Original Article

Immunological and cytokine profile (IL-25 and IL-35) in patients with *Entamoeba histolytica* infection in Southern Iraq

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ABSTRACT

Amoebiasis is an intestinal disease caused by a unicellular parasite called *Entamoeba histolytica*. Interleukin-35 (IL-35) is the youngest specific member of the IL-12 family that plays a major role in the inhibitory function of regulatory T cells (Tregs) to curb inflammatory responses. IL-25 of the IL-17 family, which is widely released by Th2 cells and epithelial cells, is a warning signal produced upon cell or tissue injury to activate immune cells. The present study aimed to determine the cytokine profile (IL-25 and IL-35) in patients with *E. histolytica* infection in southern Iraq. This hospital-based study was conducted from August 2022 to May 2023. The study participants were patients with *E. histolytica* infection admitted to the infection department of general hospitals in Thi-qar province, southern Iraq. Initially, *E. histolytica* amebiasis was detected in the patients by nested multiplex PCR. All collected sera were tested with the Human Interleukin 35 (Biotech, China, Cat.RD-IL35-Hu) and IL25 (Biotech, China, Cat.RD-IL25-Hu) ELISA kits according to the instructions of the manufacturer. A total of 80 patients, including 50 patients with *E. histolytica* infection and 30 subjects in the control group without *E. histolytica* infection, were enrolled in the present study. The results showed a significant difference ($p<0.001$) in the serum level of IL-25 in patients with *E. histolytica* infection (4275.19 pg/mL) compared to individuals in the control group without *E. histolytica* infection (2186 pg/mL). Statistical analysis showed that there was no significant difference in the serum levels of IL-35 patients with *E. histolytica* infection compared with individuals in the control group without *E. histolytica* infection. The results of the present study show that the level of IL-25 is high in patients with *E. histolytica* infection. This indicates the important role of IL-25 in the activation of the immune system during intestinal inflammation. Therefore, this cytokine can be used as a diagnostic marker for *E. histolytica* infection.

Keywords: Amoebiasis, Cytokine, ELISA, PCR

1. Introduction

Amoebiasis (AMB) is an intestinal disease caused by a unicellular parasite called *Entamoeba histolytica* (1). Studies have shown that amebiasis is the third most important disease caused by parasitic diseases after malaria and schistosomiasis, with 40,000 -100,000 deaths per year, according to the World Health Organization (WHO) (2). The prevalence of the parasite is reported to be up to 10% of the world's population. About 90% of infected individuals with amebiasis are asymptomatic, but 10% of them develop into invasive infections (3, 4). The main clinical signs of AMB are cramps or abdominal pain, diarrhea (sometimes with dysentery), nausea, colitis, and, in cases where no -treatment is given, spread to extra-internal tissues. The diseases must be distinguished from other diseases caused by diarrhea (e.g., infections with *Salmonella* spp, *Campylobacter* spp, and *Shigella* spp) and noninfectious causes ,such as involved inflammatory bowel disease, carcinoma, ischemic colitis, arteriovenous malformations, and diverticulitis) (5). The body of the host stimulates the normal immune response when infected with *E. histolytica*to employ multiple defense mechanisms, such as lymphocytic cells, mucous substances secreted by the membranes of the skeletal system, intestinal motility, and the acidic environment of the stomach (6). The cell-mediated immune response is considered the major host defense against *E. histolytica.* During the first step of infection, intestinal epithelial cells bind the carbohydrate domain of Gal/GalNAc lectin through the Toll -like receptor (TLR)-2/4 NFKB, which functions by producing inflammatory cytokines, including IL-1B, IL-6, IL-8, IL-12, IL-17, IFN-Y, and TNF-ą (7). Humoral immune responses against *E. histolytica* are fully differentiated and produced when amebiasis invades the patient and develops circular antibodies up to seven days after infection with *E. histolytica* (8). Interleukin-35 (IL-35) is the youngest specific member of the IL-12 family, that plays a major role in the inhibitory function of regulatory T cells (Tregs) to curb inflammatory responses (9). IL-25 of the IL-17 family, released by Th2 cells and epithelial cells is a warning signal produced upon cell or tissue injury to activate immune cells (10). In the present study, the cytokine profile (IL-25 and IL-35) was determined in patients with *E. histolytica* infection in southern Iraq.

