

Research Article

Assessment of CCL20, STAT1 and Nuclear factor-kappa β in patients Infected with *Cryptosporidium parvum*

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A B S T R A C T

Introduction: *Cryptosporidium parvum* is a coccidian protozoan parasite that infects humans and animals. It is one of the most commonly reported enteric parasites in both immunocompetent and immunocompromised individuals worldwide.

Aims : To detect *Cryptosporidium* infection in fecal samples using microscopic examination with modified Ziehl-Neelsen (MZN) staining and qualitative immunochromatographic assay, followed by the detection of *C. parvum* antigen using ELISA. In addition, the study aimed to evaluate serum levels of chemokine C-C motif ligand 20 (CCL20), signal transducer and activator of transcription 1 (STAT1), and nuclear factor-kappa β (NF-KB) in the studied patients.

Method: The study included 450 children under two years of age presenting with acute or persistent diarrhea and attending hospitals and private clinics in Salah Al-Din Governorate, Iraq. A total of 450 stool samples were collected between July 2022 and September 2023 and examined for *Cryptosporidium* infection. In addition 20 apparently healthy children with no history of diarrhea or gastrointestinal infection were included as a control group for immunological assay.

Results: Among the 450 stool samples examined , 40 (8.88%) tested positive for *Cryptosporidium* by MZN, while 410 (91.11%) tested negative. Using the rapid immunochromatographic assay, 65 (14.44%) samples tested positive and 385 (85.55%) tested negative. Subsequently, 90 selected stool samples previously reported positive for *Cryptosporidium* by either the rapid immunochromatographic assay or microscopic examination were analyzed using the copro-antigen ELISA assay. Of these 70 (77.8%) tested positive for *C. parvum* antigen, while 20 (22.2%) tested negative. The present immunological study recorded a highly significant increase in the levels of CCL20, STAT1, and NF-KB in the patients' serum compared to the control group (231.92 ± 70.59 pg/mL, 809.33 ± 287.74 pg/mL, 8.09 ± 2.68 ng/mL) compared to the healthy group (86.0 ± 25.48 pg/mL, 359.44 ± 94.30 pg/mL, 5.14 ± 1.15 ng/mL).

Conclusions: Modified Ziehl Neelsen staining was useful for the detection of *Cryptosporidium* oocyst in stool samples. In addition, the

copro-antigen ELISA detected *C. parvum* antigen in the selected stool samples. Children positive for *C. parvum* antigen by ELISA showed significant difference in serum levels of CCL20, STAT1, and NF- κ B compared with healthy control, suggesting an association between *C. parvum* antigen positivity and alterations in these immune related markers.

Keywords: *Cryptosporidium parvum*; MZN; ELISA; CCL20; STAT1; Nuclear Factor-kappa B

Introduction

Cryptosporidium parvum, an obligate intracellular parasite belonging to the phylum Apicomplexa, is frequently found in the small intestines of both healthy and immunocompromised people globally. *Cryptosporidium* was identified as a significant intestinal pathogen in humans in 1976.¹ In Iraq, specifically in Kirkuk, the rate of prevalence of the *C. parvum* was 61.81% in a total of 330 samples.² Since then, about 20 *Cryptosporidium* species capable of infecting humans have been identified, with *C. hominis* and *C. parvum* being the most commonly found. The infection leads to short-term gastrointestinal illness in patients with a strong immune system. These symptoms persist longer in patients with weakened immune systems. There are no established curative treatments³ for this infection. The parasites grow and multiply inside the parasitophorous vacuole located in the microvillus layer of the gastrointestinal epithelial cells. *Cryptosporidium* produces lesions in the distal small intestine, causing the host to have acute watery diarrhea that typically lasts for 5 to 10 days.⁴ CCL20, sometimes referred to as an active protein, is produced in several organs and by a diverse array of immune cells such as neutrophils, natural killer cells, Th17 cells, and B cells.^{5,6} CCL20 is typically found in inflamed tissues and areas with immunological activity, indicating its role as an inflammatory chemokine involved in the immune response to microorganisms and sporozoites.⁷ NF- κ B signaling controls induced transcriptional modifications. NF- κ B was first found in B cells because of its strong and enhanced connection to enhancer genes.⁸ Invasion of enterocytes by *C. parvum* triggers NF- κ B signaling, leading to the synthesis of chemokines that prevent immune cells from reaching the infection site.⁹ STAT1 is the primary and crucial transcription factor. STAT1 is a key transcription factor involved in interferon- γ (IFN- γ)-mediated immune responses. Following IFN- γ stimulation, STAT1 activation promotes the expression of interferon-responsive genes, including indoleamine 2,3-dioxygenase 1 (IDO1). IDO1 catalyzes the degradation of tryptophan, an essential amino acid required for the growth and proliferation of many intracellular pathogens. Therefore, activation of the STAT1–IDO1 pathway may contribute to host defense against *C.*

