histolytica is commonly transmitted through the consumption of cysts in contaminated food and water (Salit et al., 2009; Billet et al., 2019; Hassan et al., 2022). It causes digestive system inflammation and, in advanced stages, liver abscesses (Stauffer & Ravdin, 2003). It is still a major global health concern, ranking as the third leading cause of death due to parasitic infections (Dezfouli et al., 2022). The virulent variant, *E. histolytica*, can cause amoebic colitis and extraintestinal amoebiasis. *Entamoeba dispar* is considered nonpathogenic and does not cause disease symptoms (Haque et al., 2003; Shirley et al., 2018; Quail et al., 2009).

In response to inflammatory bowel disease (IBD) pathogens, neutrophils produce fecal calprotectin (FC) antibodies. Because these antibodies bind to serum albumin and form antigen-antibody complexes, serological evaluation of these antibodies is simple using an nzyme-linked immunosorbent assay (ELISA) reader. An FC antibody level greater than 50 ng/mL indicates significant gastrointestinal damage, necessitating endoscopy to determine the agents responsible. Local inflammation also causes FC degranulation within the intestinal lumen (Shirley et al., 2018). Recently, FC has emerged as an essential diagnostic tool in gastroenterology for distinguishing IBD from other non-inflammatory conditions (Mewara et al., 2023). In addition, it can be used as an indicator of the activity of the disease and also the effectiveness of treatment, especially in cases of diarrhea caused by parasitic sources (Van Rheenen et al., 2010; Spadafora et al., 2016; Hosseini et al., 2022). Amoebic colitis affects males and females equally, though, in different stages of life. In addition to the variety of stages of disease transmission, especially fecal matter, direct contact between healthy and sick people, and contamination of water and food, several risk factors can increase the incidence of severe infection and death, such as pregnancy, the use of corticosteroids, malignancy, and malnutrition (Van Rheenen et al., 2010). Amoebic liver abscesses are more likely to occur in middle-aged men, specifically those between the ages of 18 and 50, at least three times (Saidin et al., 2019; Garg et al., 2018). Neutrophils secrete FC, a calcium- and zinc-binding protein. Calprotectin levels are measured in various types of bacterial, parasitic, and viral infections, in addition to colorectal cancer and intestinal inflammatory diseases (Saidin et al., 2019). Measuring calprotectin levels in stool is a diagnostic marker for gastrointestinal diseases, especially intestinal infections. The harmful microorganisms activate the transport of neutrophils to the cells of the digestive system; the neutrophils, in turn, secrete calprotectin, which leads to tissue damage (Garg et al., 2017). Patients with gastrointestinal symptoms suffer from severe diarrhea, increased intestinal permeability, and chlorine imbalance (Garg et al., 2017; Duman et al., 2015). This study aims to estimate calprotectin and C-reactive protein (CRP) levels and their relationship to the severity and progression of intestinal inflammation.

Materials and Methods

The study design

This cross-sectional study investigated 100 samples from people infected with *E. histolytica.* The samples were collected in Al-habbobi Teaching Hospital, Thi-Qar, Al-Nasseriah, Iraq between March 10, 2023, and October 1, 2023. Fifty males and 50 females were enrolled from three different age groups: 25–29, 30–34, and more than 34 years.

Laboratory tests

Macroscopic examination

The stool's consistency, color, smell, and the presence of mucus or blood were all included in the macroscop-

ic examination. The concentration technique (formal ether), wet mount smears, and iodine-stained smears were conducted.

FC and CRP assay using ELISA test

ELISA was used to examine the calprotectin levels in the patient's feces using the immunological principle of ELISA. The sandwich technique was used to determine the antibodies according to the working principle of the kit used.

Blood sample collection and processing

A total of 5 mL of blood was drawn from each participant; 3 mL was placed in a gel tube, and 2 mL in an ethylenediaminetetraacetic acid (EDTA) tube. The first tube was left at room temperature for 10 minutes until its blood coagulated. The serum was separated to conduct the required tests, and the other tube was shaken well. After adding blood to prevent clotting, a complete blood count (CBC) test was performed, and the number of white blood cells was counted using an automated hematology machine called XP-Sysmex. Determination of the presence of Entamoeba histolytica antigens using a rapid immunoassay test (cassette test).

Statistical analysis

The statistical data analysis used the SPSS software, version 26 (IBM, SPSS Inc., USA). An independent sample t-test was used for parametric variables, and a Mann-Whitney U test was used for nonparametric variables. The correlation between dependent variables was evaluated using the Pearson correlation coefficient analysis. The result was statistically significant when $P \leq 0.05$.