2. Materials and Methods

2.1. Study area

Thi-Qar province is located at 31°14'N 46°19'E in the south of Iraq, and its capital is Nasiriyah. The province has an area of about 13,000 km^2 and a population of nearly 2 million people (Fig. 1). The weather in this province is dry and desert-like with low annual rainfall.

2.2. Study population

This hospital-based study was conducted from August 2022 to May 2023. The study participants were patients with diarrhea (before antibiotic therapy) admitted to infectious disease departments of general hospitals in Thi-qar province, southern Iraq, and the patients with immune system defects were excluded from the study.

2.3. Stool collection and microscopic examination

Stool samples were collected in a sterile container with a specific documentation number. Microscopic examination was performed using the salt and iodine wet preparation and the formalin-ether sedimentation method to detect trophozoites and cysts of *E. histolytic* (11).

2.4. Approving the *E. histolytic* amebiasis by molecular tests

DNA was clearly extracted from the ethanol-fixed stool samples using the STOOL DNA Minikit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. Then, nested multiplex PCR was performed according to the expansion of the small subunit ribosomal RNA gene (SSU rRNA) of *Entamoeba* spp, as previously explained elsewhere (11). Table 1 shows the primers used in the first and second steps of PCR. The temperature conditions for the first step were 5 min at 95°C, then 40 cycles of 95°C for 45s, 57°C for 40s and then 74°C for 45 s with a final extension at 74°C for 6 mins; whereasthe temperature conditions for the second step were 3 min at 95°C, then 35 cycles of 94°C for 40s, 56°C for 50s and then 74°C for 40 s with a final extension at 74°C for 6 mins. The PCR products obtained were then detected by electrophoresis (1.5% agarose gel), with, *E. histolytica* and *E. dispar* indicating 439 and 174 bp, respectively.

2.5. Immunological and cytokine profile study

2.5.1. Collection of blood samples

Five mL of blood was collected from all patients infected with *E. histolytica* under sterile conditions by venipuncture using 5 mL disposable plastic syringes. The collected blood samples were coagulated at room temperature for one hour. They were then centrifuged at 4000 rpm for 10 minutes after the blood had clotted. Then the serum was divided equally into two portions and put into Eppendorf tubes, which were stored at 20℃ until the immunological assays were performed.

2.5.2. Enzyme-linked immunosorbent assay test

All collected sera were tested with human interleukin using the Human Interleukin 35 (Biotech, China, Cat.RD-IL35-Hu) and IL25 (Biotech, China, Cat.RD-IL25-Hu) Sandwich enzyme-linked immunosorbent assay- based ELISA kits for quantitate *in vitro* measurement of IL35 and IL25 in human serum according to the instructions of the manufacturer. After completion of the enzyme-substrate reaction by addition of sulfuric acid solution, the color change was measured using an ELISA reader (BIOTEK ELX800TS, USA), with absorbance measured at 450 nm.