parvum by reducing the availability of tryptophan required for parasite proliferation [10]. The study was conducted due to public health importance of *Cryptosporidium* infection and its association with diarrhea among children in Iraq. The aim of this study was to detect *Cryptosporidium* infection in stool samples using microscopic examination and a qualitative immunochromatographic assay (CERTEST Crypto), followed by the detection of *C. parvum* antigen using ELISA. In addition, serum levels of CCL20, STAT1, and NF- κ B were measured to evaluate their association with *Cryptosporidium* infection among children with diarrhea in Tikrit Governorate, Iraq.

Materials and Methods

Subjects

The study population comprised 450 children under 2 years of age presenting with acute or persistent diarrhea and admitted to and private clinics in Salah al-Din City. Samples were collected from July 2022 to September 2023. All 450 samples were examined using MZN staining method and the Rapid cassette immunochromatographic assay. For further copro-antigen analysis by ELISA, a subset of 90 samples was examined. This subset comprised all 65 samples that tested positive the Rapid Cassette assay and 25 samples randomly selected from microscopy positive cases.

Fecal and blood sample collection

Both male and female children were included in the study, and the required demographic and clinical information was recorded using a standardized questionnaire. Stool samples were initially examined microscopically for the presence of *Cryptosporidium* oocysts and were also tested using a rapid immunochromatographic assay for *Cryptosporidium* antigen. For subsequent copro-antigen ELISA analysis, 90 stool samples were selected, comprising all 65 samples positive by the rapid immunochromatographic assay and additional 25 samples randomly selected from microscopy positive cases. The selected stool samples were stored at -20°C until further analysis in the parasitology laboratory.

For immunological analysis, blood samples were obtained from the 70 children whose stool samples tested positive in microscopic and rapid immunochromatographic assay and from 20 apparently healthy, age and sex matched children who served as the control group. The control children had not experienced diarrhea during the 72 hours preceding sample collection and had no clinical signs or history of gastrointestinal infection at the time of enrollment. Approximately 5-10 mL of venous blood was collected into serum separator gel tubes and centrifuged at 4000 rpm for 15 min. the separated serum was transferred into clean plastic tubes and stored at -20°C until measurement of CCL2, STAT1, and NF- κ B.

Diagnosis of *Cryptosporidium parvum* by microscopic examination

Cryptosporidium oocysts in stool samples were examined using a modified acid-fast staining technique.¹⁰ This method enables microscopic visualization of acid fast *Cryptosporidium* oocysts in fecal specimens. The stained smears were examined under light microscope using the oil immersion objective. *Cryptosporidium* oocysts were identified microscopically as pink to red, spherical to ovoid acid fast structures against a blue to purple background.¹¹

Diagnosis of *Cryptosporidium parvum* by Rapid Immunochromatographic Test

Cryptosporidium antigen in stool samples was detected using the CerTest Crypto + Giardia + Entamoeba Combo Card Test (CerTest Biotec S.L., Zaragoza, Spain), a qualitative lateral-flow immunochromatographic assay. The test was performed according to the manufacturer's instructions, and the results were interpreted based on the appearance of the corresponding control and test lines.

Cryptosporidium parvum detection by ELISA

Ninety stool samples were analyzed using a sandwich ELISA method for the detection of *Cryptosporidium parvum* antigen. The samples included stool specimens randomly selected from microscopy positive cases, together with specimens that tested positive using the Rapid cassette immunochromatographic assay. The EDI™ Fecal *Cryptosporidium parvum* Antigen ELISA kit (KTR-839; Epitope Diagnostics Inc., USA) a sandwich ELISA designed for the Qualitative detection of *C. parvum* antigen in fecal specimens, was used according to manufacturer's instructions.

