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ORIGINAL ARTICLE

Neutrophil Gelatinase-associated Lipocalin and Chemokine CXC Motif Ligand-1 as Novel Biomarkers in Patients Infected with a Co-Infection of *Escherichia coli* O157:H7 and *Entamoeba histolytica*

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ABSTRACT

Background: *The Co-infection with the E. coli O157:H7 and E. histolytica elicits a strong inflammatory response, largely driven by the effects of the toxin, which damages endothelial cells and induces the activation of immune cells.* ***Objective:*** *The study aims to evaluate the immunological role of NGAL and CXCL-1 in patients infected with a co-infection of E. coli O157:H7 and E. histolytica.* ***Methodology:*** *A case-control study was performed, including 60 healthy individuals in the control group, 30 patients with co-infection by E. coli O157:H7 and E. histolytica, and 30 patients infected by E. histolytica alone. E. coli O157:H7 was diagnosed by chromogenic agar and PCR, and E. histolytica was diagnosed by wet mount examination with 0.85% saline and Lugol's iodine. The patients' blood levels of NGAL, and CXCL-1 were assessed by the ELISA technique. Results: The patients with co-infections (E. coli O157:H7 and E. histolytica) had a significantly higher level of NGAL (346.2 ± 12.97 ng/ml) than the patients with E. histolytica infection alone (238.4 ± 14.99 ng/ml), (both significantly higher than the normal value (96.40 ± 6.78 ng/ml)) ($P < 0.0001$. Consistently, CXCL-1 levels also significantly increased in co-infected patients (48.09 ± 3.87 ng/ml) as well as in patients with E. histolytica infection alone (30.19 ± 1.41 ng/ml) compared to their normal levels (15.06 ± 0.61 ng/ml) ($P < 0.0001$). Conclusions: Infection with co-pathogens of E. coli O157:H7 and E. histolytica elicits a more robust inflammatory response, as evidenced by increased levels of NGAL and CXCL-1 in this study.*

INTRODUCTION

Bacterial- protozoa co-infections currently represent a major challenge to the public health worldwide, particularly in regions of scarcity of clean water, sanitation, and healthcare[1]. *Escherichia coli* O157:H7, a pathogenic strain linked to hemorrhagic colitis and hemolytic uremic syndrome (HUS), and *Entamoeba histolytica*, the protozoan cause of amoebiasis, are two clinically relevant enteric pathogens[2]. When these two pathogens coexist in the gastrointestinal tract, the disease becomes complicated, not only for diagnosis, prognosis or treatment[3]. The similar symptomatology between these infections of diarrhea, abdominal pain, and systemic inflammation lead to delays in diagnosis and inappropriate management. In this context, finding trustworthy non-invasive biomarkers for better patient management is a priority, especially in the case of co-infections[4].

Neutrophil gelatinase-associated lipocalin (NGAL) and chemokine C-X-C ligand 1 (CXCL-1) have been recently identified as potential novel markers of infection and inflammation[5]. Neutrophil gelatinase-associated lipocalin is a 25-kDa glycoprotein produced by neutrophils and several epithelial cells, it is an important component of the innate immune response and an iron carrier protein. Neutrophil gelatinase-associated lipocalin has been described to be increased in bacterial infection, especially with Gram-negative bacteria such as *E. coli*, which can be explained by its bacteriostatic effects mediated through iron chelation[6]. Moreover, NGAL is a well-studied marker of renal injury and sepsis, rendering it a versatile marker of systemic inflammation[7].

In contrast, CXCL1, a member of the family of CXC chemokines, plays a major role in neutrophil recruitment toward sites of infection. It's up-regulation has been reported following bacterial endotoxins and protozoal infections like other PKR family members, which suggests its function in the regulation of the host immunity[8]. An increase in the CXCL1 level in enteric infections is thought to depend on both the pathogen load and the degree of enteric inflammation[9]. This desirably renders it a potential biomarker for discriminating among various kinds of GI infections and their severity.

The interconnection of interaction between bacterial and protozoal pathogens during co-infection creates a host that is presented with a unique challenge for the immune system, leaving them with exaggerated

inflammation and more tissue damage[10]. This leads to the question whether they are synergistically increased during co-infection, providing a specific biomarker signature for co-infection. Such a pattern of co-infections would have significant implications for early diagnostics as well as therapeutic interventions, particularly in endemic co-infection areas[11].