Results

The sociodemographic characteristics of the study groups

The results showed no statistically significant difference in age between the groups infected with *E. histolytica* with the control group (P=0.80) (Table 1). While white blood count (WBC) increased significantly in patients compared to the control group (P<0.001). The 95% confidence intervals of WBC in the control group were 6.25±0.149, while in the patients' group, they were 10.88±0.135.

Age, gender, clinical signs and symptoms, and diagnosis of *E. Histolytica* in GSE among the study groups

Regarding the males, they were 21 in the age range of 25–29, accounting for 43.8% of the total sample, 47 in the 30-34 years age group, accounting for 52.2%, and 32 in the age category of 35 and older, accounting for 51.6% of the total sample. Regarding the females, they comprised 27 in the 25-29 years age group, which accounts for 56.3% of the total; 43 in the 30-34 years age group accounted for 47.8%, and 30 in the age range of 35 and older, accounting for 48.4% of the total (Table 2 and Figure 1). Diarrhea and abdominal pain was observed in 24 cases within the age group of 25–29, accounting for 50% of the total. Additionally, there were 44 cases within the age group of 30-`34, representing 48.9% of the total and 32 cases in the age group of 35 and older, constituting 51.6% of the entire amount. There were 24 cases in the age group 25–29, accounting for 50% of the total, 46 instances in the age group of 30-34, accounting for 51.1% of the total, and 30 instances within the age category of 35 and older, accounting for 48.4% of the total (Table 2 and Figure 2). There were 24 cases of *E. histolytica* cyst formations in people aged 25–29, accounting for 50% of the total, and 24 cases of people with normal formations in the same age group, accounting for 50%. Also, 44 cases of *E. histolytica* cyst formations occurred in people aged 30-34, accounting for 48.9%, and 46 people with normal formations in the same age group, accounting for 51.1%. Finally, 32 cases of *E. histolytica* cyst formations in people aged 35 and up account for 51.6%, and 30 cases in people with normal formations in the same age group, accounting for 48.4% (Table 2 and Figure 3).

The frequency and percentage of diagnosing of *E. histolytica* in immunology rapid test among the study groups

In the 25-29 years age group, there were 24 *E. histolytica* antigen (Ag)-positive cases, representing 50% of the total. In the same age group, there were also 24 *E. histolytica* Ag-negative cases, representing 50% of the total. In the 30-34 years age group, there were 44 *E. histolytica* Ag-positive cases, representing 48.9% of the total. Also, 46 *E. histolytica* Ag-negative cases were present in the same age group, representing 51.1% of the total. In the age group of 35 years and older, there were 32 *E. histolytica* Ag-positive cases, representing 51.6% of the total. There were also 30 *E. histolytica* Ag-negative cases in the same age group, representing 48.4% of the total (Table 3 and Figure 4).

Table 1. The relationship between the age and level of WBC in the control and patient groups

Characteristic	Mean±SD (95% CI)		P
	Control Group (n=100)	Patient Group (n=100)	
Age (y)	32.37±3.12	32.48±3.19	0.80 NS
WBC (Cells/µL)	6.25±0.76 (6.25±0.149)	10.88±0.69 (10.88±0.135)	<0.001

NS: Not significant.

The frequency and percentage of calprotectin and CRP test among the study groups

There were 24 cases with a calprotectin value of less than 50 ng/mL in the 25–29 years age group, accounting for 50% of the total. In the same age group, there were 24 cases with a calprotectin value greater than 50 ng/mL, accounting for half of the total. There were 46 cases with a calprotectin value of less than 50 ng/mL in the 30-34 years age group, accounting for 51.1%. In the same age group, there were 44 cases with a calprotectin value greater than 50 ng/mL, accounting for 48.9% of the total. There were 30 cases with a calprotectin value of less than 50 ng/mL in the age group of 35 and older, accounting for 48.4% of the total. In the same age group, there were 32 cases with a calprotectin value greater than 50 ng/mL, accounting for 51.6% of the total (Table 4 and Figure 5). There were 24 cases with a positive CRP result in the age group of 25–29 years, accounting for 50% of the total. In the same age group, 24 cases had a negative CRP result, accounting for 50% of the total. There were 44 cases with a positive CRP result in the 30-34

Table 2. The relationship between the ages and genders, clinical signs and symptoms and diagnosis of *E. histolytica* in general stool examination (GSE)