Human, STAT1, CCL20, NF-kB ELISA test

Serum concentration of STAT1, CCL20, and NF-kB were measured in 70 children whose stool samples tested positive for *C. parvum* antigen by ELISA and in 20 apparently healthy children serving as controls. The three biomarkers were measured separately using quantitative sandwich ELISA kits (SunLong Biotech, China), according to manufacturer instructions, and the absorbance was measured at 450 nm using a microplate reader. Biomarker concentrations were calculated from the corresponding standard curves. The assay ranges were 10-800 pg/mL for STAT1, 1-100 pg/mL for CCL20, and 0.06-4 ng/mL for NF-kB.

Statistical analysis

statistical analyses were performed using R software (version 4.5.3). The normality of data was assessed using ShapiroWilk test . Differences between the *C. parvum* antigen-positive patient and control group were evaluated using Mann-Whitney U test. Descriptive data were summarized using the mean and standard deviation (SD).

A p-value <0.05 was considered statistically significant. Graphical representations were generated using the ggplot2 package in R.

Results

Detection of *Cryptosporidium* oocyst using Modified Ziehl-Nelsen Stain

Using the MZN staining method, 40 of 450 stool samples (8.88%) tested positive for *Cryptosporidium*, whereas 410 (91.11%) tested negative, as shown in Table 1. Microscopically, *Cryptosporidium* oocyst appeared as spherical, reddish-purple acid fast against a blue stained background (Figure 1).

Table 1. Diagnosis of *Cryptosporidium* oocyst using MZN stain

Assay	Total number of examined sample	Negative result (-ve)	Positive result (+ve)
Modified Ziehl-Neelsen stain	450	410 (91.11 %)	40 (8.88%)

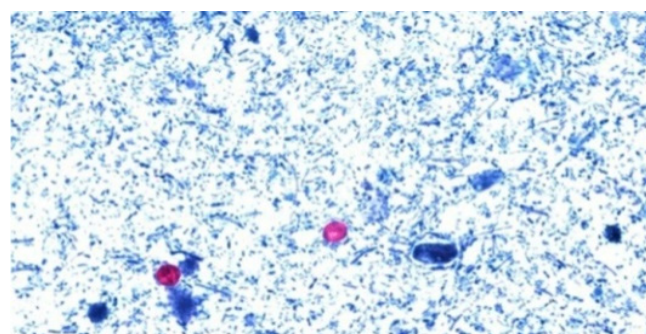


Figure 1. *Cryptosporidium* oocysts detected in stool samples from children using MZN staining (100x oil immersion objective)

Detection of *Cryptosporidium* antigen using immunochromatographic assay in stool samples

Using the rapid immunochromatographic assay, 65 of the 450 stool samples (14.44%) tested positive for *Cryptosporidium* antigen, whereas 385 (85.55%) tested negative, as shown in Table 2 and Figure 2.

Table 2. Detection *Cryptosporidium* antigen rapid immunochromatographic assay

Assay	Total number of examined sample	Negative result (-ve)	Positive result (+ve)
Chromatography Assay	450	385 (85.55 %)	65 (14.44%)

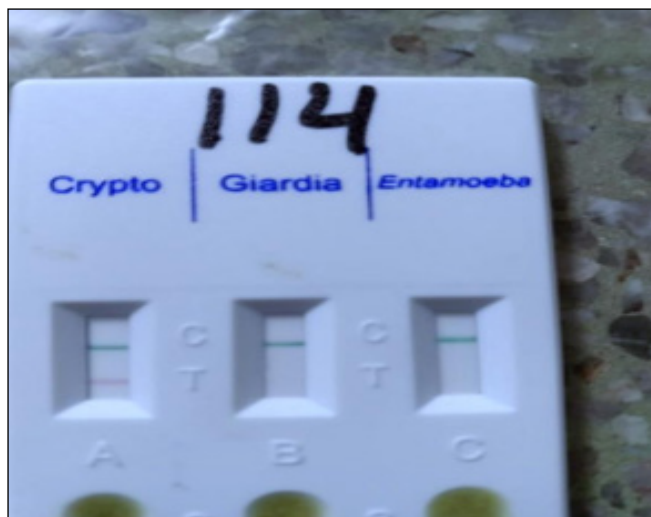


Figure 2. Positive result for *Cryptosporidium* antigen using rapid immunochromatographic assay



Figure 3. Negative result for *Cryptosporidium* antigen using rapid immunochromatographic assay

The ELISA assay detected 70 positive samples (77.8%) out of 90 examined samples. In contrast, 20 samples (22.2%) were negative. The results are presented in Table (3) and Figure (4).