The present study analyzed the serum levels of NGAL and CXCL1 in patients with *E. coli* O157:H7 and *E. histolytica* infections and assessed the use of these two markers in combination for the early diagnosis and monitoring of infection. The ultimate goal was to increase knowledge of the inflammatory and immunologic responses occurring during co-infection and ultimately contribute to the design of more focused diagnostic tools.

METHODOLOGY

Participants in the study

It was a case–control study carried out at Gastroenterology Department of Al-Najaf Teaching Hospital, Iraq. It covers the span from 1 October 2023 to 1 March 2025. Sixty non-COVID healthy individuals were included as the controls. There were 30 patients with co-infection by *E. coli* O157:H7 / *E. histolytica* and 30 patients had *E. histolytica* infection only.

Ethical Considerations

The study was approved by the Institutional Ethics Committee of Faculty of Science—University of Kufa and College of Medicine.

Detection of *E. coli* O157:H7 and *E. histolytica*

E. coli O157:H7 were confirmed by Polymerase Chain Reaction (PCR) and chromogenic agar[12]. *E. histolytica* was diagnosed by wet mount examination with 0.85% saline and Lugol's iodine[13].

Quantitative ELISA Assay to measure NGAL and CCXL-1 levels in blood for patients

Blood sample (5 ml) were collected from all the patients and 2 ml of serum for each patient has been collected after centrifugation at 8000 rpm /10 min. The immune parameters NGAL and CCXL-1 were assessed in the peripheral blood[14]. The levels of using enzyme-linked immunosorbent assay (ELISA) technique (Accubiotech, China) with Kits (Bioassay Technology Laboratory. Shanghai, China), and a series concentration standards were set up to derive absorbance[15]. This enabled the concentrations to be calculated from absorbance measurements using the Beer-Lambert law for each of the two groups. Plates are coated with human NGAL monoclonal antibodies, which capture the sample's BDNF protein[16]. Biotinylated NGAL antibody is captured by bound streptavidin-HRP, and absorbance at 450 nm is determined. The identical is true for CCXL-1 concentration.

Statistical analysis

Values are means (±SE) with from triplicate determinations with GraphPad Prism. The threshold of $p<0.05$ was considered statistically significant for the analysis.

RESULTS

Escherichia coli O157:H7 diagnosis

Escherichia coli strains were identified by biochemical tests and grown on chromogenic agar (Fig. 1), with the formation of blue colonies corresponding to root containing the bacteria. The bacterial species was reconfirmed by PCR technique in which 614 and 779 bp bands (stx1 and stx2, respectively) were detected (Fig. 2)

Fig.1: *Escherichia coli* O157:H7 grown on chromogenic agar showed blue colonies

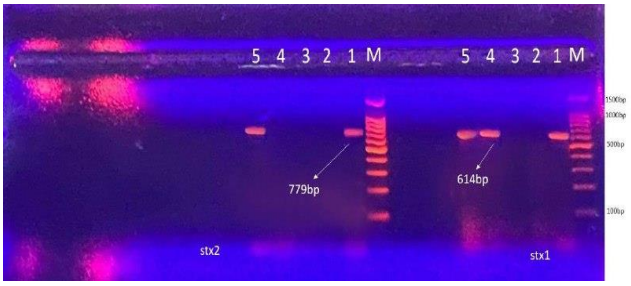

Fig.2: Detection of marked bands at 614 and 799 bp was observed in positive samples of *Escherichia coli* O157:H7 using the PCR technique.

Neutrophil gelatinase-associated lipocalin

Co-infected patients *E. coli* O157:H7 and *E. histolytica* had significantly higher serum NGAL levels (346.2 ± 12.97 ng/mL) compared with control group (96.40 ± 6.78 ng/mL) ($P < 0.0001$). Likewise, patients with *E. histolytica* infection only also presented significantly higher NGAL concentrations (238.4 ± 14.99 ng/mL) compared with controls ($P < 0.0001$). In addition NGAL levels were also significantly elevated in co-infected compared to the *E. histolytica* only-infected group ($P < 0.0001$) (Fig 3).