Parameters		No. (%)	
		Sex	
		Male	Female
Age (y)	25-29	21(43.8)	27(56.3)
	30-34	47(52.2)	43(47.8)
	>35	32(51.6)	30(48.4)
Parameter		**No. (%)**	
		Clinical Sign and Symptoms	
		Diarrhea and Abdominal Pain	**Non-clinical Signs and Symptom**
Age (y)	25-29	24(50)	24(50)
	30-34	44(48.9)	46(51.1)
	>35	32(51.6)	30(48.4)
Parameter		**No. (%)**	
		GSE	
		***E. histolytica* Cyst**	**Normal**
Age (y)	25-29	24(50)	24(50)
	30-34	44(48.9)	46(51.1)
	>35	32(51.6)	30(48.4)

Table 3. The association between age and *E. histolytica* antigen infection

Parameter		No. (%)	
		E. histolytica Antigen	
		Positive	Negative
Age (y)	25-29	24(50)	24(50)
	30-34	44(48.9)	46(51.1)
	35>	32(51.6)	30(48.4)

years age group, accounting for 48.9% of the total. There were 46 cases with a negative CRP result in the same age group, accounting for 51.1%. There were 32 cases with a positive CRP result in the age group of 35 years and older, accounting for 51.6% of the total. In the same age group, 30 cases had a negative CRP result, accounting for 48.4% of the total (Table 4 and Figure 6).

Discussion

There are no statistically significant differences (P=0.80) between the two groups regarding the age. Therefore, it can be believed that age does not significantly impact the other characteristics studied. For the number of WBC, there are no statistically significant differences (P<0.001) between the two groups. The Mean±SD number of white blood cells in the patient population (10.88±0.69) is markedly higher than the control group's average (6.25±0.76). This finding indicates an increase in white cells in patients compared to healthy people. That is why *E. histolytica* causes gastrointestinal infections, leading to a high white blood cell rate (Saidin et al., 2019). Data are provided in frequency and percentage per age group for clinical and non-clinical symptoms. For clinical symptoms, diarrhea and abdominal pain were found in 50% of patients in each age group (25–29, 30-34, and more than 34 years). For non-clinical symptoms, the percentages show no significant difference between the two groups. The percentage of non-clinical symptoms ranges from 48.4% to 51.6% in different age groups (Kantor et al., 2018).

Colonic epithelial cells stick to trophozoites because of a certain galactose-N-acetyl galactosamine lectin, which leads to infection. When trophozoites adhere to colonic epithelial cells, they cause cytolysis and apoptosis, releasing interleukin (IL)-1α and the precursor interleukin-1 beta (IL-1β). Distal cells produce cytokines

Table 4. The correlation between calprotectin and CRP levels and age

Parameter		No. (%)	
		Calprotectin	
		<50 ng/mL	>50 ng/mL
Age (y)	25-29	24(50)	24(50)
	30-34	46(51.1)	44(48.9)
	>35	30(48.4)	32(51.6)
Parameter		**No. (%)**	
		C-reactive Protein	
		Positive	**Negative**
Age (y)	25-29	24(50)	24(50)
	30-34	44(48.9)	46(51.1)
	>35	32(51.6)	30(48.4)

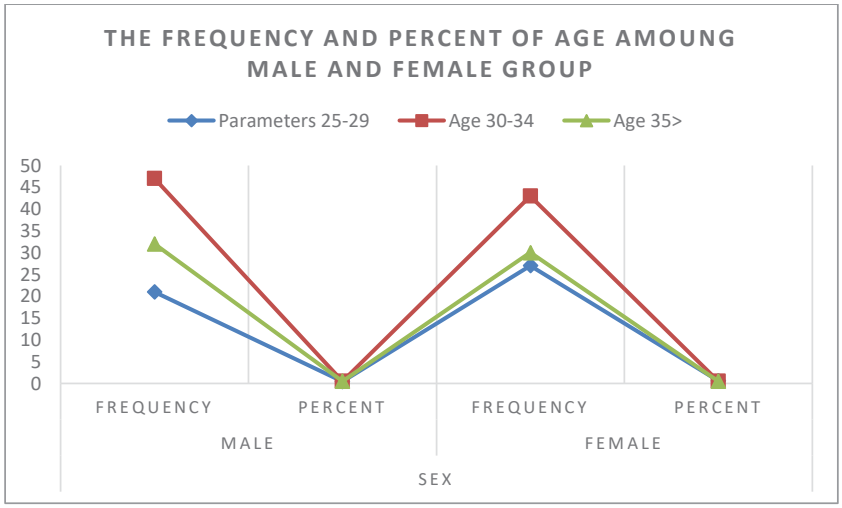

Figure 1. The frequency and percentage of age groups among male and female samples

