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Immuno-Molecular Study of Cryptosporidiosis among Diarrheic Children in Erbil City, Kurdistan Region-Iraq

Lana Othman Kanabe[1], Suhaila Nafea Darogha[2*]

[1,2]*Department of Biology, College of Education, Salahaddin University-Erbil, Erbil, Iraq.*
[2*]*E-mail: suhaila.darogha@su.edu.krd*

Abstract: ***This study was carried out in Raparin Pediatric hospital in Erbil city. Stool samples (No= 548) were collected from children of less than 1 month to 13 years of age of both genders. The study was designed to assess some immunological parameters with molecular characterization based on sequence analysis of Cryptosporidium spp. positive group compared to the control group. The level of C-reactive protein in cryptosporidiosis positive group showed an extremely significant (p < 0.01) elevation with the mean value of (16.14 ± 3.41) when compared to control group with the mean value of (4.80 ± 1.30). Complement components C3 and C4 showed a significant increase in cryptosporidiosis positive group with mean values of 209.30 ± 36.97 and 1.74 ± 0.016) respectively compared to control group with mean values of 136.00 ± 6.59 and 1.59 ± 0.025 respectively. The results of IgG mean value in the present study showed statistically a non-significant difference among cryptosporidiosis positive group (1751.0 ± 101.5) compared to control group (1586.0 ± 116.0). On the other hand, serum levels of the IgE, IgA and IgM among cryptosporidiosis positive group showed a significant increase (384.0 ± 35.7, 447.0 ± 21.1 and 298.3 ± 16.25) respectively in compared to control group (107.0 ± 24.8, 308 ± 32.9 and 235.2 ± 24.97 respectively). A significant increase (p<0.0001) of IL-18 was observed in cryptosporidiosis positive group (84.11 ± 6.098 ng/L) compared to control group (32.37 ± 4.668 ng/L). A significant increase of TNF-α was observed in cryptosporidiosis positive group (2.68 ± 0.03 ng/L) when compared to control group (2.30 ± 0.06 ng/L).Out of 548 stool samples examined with modified Zhiehl-Neelson (ZN) technique, 59 (10.77%) of them were positive for Cryptosporidium spp. followed by formalin-ether ZN technique with the rate of 10.45% from the total 276 examined stool samples. Then 70 and 21 stool samples with the previously mentioned methods were subjected to immunochromatographic (IC) and PCR-sequence methods respectively and the results showed that the rate 100% of infection with Cryptosporidium spp. for 50 and 15 positive stool samples respectively that previously identified with ZN techniques. On the other hand, 20 and 6 negative samples were obtained for both IC and PCR-sequence methods respectively. The expected amplicon size for CPB-DIAG (18S rDNA) of Cryptosporidium spp. is 450bp, applying molecular procedure based PCR sequencing tool results got from sequencing were only 400 bp (missing 50 bp due to the quality of nucleotide sequence). BLAST program from Genbank was used to compare the results of amplified sequences of the present study with other stored species of Cryptosporidiumsequences. The results indicated that the query sequence was 100% identical to Cryptosporidiumparvum.***

Keywords: Cryptosporidiosis, Complement, Antibodies, Molecular Methods.

1. INTRODUCTION

The parasitic infection is a common cause of morbidity and mortality in pediatrics population in tropical countries. The prevalence of intestinal parasites in children varies in different regions of the world (Wadood *et al*., 2005).*Cryptosporidium parvum, Giardia lamblia and Entamoebahistolytica* are the most common diarrhea causing parasitic protozoa (Ali *et al*., 2005).

Cryptosporidiosis is a zoonotic disease of medical and veterinary importance, caused by the intestinal apicomplexan protozoan parasite of the genus *Cryptosporidium*, an epidemiological study investigated that the cause and effect of diarrhea in over 22,000 children (under 5 years of age) identified cryptosporidiosis as the second most common pathogen after rotavirus that responsible for diarrhea and 30-50% of globally death in children (Striepen, 2002; Snelling *et al*., 2007; Kotloff *et al*., 2013).