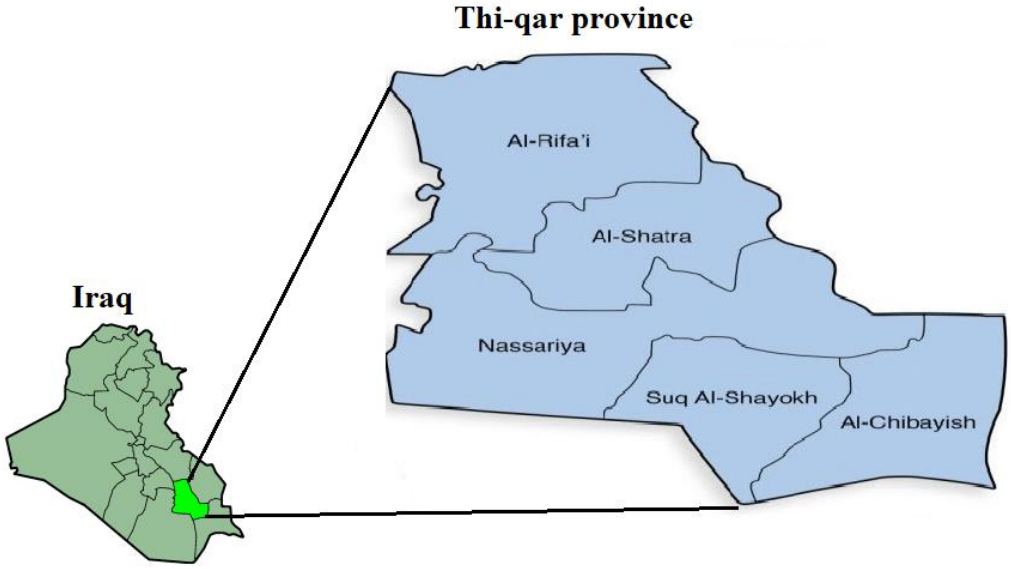

Figure 1. The geographical characteristics, where study was conducted.

Table 1. The list of the primers used in this study.

PCR round	Primers	Sequence (5′→3′)
First step	E-1	F: TAAGATGCACGAGAGCGAAA R: GTACAAAGGGCAGGGACGTA
Second step	EH-1	F: AAGCATTGTTTCTAGATCTGAG R: AAGCATTGTTTCTAGATCTGAG
	ED-1	F: TCTAATTTCGATTAGAACTCT R: TCCCTACCTATTAGACATAGC

2.6. Statistical analysis

SPSS software (ver. 24) was used for data analysis. Chi-square and logistic regression tests were used to compare the relationship between variables. $P < 0.05$ indicated a significant difference.

3. Results

3.1. Participants

A total of 80 patients ,including 50 patients with *E. histolytica* infection and 30 subjects in the control group without *E. histolytica* infection, were included in the present study. Among the patients with *E. histolytica* infection, 22 patients (44 %) were male while, 28 patients (56%) were female. In the control group, 14 subjects (46.66 %) were male while, 16 patients (53.33%) were female.

3.2. Immunological study

Interleukin concentration was investigated in the current study in patients with *E. histolytica* infection in Thi-Qar province, including IL-25 and IL-35. Table 2 shows the serum level of IL25 in patients with and without (control group) *E. histolytica* infection. The results showed a significant difference ($p<0.001$) in the serum level of IL-25 in patients with *E. histolytica* infection (4275.19 pg/mL) compared to individuals in the control group without *E. histolytica* infection (2186 pg/mL). The serum level of IL-35 was also checked in patients with *E. histolytica* infection. As shown in Table 3, the mean serum level of IL-35 in patients with *E. histolytica* infection was 7.05 pg/m in contrast, the mean serum level of IL-35 in the control group without *E. histolytica* infection was 6.87 pg/mL. Statistical analysis showed that there was no significant difference in the serum level of IL-35 patients with *E. histolytica* infection compared with individuals in the control group without *E. histolytica* infection.

Table 2. indicates the serum level of IL-25 among patients with or no (control group) *E. histolytica* infection. * highly significant difference (P<0.001) in comparison with the control group

Group	IL-25 level (pg/mL) Mean±SD	Male No. (%)	Female No. (%)	Total No. (%)
Patients	4275.19±498.4	22 (44.0)	28 (56.0)	50 (100)
Control	2186.6±147.1	14 (46.6)	16 (53.3)	30 (100)
P value	0.0272*	-	-	-

Table 3. indicates the serum level of IL-35 among patients with or no (control group) *E. histolytica* infection. * No significant differences (P<0.05).