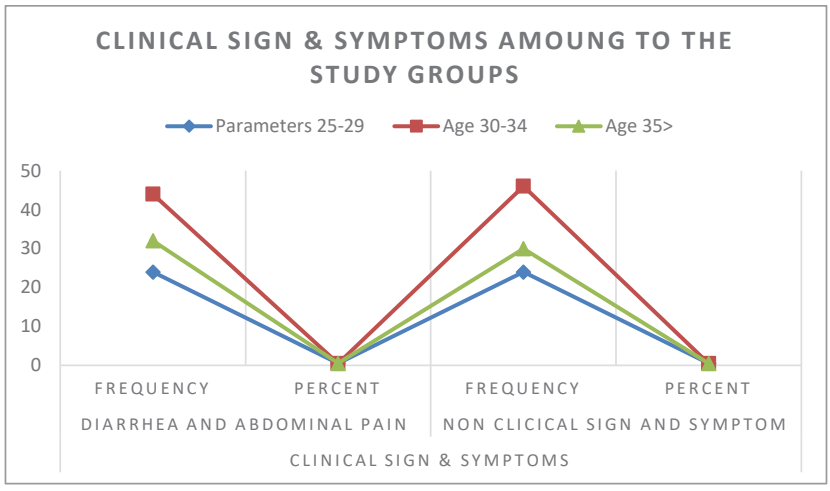

Figure 2. Signs and symptoms among the research groups

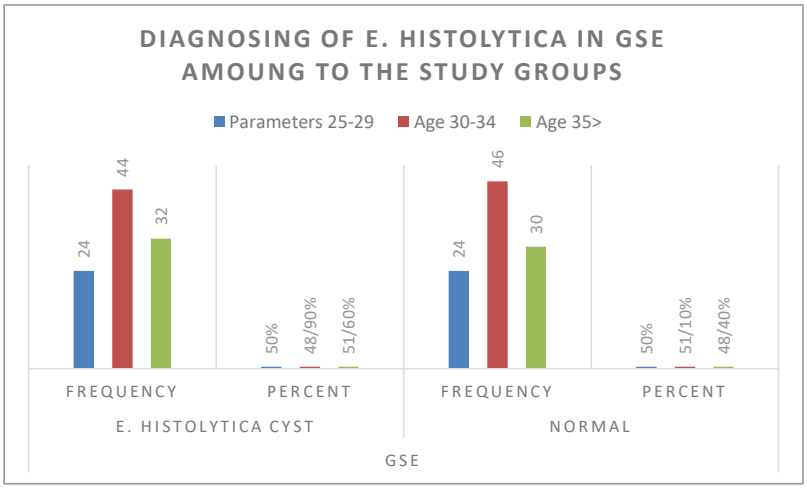

Figure 3. Detection of *E. histolytica* in GSE for the research groups

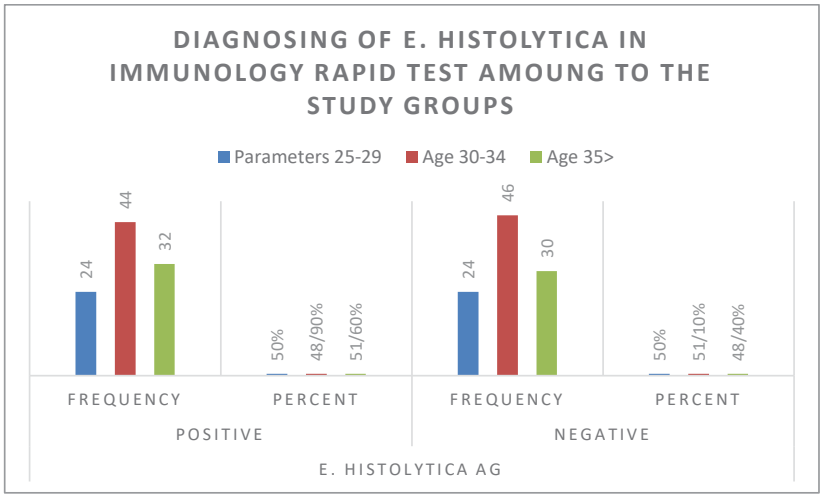

Figure 4. Identification of *E. histolytica* in immunology rapid test among research groups