Table 3. *C. parvum* infection rate using ELISA – copro antigen technique

Assay	Total number of examined sample	Negative result (-ve)	Positive result (+ve)
ELISA copro-antigen	90	20 (22.2 %)	70 (77.8%)

Assessment of serum CCL20 levels in children positive for *C. parvum* antigen

The current immunological study recorded a highly significant increase in the level of CCL20 in infected children (231.6 ± 11.1 pg/ml) compared to the control group (86.0 ± 25.48 pg/ml). Table 4 and Figure 4.

Table 4. Serum CCL20 levels in children positive for *C. parvum* antigen and healthy controls

CCL20 pg/ml	Patients group N=70	Control group N=20	P. value
Mean $\pm$ SD	231.92 ± 70.59	86.0 ± 25.48	<0.001

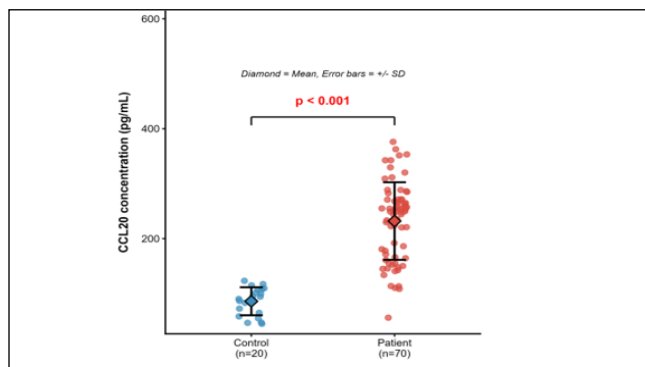


Figure 4. Serum CCL20 concentrations in *C. parvum* antigen-positive patients and healthy controls. Individual observations are shown together with mean $\pm$ SD. The p-value was obtained using the Mann-Whitney U test

Assessment of serum STAT1 levels in children positive for *C. parvum* antigen

The current immunological study recorded a highly significant increase in the level of STAT1 in the infected children (809.33 ± 287.74 pg/mL) compared to the control group (359.44 ± 94.30 pg/mL), as shown in Table 5 and Figure 5.

Table 5. Serum STAT1 levels in children positive for *C. parvum* antigen and healthy controls

STAT1 pg/mL	Patients group N=70	Control group N=20	P. value
Mean $\pm$ SD	809.33 ± 287.74	359.44 ± 94.30	<0.001

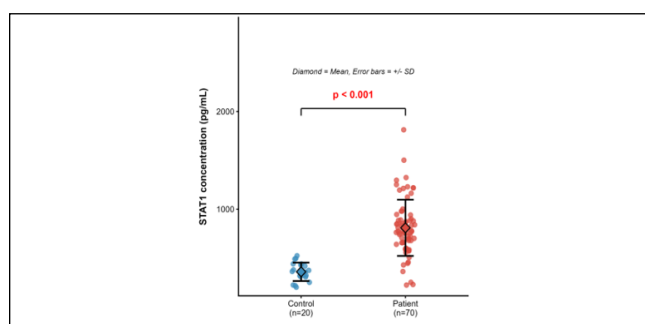


Figure 5. Serum STAT1 concentrations in *C. parvum* antigen-positive patients and healthy controls. Individual observations are shown together with mean $\pm$ SD. The p-value was obtained using the Mann-Whitney U test

Assessment of serum NF-kB levels in children positive for *C. parvum* antigen

The current study recorded a highly significant increase in the level of NF-kB in the infected individuals (8.09 ± 2.68 ng/mL) compared to the control group (5.14 ± 1.15 ng/mL) as shown in Table 6 and Figure 6.