The host immune response to *Cryptosporidium* involves components of both the innate and adaptive immune systems. The presence of *Cryptosporidium*-specific antibodies (Abs) is indicative of exposure to the parasite and is widely used to estimate seroprevalence. Titers of IgM, IgG, IgA and IgE increase during infection and decline after recovery, although IgG in serum may persist for several months longer than IgM (Hill *et al*., 1993; Priest *et al*., 2006). Cytokines are secreted or membrane-bound proteins which are produced by both innate and adaptive immune cells in response to microbes and tumor Ag, and can stimulate cell activation, growth and differentiation (Kaplan *et al*., 1998). In intestinal parasitic protozoan infection, levels of several cytokines, including TNFα and IL-6, as well as CRP was even more elevated in-patient sera (Bayraktar *et al*., 2005). Interleukin-18 is a proinflammatory important cytokine in the control of *Cryptosporidium* infection, which is secreted by IECs, macrophages, and dendritic cells at sites of infection (Choudhry *et al*., 2012).Although the cytokine is associated with pathology of numerous diseases, IL-18 produced by IECs has been demonstrated to play a key part in maintaining epithelial integrity during inflammation (Zaki *et al*., 2010). McDonald *et al*. (2006) demonstrated that IL-18 inhibited parasite reproduction in human intestinal cell lines and that the cytokine increased IEC production of a β-defensin shown to inactivate *C. parvum* sporozoites.

Tumor necrosis factor-α is an important inflammatory cytokine in immune regulation and resistance to various microbes including protozoa (Burns *et al*., 1996). TNF-α is believed to be involved in recruiting inflammatory cells to the intestinal mucosa (Gibson, 2004). Infected children with cryptosporidiosis expressed higher levels of TNF-α up to 6 months after infection when they were restimulated *in vitro*, suggesting that cryptosporidiosis triggers a systemic immune response which may lead to enhanced inflammation and possibly be responsible for worsening of malnutrition and stunting (Kirkpatrick *et al*., 2006).

To date, there is few data available on the immune response and molecular based characterization of children with *Cryptosporidium* spp. in Iraq, so the present study aimed to determinate the efficacy of some laboratory techniques in diagnosing of *Cryptosporidium* spp. and evaluation of certain immunological parameters to provide an understanding of the immune response towards the parasite such as IL-18, TNF-α, C3, C4, IgG, IgM, IgA, IgE and CRP titer.

2. MATERIALS AND METHODS

The study was achieved on 740 children suffering from diarrhea attending Raparin Pediatric Hospital in Erbil province from both genders, but the respond rate examined group was 74.1

%.Total of 20 asymptomatic (apparently healthy) children, results of cryptosporidiosis were negative.

Stool Sample

Stool samples were collected from the children and control in a clean, sterile, disposable plastic, tightly screw-cap containers, with and without preservatives. The stool samples with preservatives were mixed with potassium dichromate ($K_2Cr_2O_7$) solution 1/10 (one part of stool sample was mixed in 9 parts of potassium dichromate solution), while the samples without preservatives were stored at -20°C.

Blood Samples

Venous blood samples were collected aseptically from infected children with cryptosporidiosis and control group, 3-4 ml of blood was collected into plain universal tubes allowed to clot and then centrifuged at 2500-3000 rpm for 15 min. Obtain serum used for determination of the immunological parameters by ELISA, which include (IL-18, TNF-α), the serum level of immunoglobulins (IgA, IgG and IgM) complement proteins (C3 and C4) were evaluated by immunodiffusion plate method, IgE and CRP evaluated by automated cobse E411 analyzer.

Stool Sample Examination

For identifying *Cryptosporidium* spp. oocysts the following laboratory methods were performed: Direct wet-mount examination, Saline wet-mount, Iodine wet-mount Indirect (concentration) techniques, Modified Ziehl-Neelson staining technique, Immunochromatographic assay, Rotavirus and Adenovirus detection by IC assay and Molecular-based identification of *Cryptosporidium* spp. (Brown, 1969; Faust *et* al., 1978; WHO, 1991; Markell *et al*., 1999).

Molecular-based Identification of Cryptosporidium spp

Genomic DNA from 15 positive and 5 negative stool samples with *Cryptosporidium* spp.by ZN and IC methods were randomly selected and subjected to DNA extraction by employing kit of extraction (PrimePrep^{TM}). Both positive (*Cryptosporidium* DNA) and negative (containing all PCR reagents without DNA template) controls were applied in each PCR round to validate the results. PCR was performed to amplify *Cryptosporidium* target genes by amplifying of 450 bp of the 18S small subunit ribosomal RNA (18S SSU rRNA) gene, which was carried out using forward primer 5' –AAG CTG GTA GTT GGA TTT CTG- 3' (F, 21 mer), and reverse primer 5' –TAA GGT GCT GAA GGA GTA AGG- 3' (R, 21 mer). In the amplification process the master mixture was involved 1.5µl for the primer, 1.5µl of DNA sample and 47µl of one PCR^{tm}. One PCR^{tm} was a ready to use PCR reaction mixture and consisted of taqDNA polymerase, PCR buffer, dNTPs, gel loading dyes and novel green, it contained the fluorescence dye which was directly detect on the light trans illuminator or UV epi-illuminator after the DNA electrophoreses.