Group	IL-35 level (pg/mL) Mean±SD	Male No. (%)	Female No. (%)	Total No. (%)
Patients	7.05±1.08	22 (44.0)	28 (56.0)	50 (100)
Control	6.87±0.58	14 (46.6)	16 (53.3)	30 (100)
P value	0.960*	-	-	-

4. Discussion

E. histolytica is considered one of the main health problems and it is the main reason for amoebiasis. Symptoms of this disease consist of several diagnostic signs, namely, fever, dysentery or diarrhea, dehydration, and abdominal pain (13). These results pointed to the effect of IL-25 on *E. histolytica* infection. The following study detected the role of IL-25 with amoebiasis by observing some objectives, increasing concentrations of IL-25 when the examination of patients infected with amoebic dysentery and healthy control. The study conducted by Moonah et al (14), indicates which epithelial cells in the human intestine can produce IL-25, that have a significant role in comparison to protective barriers in the bowel, also the existence of bacteria in the intestine, can induce intestinal epithelial cells for products IL-25. A similar study conducted by Feng and Mao (15) showed that a high concentration of IL-25 allows for the defense of *E. histolytica* as eosinophil-dependent (15).

The study carried out by Al-Salehy et al (16) in Thi-Qar province, Iraq reported a significant increase in WBC involving eosinophil-for male and female patients with diarrhea when compared with healthy. Another study conducted by Buonomo et al (17) showed that IL-25 increasing leads to induce and support from eosinophils in the intestine and protection against pathogens, thus the protecting role for eosinophils at amebiasis is the appearance which decreased eosinophil results (Charcot-Leiden crystals) are present over with trophozoites in the stool of patients with amoebiasis (18). Recently, Von Moltke et al (19) showed that IL-25 has been increased for developing mucus production in the intestine. Guo et al (20) reported that IFN-y, IL-17, and IL-25 participate in vaccine-induced protection in murine studies, and they detected propose a significant role for cell-mediated cytokine production in the protection of amoebiasis. Liu et al (21) demonstrated that the increased concentrations of

IL-25 can be results in inserted lymphoid type 2 cells (ILC2s) to induce IL-4, IL-5, IL-13, and other cytokines with the cytokines eosinophils produced in infectious disease. IL-25-produced inflammation is typically characterized by elevated concentration of type -2 cytokines that show pathological changes in the lungs and digestive tract like high serum IgE and IgG 1, rising mucus secretion, and epithelial cell hyperplasia (22). The current study showed that the concentration of IL-35 is low in patients this is no significant differences. Explain IL-35 showed a high concentration of 7.05 +/- 1.08 in serum samples of patients infected *E. histolytica*. The study conducted by Cao et al (23) in Chongqing, China reported that IL-35 concentration in serum specimens of adult and child patients and significantly higher compared with healthy and progressively increased according to sepsis severity. Another study conducted by Du et al (24) in Chongqing, PR China showed that the serum focuses of IL-35 were highly significant differences in the Neonate sepsis group compared with the healthy controls group. The study in Mexico City reported results proposal under a system of inflammation in work inflammatory bowel disease (IBD) in patients can be established in high concentration appearances of IL-35 (25). According to widespread studies in immunology, such as a study conducted by Banchereau et al (26) showed T cell that produces IL-35 and has a protected function can be produced in the intestine of mice infected with the intestine infected by *Trichuris muris* parasite. Choi et al (27) also found that IL-35 has an immunosuppressive impact on inflammation through induction from Treg cells and protection of Th 1 and Th 17. In line, Dong et al (28) reported when appear IL-35 in another chronic inflammatory disease and parasitic/bacterial infections was the inhibitor cytokines. The findings of the present investigation revealed the high levels of IL-25 in patients with *E. histolytica* infection; indicating the important role of IL-25 in activating the immune system during intestinal inflammation. Therefore, this cytokine can be used as a diagnosis marker for *E. histolytica* infection.

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Authors' Contribution

AKK and FAH contributed to the study concept and design. AKK and FAH collected the data. AKK drafted the manuscript. All authors read and approved the final manuscript.

Ethics

The work was carried out in line with the 1964 Helsinki declaration. The participants were notified about the aims and processes of the study and the description of their participation. Subsequently, written and signed informed consents were gathered from the participants (or parents or guardians of young children) before the beginning of the study. The study was reviewed and received the ethical approval from the ethics committee of University of Thi-qar, Iraq (No. 2022260).

Conflict of Interest

The authors declare that they have no competing interests.

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