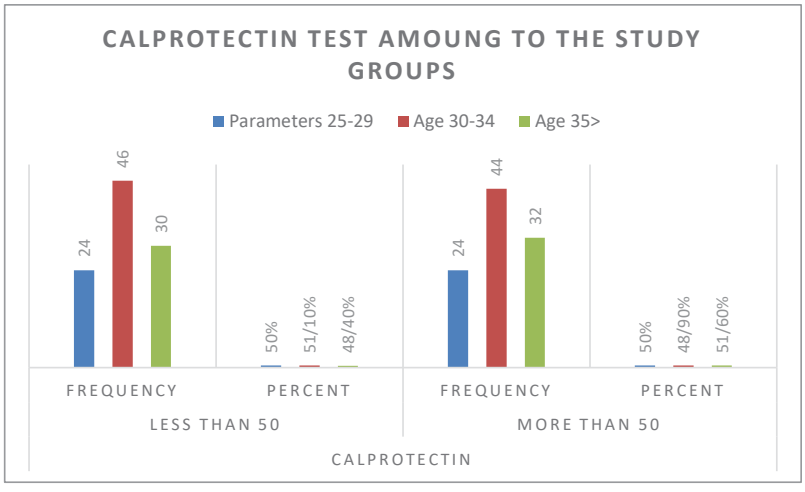

Figure 5. Testing for calprotectin in the research groups

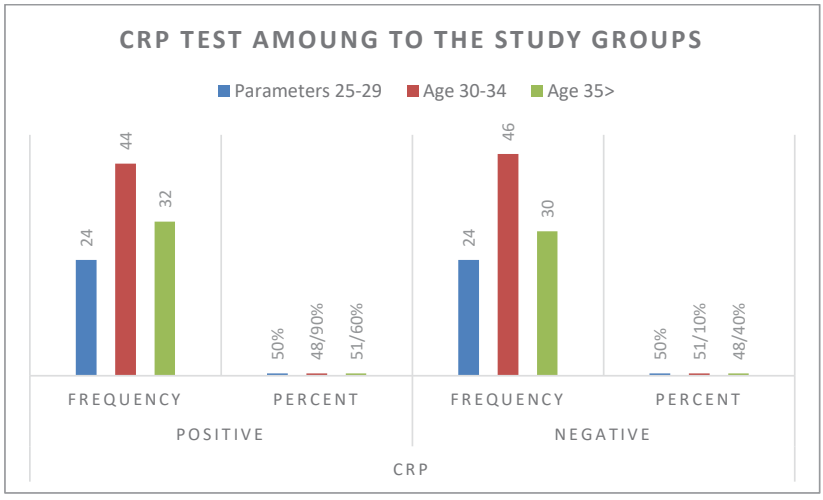

Figure 6. CRP test amongst the research groups

and inflammatory mediators like cyclooxygenase-2 inhibitor (COX-2), IL-1, and IL-8 when IL-1β activates nuclear factor-kappa B (NF-κB). Also, amoebic cysteine proteinases can convert precursor IL-1β to active IL-1β, enhancing the process (Kantor et al., 2018). Cytokines and inflammatory mediators attract neutrophils and macrophages. Direct contact with trophozoites damages neutrophils, which damages colonic epithelial cells and releases more mediators. Tumor necrosis factor-alpha (TNFα) and other mediators released by macrophages further contribute to inflammation (Stanley, 2003).

Data were provided in frequency and percentage per age group and each calprotectin level (less than 50 ng/mL and higher than 50 ng/mL). It can be noted that there is an equal distribution of calprotectin levels among patients in different age groups. In each age group, 50% of patients have a calprotectin level below 50 ng/mL, and 50% have a calprotectin level above 50 ng/mL. Thus, it can be said that the level of calprotectin does not differ significantly among the age groups included in the study. This finding indicates that the calprotectin level is not significantly affected by age and maybe a strong indicator of intestinal inflammation, regardless of age group. Calprotectin levels were higher in all people infected with *E. histolytica* than in the control group. Calprotectin can affect the severity of inflammation because neutrophils release it and have an important role in the inflammatory immune response in the body (Ali et al., 2018; Salman et al., 2017).