3. RESULTS AND DISCUSSION

The level of CRP-titer in cryptosporidiosis positive group showed an extremely significant ($p < 0.01$) elevation with the mean value of (16.14 ± 3.413) when compared to healthy controls with the mean value of (4.800 ± 1.303) as shown in Table (1) and Fig. (1).

Table 1: Mean ± SEM of CRP-titer in Healthy Controls and Cryptosporidiosis Positive Group

Parameter	Healthy controls (Mean ± SEM)	Cryptosporidiosis positive group	p-value
CRP-Titer	4.800 ± 1.303	16.14 ± 3.413	< 0.01

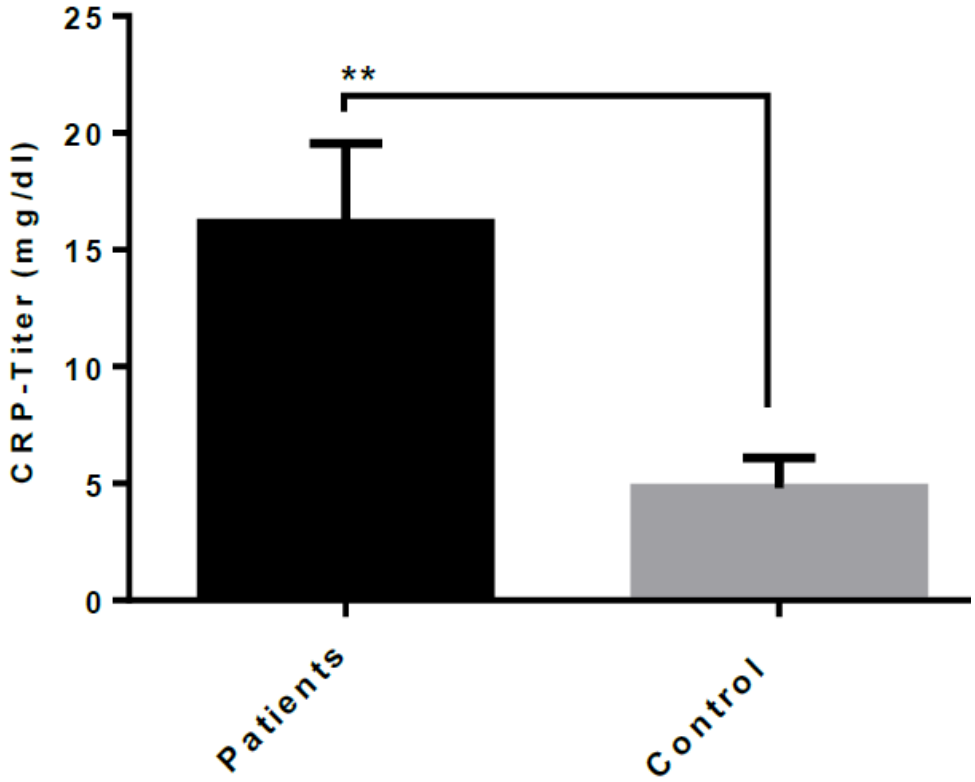

Fig. 1 Mean ± SEM of CRP-titer in Healthy Controls and Cryptosporidiosis Positive Group

C-reactive protein binds to phosphoryl choline on microbes. It is thought to assist in complement binding to foreign and damaged cells, and it enhances phagocytosis by macrophages, which express a receptor for CRP. It is also believed to play an important role in innate immunity, as an early defense system against infections (Pepys and Hirschfield, 2003). In a study conducted by Eriksson *et al.* (2013) showed high rate of CRP level, they mentioned that parasite load associates with elevation of CRP levels among asymptomatic individuals. CRP has also been proposed to be implicated in the susceptibility to certain parasite species (Israelsson *et al*., 2009). Barbai *et al.* (2010) and Juris *et al*. (2014) reported high rate of CRP among *Cryptosporidium* positive group, which was in agreement with the results of the present study, but statistically non-significant differences was observed by them.