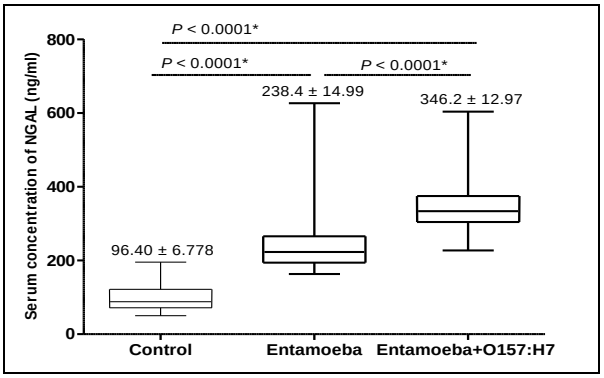

Fig.3: Serum NGAL levels in patients co-infected with *E. coli* O157:H7 and *E. histolytica*, as well as in those infected and with *E. histolytica* alone, compared to the control group

Chemokine CXC motif ligand-1

The highest concentration of serum CXCL-1 was recorded (48.09 ± 3.869 ng/mL) and significantly higher compared with the control group (15.06 ± 0.6051 ng/mL) (P < 0.0001) in *E. coli* O157:H7-Patients that were co-infected with *E. histolytica*. Likewise, patients with *E. histolytica* alone presented significantly higher levels of CXCL-1 (30.19 ± 1.408 ng/mL) than controls (P < 0.0001). Additionally, concentrations of CXCL-1 were significantly higher in co-infected as compared to *E. histolytica* infected (P < 0.0001) (Fig. 4).

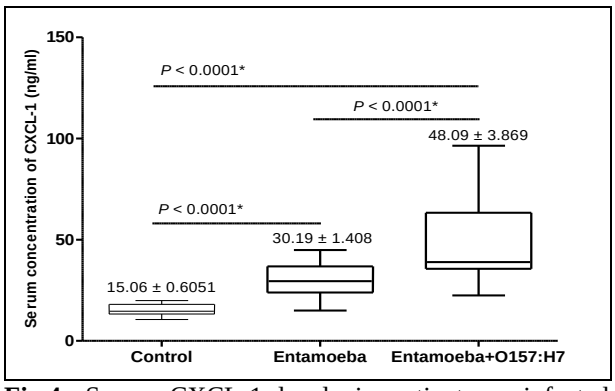

Fig.4: Serum CXCL-1 levels in patients co-infected with *E. coli* O157:H7 and *E. histolytica*, as well as in those infected and with *E. histolytica* alone, compared to the control group

The results showed positive correlation (Slope 2.606) between NGAL and CXCL-1 (Fig.5) in blood patients infected with co-infection of *E. coli* O157:H7 and *E. histolytica* (P 0.5471). Also, positive correlation was observed (Slope 2.010) (P 0.8063) in blood patients infected with *E. histolytica* (Fig.6).

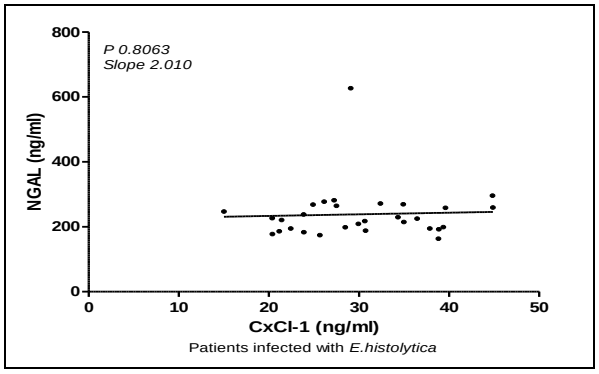

Fig.5: Correlation between total serum's levels of NGAL and CXCL-1 in patients infected with *E. histolytica*

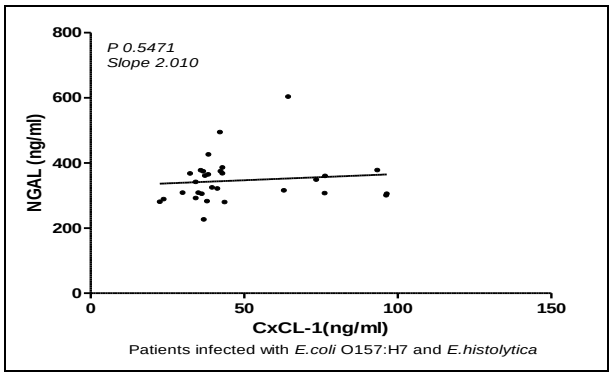

Fig.6: Correlation between total serum's levels of NGAL and CXCL-1 in patients infected with co-infection of *E. coli* O157:H7 and *E. histolytica*

DISCUSSION

Neutrophil gelatinase-associated lipocalin (NGAL), a 25-kDa glycoprotein, is emerging as a highly sensitive marker of inflammation and infection within the setting of epithelial injury and immune response[17]. Increased serum NGAL levels have also been described in various infections and inflammation. In gastrointestinal infections, NGAL mirrors neutrophil activation and mucosal damage, qualifying NGAL as a promising parameter for evaluating disease severity and systemic inflammation. This is particularly true for infections with *Escherichia coli* O157:H7 and *Entamoeba histolytica* that can produce severe gastrointestinal disease[18].