Table 6. Serum NF-kB levels in children positive for *C. parvum* antigen and healthy controls

NF- kB ng/mL	Patients group N=70	Control group N=20	P. value
Mean $\pm$ SD	8.09 ± 2.68	5.14 ± 1.15	<0.001

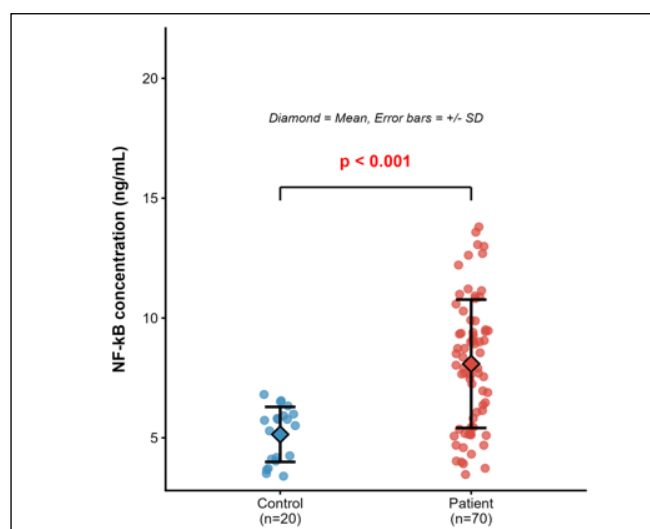


Figure 1. Serum NF-kB concentrations in *C. parvum* antigen-positive patients and healthy controls. Individual observations are shown together with mean $\pm$ SD. The p-value was obtained using the Mann-Whitney U test

Discussion

Cryptosporidium spp. are enteric protozoan parasite that infect epithelial cells of the small intestine, and are an important cause of diarrhea.¹² In the present study, MZN staining detected *Cryptosporidium* oocyst in 8.88% (40/450) of the examined stool samples, whereas the rapid immunochromatographic assay detected *Cryptosporidium* antigen in 14.44% (65/450) of the samples. Variations in *Cryptosporidium* positivity rates among different studies may attributed to several factors, including differences in geographical and environmental conditions, study populations, exposure to potential sources of infection, and the diagnostic methods employed.¹³ Previous studies conducted in different Iraqi cities have reported variable rates of *Cryptosporidium* detection including 39.13% in Ramadi¹⁴, 14.3% in Mosul¹⁵, 6.6% in Al-Hilla¹⁶, 6.6% in

Diwaniyah¹⁷, and 8.2% in Samarra.¹⁸ Higher rates were also reported in Baghdad (34%)¹⁹ and Kirkuk (16.28%).²⁰ Studies from neighboring countries have similarly shown considerable variation, with reported rates of 1.7% in Iran²¹ and 17% in Turkey.²

The modified Zielh-Neelson procedure is widely used conventional technique for the microscopic detection of *Cryptosporidium* oocysts in stool samples because of its relatively inexpensive and simple to perform. The method typically demonstrates the oocyst as red to pink acid-fast structures against blue background. However, microscopic examination can be labor-intensive, require experienced personnel, and does not allow reliable species level identification of *Cryptosporidium*.

In the present study, the rapid immunochromatographic assay showed a higher positivity rate for *Cryptosporidium* antigen (14.44%) than MZN microscopy (8.88%). Variation in positivity rates according to the diagnostic method has been reported in Iraq. Younis and Alkhasha¹⁵ reported positivity rates of 40% using stool ELISA, 36% using blood ELISA, 35% using MZN staining, and 30% using the flotation method among children in Nineveh Province. These variations may be related, at least in part, to differences in the detection principles of the methods used; antigen based assays detect parasite antigens in stool, whereas MZN microscopy depends on the direct visualization of *Cryptosporidium* oocysts.

In the present study, serum CCL20 levels were significantly higher $p < 0.05$ in children positive for *C. parvum* antigen (231.92 ± 11.1 pg/mL) than in healthy controls (86.0 ± 25.48 pg/mL). This finding is consistent with previous evidence demonstrating increased CCL20 expression in response to inflammatory and microbial stimuli.⁷ CCL20 is constitutively expressed in the intestinal mucosa and possesses both chemotactic and antimicrobial properties, while its expression can be enhanced by various danger signals.²² During microbial infection, increased intestinal CCL20 production contributes to the recruitment of immune cells and may participate in the development of Th17 associated mucosal immune responses.²³ These mechanisms may help explain the altered CCL20 levels observed in children positive for *C. parvum* antigen in the present study.