Complement components C3 and C4 were statistically significant increase in cryptosporidiosis positive group with the mean values (209.3 ± 36.97 and 1.749 ± 0.01680) respectively in compared to healthy controls with the mean values (136.0 ± 6.590 and 1.592 ± 0.0255) respectively (Table: 2 and Fig.: 2 A, B).

Table 2: Mean ± SEM of Serum Complement Components (C3 and C4) of Healthy Controls and Cryptosporidiosis Positive Group

Parameter	Healthy controls (Mean ± SEM)	Cryptosporidiosis positive group	p-value
C3	136.0 ± 6.590	209.3 ± 36.97	< 0.05

C4	1.592 ± 0.02553	1.749 ± 0.01680	< 0.0001

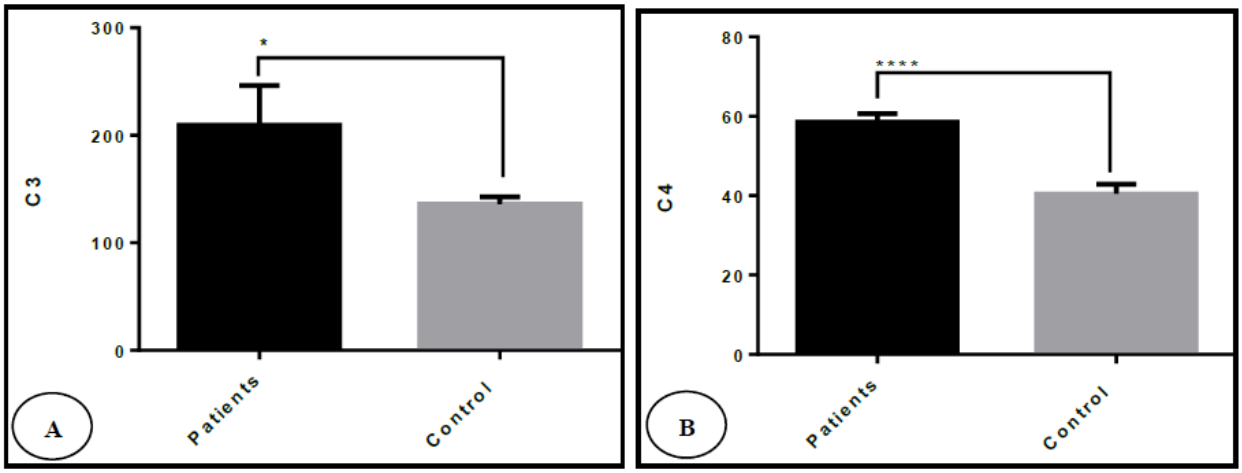

Fig. 2 Mean ± SEM of Serum Complement Components of Healthy Controls and Cryptosporidiosis Positive Group. A: Complement Component C3 B: Complement Component C4

These results were agreed with that reported by Mahdi *et al*. (2007) in Iraq,but they were reported statistically insignificant. Some complement components may be produced by enterocytes, but the role of complement in control of cryptosporidial infection is unclear. Classical activation of the complement cascade can be triggered by two mechanisms that lead to enzymic cleavage of C4 and C2 to form C3 convertase. Cleavage may be initiated by enzymatic action of C1 induced by antibody–antigen complexes or by serine proteases associated with mannose-binding lectin (MBL) following attachment of the latter to microorganisms (Moon *et al*., 1997; Kelly *et al*., 2000).

Mannose-binding protein (MBP) deficiency in the serum of Haitian children was also found to correlate with increased incidence of cryptosporidiosis. Both MBL and C4 have been shown to adhere to the surface of sporozoites, so it is possible that MBL may block parasite attachment to epithelial cells or activate the complement membrane attack complex (Kirkpatrick *et al*., 2006).

Kelly *et al*. (2000) showed that MBL / mannose-binding lectin–associated serine proteases (MBL-ASP) was able to initiate complement activation on the surface of *C. parvum*, when sporozoites were incubated with purified C4, after incubation with the MBL preparation, C4 binding to sporozoites was observed with MBL, and this appeared to be dependent on MBL concentration, but C4 binding was not observed without preincubation with MBL. The generation of a C3 convertase (C4b2a) by MBL/MBL-ASP should result in the deposition of C3 fragments on the sporozoite surface, which ordinarily would be sufficient to opsonize particles for phagocytic cells. MBL/MBL-ASP activation could also result in the formation of the membrane attack complex on the sporozoite surface, which could reduce the viability of the parasites. They hypothesized that the mechanism of MBL protection against *C. parvum* would be an interaction between MBL and the parasite in the gut lumen or in the brush border, and the possibility of complement-mediated inactivation is intriguing.