The specific mechanisms that cause the occasional invasion of the mucosal epithelium by *E. histolytica* are still not understood, and neither are the elements from the host-parasite relationship that contribute to this invasion. The prognosis of an invasive *E. histolytica* infection is unpredictable. It might result in symptoms such as amebic diarrhea, colitis, or the spread of parasites through the portal circulation, leading to hepatic abscesses. An invasion by *E. histolytica* causes a profound pro-inflammatory response and the destruction of host tissue, both exacerbating the illness. Currently, no vaccination effectively counteracts this condition. However, parasites that live in tissues can be successfully treated using nitroimidazoles, such as metronidazole. Administration of metronidazole can lead to the emergence of severe side effects and an elevated susceptibility to drug resistance. Neutrophils, part of the host immune system, release significant amounts of calprotectin protein near the infection site as a defensive response to kill the parasite. However, continued morbidity and mortality indicate that this parasite can escape host defense responses to maintain its survival. Thus, an understanding of the human immune response to the parasite and the strategies used by the parasite to evade host defense will deeply improve the development of effective immunotherapies (Salman et al., 2017). Calprotectin levels are high in people infected with *E. histolytica*, as well as other intestinal infections such as worms, because the mechanism by which calprotectin protein increases is similar among all infections because parasitic intestinal infections lead to inflammation and damage to the intestinal tissue, which leads to increased levels of blood cells. White blood, especially neutrophils, raises calprotectin levels as a defense method to eliminate infection or disease developments (Ali et al., 2018).

There is no statistical significance in the percentage of positive and negative results between age groups. CRP levels were also higher in all patients infected with *E. histolytica* than in the control group. Due to the pathological condition caused by the parasite, in addition to inflammation, the body produces CRP as a first stage of innate immunity to get rid of the infection and protect the damaged tissue. Therefore, CRP levels are higher in patients compared to the control group, consistent with results from Saheb et al. (2020). One study also found that CRP levels are high in patients infected with *Giardia lamblia* and *E. histolytica,* making it a non-specific immunological marker to evaluate clinical symptoms during infection. In reaction to IL-6, the liver produces the pentameric protein known as a CRP during inflammation. When inflammation and infection are at their most severe, they are released (Saheb et al., 2020). CRP is produced as soon as membrane-associated macrophages get activated during inflammation, particularly when infections like *E. histolytica* are present (Tinuade et al., 2006; Haque, 2007). There are several reasons why CRP levels could be low, but the amount of antigen or the causative agent that enters the body is the primary one (Tinuade et al., 2006).

The liver releases CRP in response to intestinal parasite attachment to the intestinal wall or membrane. This protein then combines with macrophages and mucosal immunoglobulin A (IgA) to combat infection. Thus, CRP levels significantly rise during the acute phase of *G. lamblia* and *E. histolytica*-related diarrhea (Saheb et al., 2020).

Age does not significantly impact the other characteristics studied. The increased number of WBCs may be related to the defense mechanism and immunological responses against parasites (Safi, 2012; Macy et al., 1997). In addition to intestinal parasitic infections that lead to increased calprotectin levels, other parasitic infections, especially Helminth, can increase the release of the protein calprotectin. Besides inflammatory bowel disease,

infections are another significant contributor to elevated calprotectin levels. These infections include bacterial infections like *Salmonella* and *Clostridioides difficile*, which often lead to higher calprotectin levels than viral infections such as rotavirus or norovirus. Infection with some parasite protozoa, such as *Dientamoeba fragilis*, can lead to the infiltration of neutrophils in the intestines, resulting in an elevation of FC levels. FC is a chemical associated with inflammation. Levels of FC were higher in adults and children with acute bacterial diarrhea than in viral diarrhea. Furthermore, the severity of the diarrhea was directly proportional to the increase in these levels.

Conclusion

Age does not have a significant impact on the other characteristics studied. The number of white cells in the patient population was significantly higher than in the control group, indicating gastrointestinal infections. The study also found that calprotectin levels are higher in all age groups infected with *E. histolytica* than in the control group. The CRP test results were higher in all patients infected with *E. histolytica* than in the healthy, uninfected control group. It suggests that gastrointestinal tract inflammation caused by *E. histolytica* affects immune responses and high CRP levels. Changes in C-reactive protein and calprotectin levels were monitored as a marker to identify and define the host immune role in parasitic infections. These studies can be expanded to understand the biochemical and cellular mechanisms that drive these processes and how these elements interact with various factors such as diet, overall body health, and environmental factors.

Ethical Considerations

Compliance with ethical guidelines

The Ethics and Research Committee of the Department of Medical Laboratory Techniques at Shatra Technical College, Southern Technical University, approved this study (Code: 398, in 5/3/2023). Oral consent was obtained from all participants in the research and they signed the ethical consent form.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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