The elevated CCL20 levels observed in patients with *C. parvum* infection may reflect activation of the innate immune response and the associated intestinal inflammatory response. CCL20 is produced by a diverse array of immune cells, including neutrophils, natural killer cells, T-helper type 17 (Th17) cells, B cells, and a variety of antigen-presenting cells, such as dendritic cells (DCs), Langerhans cells, and macrophages.²⁴ CCL20 is typically detected in tissues that are characterized by immune activation and inflammation,

which implies that it is an inflammatory chemokine and is involved in the immune response to microorganisms.⁸ Research has demonstrated that the host immune response is influenced by parasitic infection and microorganisms, which results in an increase in the production of CCL20 through a mechanism that is dependent on the Toll-like receptor 10 (TLR10).²⁵ Furthermore, the recruitment of DCs necessary to control infection has been demonstrated to be facilitated by the increased production of CCL20 during infection by *Yersinia enterocolitica*.²³

The present study demonstrated significantly higher serum levels of NF- κ B (8.09 ± 2.68 ng/mL vs. 5.14 ± 1.15 ng/mL) and STAT1 (809.33 ± 287.74 pg/mL vs. 359.44 ± 94.30 pg/mL) in children positive for *C. parvum* antigen compared with healthy controls, these findings may reflect alterations in the host immune and inflammatory responses associated with *Cryptosporidium* infection. However, the measured serum concentrations of NF- κ B and STAT1 represent circulating protein levels; further studies assessing NF- κ B and STAT1 are required to determine the activation status of these signaling pathways.

Intestinal epithelial cells are essential for both innate and adaptive immune responses during a microbial infection of the gastrointestinal tract. Toll-like receptors (TLRs) and other pathogen-recognition receptors are expressed by these epithelial cells. When TLRs identify a pathogen, they trigger a series of adaptor proteins (such as myeloid differentiation protein 88 (MyD88), which in turn trigger the activation of several intracellular regulatory signals, such as the signal transducers and activators of transcription (STAT) signaling pathway and nuclear factor κ B (NF- κ B). A sequence of host cell defensive responses against infection is triggered by the activation of various signaling pathways. These precisely regulated epithelial defense responses reflect a delicate balance between effective immune defense against *C. parvum* and the preservation of intestinal epithelial integrity.²⁶

Epithelial cells harboring *Cryptosporidium* infection have the ability to trigger antiapoptotic cell death signaling through NF- κ B signaling, potentially promoting parasite survival.²⁷ IFN- γ -dependent gene transactivation in the intestinal epithelium may be suppressed by the ability of *C. parvum* to reduce STAT1 α levels, a crucial transcription factor involved in IFN- γ signaling.²⁸ The expression of C-C motif chemokine ligand 20 (CCL20), a chemokine with potential antiparasitic properties that may contribute to parasite clearance, is suppressed when host epithelial cells become infected with *C. parvum*.⁷

Conclusion

Cryptosporidium was detected among children <2 years of age with diarrhea in Salah al-Din Governorate using MZN

microscopy and a rapid immunochromatographic assay, with positivity rates of 8.88% and 14.44%, respectively. Among the 90 selected stool samples examined by ELISA, 70 (77.8%) tested positive for *C. parvum* antigen. In addition, children positive for *C. parvum* antigen showed significant alterations in serum levels of CCL20, STAT1, and NF- κ B compared with healthy control, suggesting an association between *C. parvum* antigen positivity and changes in these immune related markers.

Abbreviations

MZN: modified Ziehl-Neelson; CCL20: CC motif Ligand 20; STAT1: Signal transducer and activator of transcription1; NF- κ B: Nuclear factor-kappa B cell; IFN γ : interferon γ ; IDO: Indolamine 2,3 Dioxygenase; *C. parvum*: *Cryptosporidium parvum*

Declarations

Ethics approval and consent to participate

In accordance with administrative order 13896, the study was authorized on September 21, 2022, by the Scientific Committee of the Salah al-Din Health Department and the Scientific Committee of the College of Science/Tikrit University. In order to gather samples from the children, the questionnaire was filled out after their parents gave their consent for the study. The usual symptoms, name, and address were filled out with the consent of the parents.

Conflict of interest: None

Source of Funding: None

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mohammed Abbas Saab, and Ali Mohammed Abed. The first draft of the manuscript was written by Mohammed Abbas Saab, and Ali Mohammed Abed commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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