The result of IgG mean value (1751 ± 101.5) in the present study showed statistically non-significant difference ($p>0.05$) among cryptosporidiosis positive group (1751 ± 101.5) in compared to healthy controls (1586 ± 116.0). On the other hand, serum levels of the IgE, IgA and IgM among cryptosporidiosis positive group were statistically significant increased with the mean values (384 ± 35.7, 447 ± 21.1 and 298.3 ± 16.25) respectively in compared to

healthy controls (107 ± 24.8, 308 ± 32.9 and 235.2 ± 24.97) respectively (Table: 3 and Fig.: 3 A, B, C, D).

Table 3: Mean ± SEM of Serum Immunoglobulins of Healthy Controls and Cryptosporidiosis Positive Group

Parameter	Healthy controls (Mean ± SEM)	Cryptosporidiosis positive group	p-value
S. IgG	1586 ± 116.0	1751 ± 101.5	> 0.05
S. IgM	235.2 ± 24.97	298.3 ± 16.25	< 0.05
S. IgA	308 ± 32.9	447 ± 21.1	< 0.001
S. IgE	107 ± 24.8	384 ± 35.7	< 0.0001

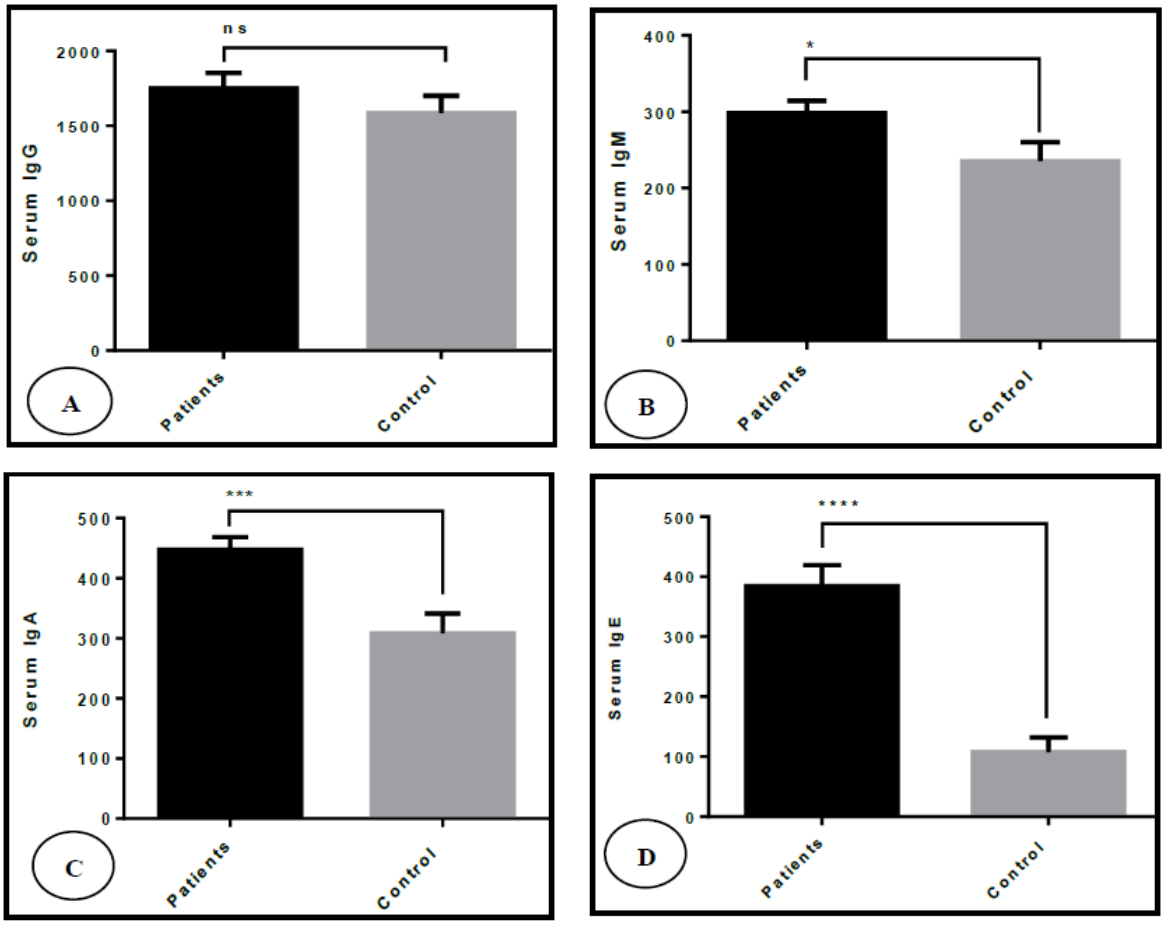

Fig. 3 Effect of Cryptosporidiosis on Serum Immunoglobulins. A: Serum IgG. B: Serum IgM. C: Serum IgA. D: Serum IgE.