More recent prospective studies compared levels of serum NGAL of co-infected *E. coli* 157:H7 and *E. histolytica*, *E. histolytica* only infected and healthy subjects. Results consistently show that patients with co-infections have significantly higher serum NGAL levels than those with mono-infection or non-infection[19]. This increase is considered to be the result of the combined action of bacterial and protozoal

pathogens on the gut mucosa, resulting in increased neutrophil activity and increased epithelial damage[20].

In co-infected patients, the levels of serum NGAL were significantly higher than in patients infected with *E. histolytica* alone. This is perhaps due to the synergistic pathogenic mechanisms of the two pathogens. *E. coli* O157:H7 release Shiga toxins and cause endothelial destruction and an intense systemic immune response[21]. Meanwhile, *E. histolytica* penetrate the colonic epithelium leading to tissue damage and ulceration. This two-pronged insult to the GI tract is associated with augmented neutrophil recruitment and NGAL release, indicating an increased inflammatory load[22].

Mono-infected *E. histolytica* patients possessed higher levels of NGAL compared to healthy subjects, but to a lesser extent than co-infected subjects. The infection with *E. histolytica* would however be able to induce itself a strong local inflammation, reflected by neutrophils influx and oxidative stress[23]. The consequent epithelial injury and inflammation is reflected by the modest increase in serum NGAL levels, which are lower than those in co-infection[24].

By contrast, baseline circulating levels of NGAL in healthy control subjects were within the normal physiological range, which supports a relation between an increased level of NGAL and inflammation caused by infection[25]. These baseline concentrations constitute a reference determining the diagnostic value of NGAL as a tool for trying to differentiate between infected and uninfected status[26].

Serum CXCL-1 concentrations in patients with gastrointestinal infections were significantly different between the groups. Notably, in infected patients co-infected with *Escherichia coli* O157:H7 and *Entamoeba histolytica*, serum CXCL-1 levels are significantly higher than in patients infected solely with *E. histolytica* or in healthy control individuals[27]. The co-infection causes highly inflammatory response, possibly as a result of the syntropic actions of the bacterial and protozoal agents. O157:H7 produces Shiga-like toxins, leading to endothelial damage and release of cytokines, and *E. histolytica* invades the colonic mucosa, resulting in ulceration and direct damage to epithelial cells[28].

The synergy of these two pathogens seems to enhance neutrophil recruitment and pro-inflammatory cytokine expression, generating a marked increase in the concentration of CXCL-1. Those elevated levels seen in co-infected hosts probably are mirror local immune activation in the intestinal mucosa as well as systemic immune activation[29]. Furthermore, the amplitude of these responses is proportional to the level of symptoms, such as diarrhea, abdominal pain, and mucosal ulceration, supporting CXCL-1 as a possible marker for disease severity[30].

Patients with *E. histolytica* alone have higher CXCL-1 levels compared to the control group. The parasite causes inflammation in the vicinity that is characterized by neutrophil migration to the site, tissue necrosis, and release of cytokines[31]. This inflammatory effect alone already up-regulates the CXCL-1 expression implying its contribution to the early antiprotozoal innate immune response[32]. However, the lack of Shiga toxin-associated endothelial injury in mono-infection suppresses the systemic inflammatory magnitude such that the serum CXCL-1 concentration is somewhat elevated, rather than severely elevated as observed[33].

CONCLUSION

In conclusion, the serum levels of NGAL and CXCL-1 are promising candidate biomarkers for the grading of the severity of GI infections. NGAL is also significantly higher in co-infected patients and may therefore be helpful in identifying infection, tissue injury, and disease severity. Also, CXCL-1 correlates with severity of inflammation, particularly in double infections like *E. coli* O157:H7+*E. histolytica*, suggesting its involvement in neutrophilic-derived response. Both markers might be useful for diagnosing, to follow response to therapy and for clinical management. Nevertheless, future studies are required to validate their prognostic significances and benefit in the daily practical clinic.

Conflict of interest

The authors insured there was no conflict of interest in this study

Patient Declarations

All patients in the study (including controls for blood sampling from healthy individuals) provided written consent. This research was completed with great cooperation between them.

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