In the present study, humoral immunity that includes IgM, IgA, and IgE tended to be higher in cryptosporidiosis positive children compare to control group. Parasite-specific Abs appears in the circulation and in the mucosa during infection of different mammalian hosts, including humans (Peeters *et al*., 1992). In a study with *C. parvum*-infected immunocompetent human volunteers, the level of parasite-specific sIgA was higher in individuals excreting oocysts or with diarrhea, but IgG was not found (Dann *et al*., 2000).

In a longitudinal analysis of serological responses in Peruvian children, IgG responses to cryptosporidial-Ag were detected following infection and tended to be higher in older children, possibly indicating recurrent exposure (Priest *et al*., 2006), which was agreed with

the results of the present study,, in which the level of IgG was elevated among cryptosporidiosis positive group, but showed no significant differences (P>0.05). In addition, the increase in IgG level was significantly greater in cryptosporidiosis positive group as compared to controls (Borad *et al*., 2007), which was agreed with the results of the present study.

Regarding to the level of IgM for the present study was agreed with that reported by Allison *et al*. (2005) in case-control study of cryptosporidiosis in Bangladesh children, case had significantly higher IgM levels compared to the control group. Cryptosporidial infection leads to a parasite-specific antibody response in both serum and intestine but murine studies suggest that B cells are not essential for recovery from infection. A study suggested that colostrum or parasite-specific colostral Abs may provide a degree of passive immunity to suckling human offspring (Fayer and Xiao, 2008).

The current study revealed increased at level of IgA during infection with *Cryptosporidium* spp., which was agreed with the reports of Hussein and Shakir (2014), they showed that IgA conferred protection against these mucosal pathogens and Ag-specific IgA responses occur in hosts with cryptosporidiosis, the study was hypothesized that IgA directed to neutralization-sensitive epitopes may be useful in passive immunization against *C. parvum*. Many investigations have documented that IgA has been associated with resistance to a number of mucosal pathogens (MunozCruz *et al*., 2010; Nazeer *et al*., 2013).

Immunoglobulin E activates platelets and induced cytotoxic functions against parasites. An elevation in serum IgE level was also reported even in helminthes and protozoan infectious diseases such as cryptosporidiosis and giardiasis (Rojas Cartagena *et al*., 2005; Vieira *et al*., 2012).The level of IgE for the present study was significantly elevated among cryptosporidiosis positive group compared to control group, which was disagreement with that reported by Hussein and Shakir (2014). On the other hand, the elevation of IgE level was agreed with that reported by (Kennedy, 2000; Zarebavani *et al*., 2012; Juris *et al*., 2014).

A significant increase of IL-18 was observed in cryptosporidiosis positive group with mean value of (84.11 ± 6.098 ng/L) when compared to healthy controls with the mean value (32.37 ± 4.668 ng/L), statistical analysis showed significant differences ($p < 0.0001$) in distribution of cryptosporidiosis during the studied period as shown in Table (4) and Fig. (4).

Table 4: Mean ± SEM of IL-18 in Healthy Controls and Cryptosporidiosis Positive Group

Parameter	Healthy controls (Mean ± SEM)	Cryptosporidiosis positive group	p-value
IL-18	32.37 ± 4.668	84.11 ± 6.098	< 0.0001

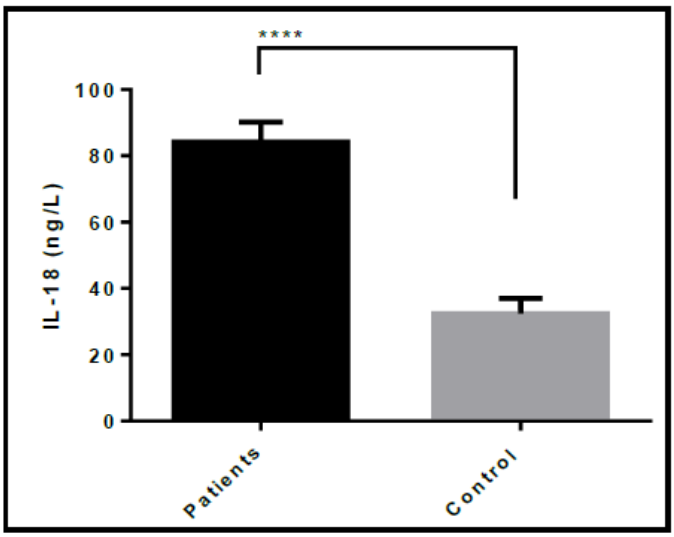

Fig. 4 Mean ± SEM of IL-18 in Healthy Controls and Cryptosporidiosis Positive Group

The results of the present study of IL-18 was agreed with that reported by McDonald *et al*. (2006) and Choudhry *et al*. (2012) in which they reported the murinal intestinal cells had increased production of mature IL-18 following infection with *C. parvum*. The cytokine partially inhibited *C. parvum* infection of human intestinal cell lines, and this increased expression of antimicrobial peptides, which lead to kill sporozoites of *C. parvum* (Zaalouk, *et al*., 2004). IL-18 therefore plays a protective innate immunological role against *C. parvum* infection and one possible mechanism is by promoting IFN-γ production by macrophages (Choudhry *et al.*, 2012).

Intestinal epithelial cells act as critical sensor of infection and provide the first line of defense by producing chemokines, and proinflammatory cytokine such as IL-18 (Kagnoff and Eckmann, 1997). IL-18 which belongs to IL-1 family of proinflammatory cytokines is a key inducer of IFN-γ (Dinarello and Fantuzzi, 2003) and is expressed by IECs, macrophages and DCs in the response to infection. Recent studies have shown that IL-18 is important in immunity to *C. parvum* in immunocompetent mice and IL-18 deficient mice are susceptible to infection, supporting a protective role for this cytokine in Cryptosporidial infection. (Ehigiator *et al*., 2007; Tessema *et al*., 2009; Lamb, 2012). The results of the present study demonstrate that intestinal mucosal inflammation, as measured by the proinflammatory cytokine IL-8, is increased among children with cryptosporidiosis, compared with healthy controls. At enrollment, was agreed with that reported by Kirkpatrick *et al*. (2006) for the higher rates of IL-18 production in case patients compare to control subjects.

Intestinal protozoan infections will lead to a robust inflammatory process and production of a wide range of cytokines including TNF-α that is elevated in the gastrointestinal tract of some forms of inflammatory colitis, cryptosporidiosis and giardiasis (Jimenez *et al*., 2009; Hussein and Shakir, 2014). A significant increase of TNF-α was observed in cryptosporidiosis positive group with mean value of (2.689 ± 0.03194 ng/L) when compared to healthy controls (2.309 ± 0.06805 ng/L), statistical analysis showed significant differences ($p<0.0001$) in distribution of cryptosporidiosis during the studied period as in Table (5) and Fig. (5).

Table 5: Mean ± SEM of TNF-α in Healthy Controls and Cryptosporidiosis Positive Group.

Parameter	Healthy controls (Mean ± SEM)	Cryptosporidiosis positive group	p-value
TNF-α	2.309 ± 0.06805	2.689 ± 0.03194	< 0.0001

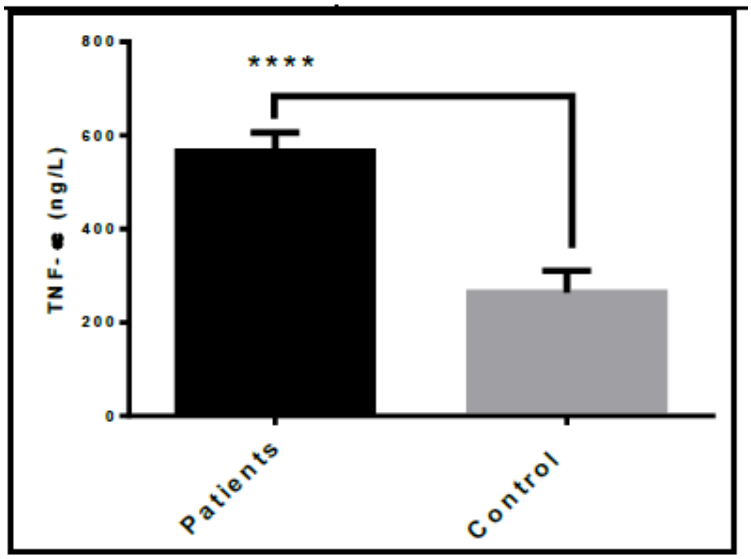

Fig. 5 Mean ± SEM of TNF-α in Healthy Controls and Cryptosporidiosis Positive Group

Tumor-necrosis factor-α is a proinflammatory cytokine that is commonly produced by T-cells and other cells during infections and is important in the stimulation of antimicrobial killing mechanisms as well as in inflammation, and it can also be involved in immunoprotective mechanisms against intracellular infections (Murray *et al*., 2000; Mizuki *et al*., 2002; Zganiacz *et al*., 2004). Tumor-necrosis factor -α may have a redundant role in establishing resistance to infection. It was shown to act mainly to prevent parasite invasion and appeared to have little effect on intracellular parasite development (Lean *et al*., 2006).

In human study, *C. parvum* infection results in expression of the chemokines, such TNF-α (Lacroix-Lamande, *et al*., 2002; Deng, *et al*., 2004). This hypothesized to signal PMN's influx into intestinal mucosa in *C. parvum* infection (Segui *et al*., 2004; Molla *et al.*, 2005).In addition TNF-induced NO production by macrophages leads to cytotoxicity of intestinal parasitic protozoa (Kristine *et al*., 2010; Ekhlas *et al*., 2013).

The results of the study done by Lean *et al*. (2006) indicated that TNF-α can inhibit *C. parvum* reproduction in enterocytes by prevention of host cell invasion, but clearance of the infection in the murine host may still be readily accomplished without the involvement of this cytokine.

In agreement with the results of the present study, studies done by Kirkpatrick *et al.* (2006) in Brazil and Hussein and Shakir (2014) in Iraq reported that TNF-α production significantly increased to cryptosporidiosis for case patients, compared with control subjects. Children with parasitic diarrhea had significantly higher TNF-α levels than those without diarrhea (Peterson *et al*., 2010), which was agreed with the results of the present study.

Proinflammatory cytokines attract leukocytes to the intestinal tract, contributing to the clinical manifestations of Cryptosporidial diarrhea. IL-18 and TNF-α have been studied in animal and human xenograft models, and they are consistently elevated in association with the inflammatory changes stimulated by *Cryptosporidium* infection, and these cytokines directly produce from the *Cryptosporidium*- infected intestinal epithelial cell (Kandil *et al*., 1994; Lacroix *et al*., 2001).

Immunochromatographic assay and PCR technique has been described previously for the detection of *C. parvum* in faecal samples (Goni *et al*., 2012; Elsafi *et al*., 2013). In the present study, the results obtained using these methods were compared prospectively with the results obtained by routine ZN microscopy in clinical laboratory practice for patients with cryptosporidiosis.

Out of 548 and 276 stool samples examined with modified ZN and formalin-ether ZN techniques respectively, 59 and 28 of them were positive for *Cryptosporidium* spp. respectively. Then 70 and 21 stool samples were subjected to IC and PCR-sequence methods and the results showed that the rate 100% of infection with *Cryptosporidium* spp. for 50 and 15 stool samples respectively that previously identified with ZN techniques (Table 6).

Table 6: Prevalence of Cryptosporidiosis among Children Attending Raparin Pediatric Hospital Using different Diagnostic Techniques

Diagnostic Methods	**No. ofexamined samples**	**No. ofpositive samples (%)**	**No. ofnegative samples**
Direct ZN technique	548	59 (10.77)	489
Formalin-Ether ZN technique	276	28 (10. 45)	248
IC assay	70	50[a] (100)	20[b]
PCR-sequencing method	21	15[a] (100)	6[b]

a: positive samples with ZN. b: Negative samples with ZN.

Out of 59 positive stool samples for cryptosporidiosis by direct ZN technique, 28 positive samples by formalin-ether sedimentation ZN technique and 50 positive stool samples by IC techniques, only 15 of them were subjected to PCR and nucleotide sequencing technique for confirming the previous routine methods and species identification from children with gastroenteritis, and 6 healthy control which were negative for cryptosporidiosis by direct ZN, formalin ether sedimentation ZN and IC techniques. As shown in Fig. (6) the Lane 1 was loaded standard ladder used as a control for comparing other loaded DNA samples, from the Lane 2-4 there are 450bp bands that were specific for selected primers of *Cryptosporidium* spp., but the Lane 5-8 were negative for *Cryptosporidium* spp.

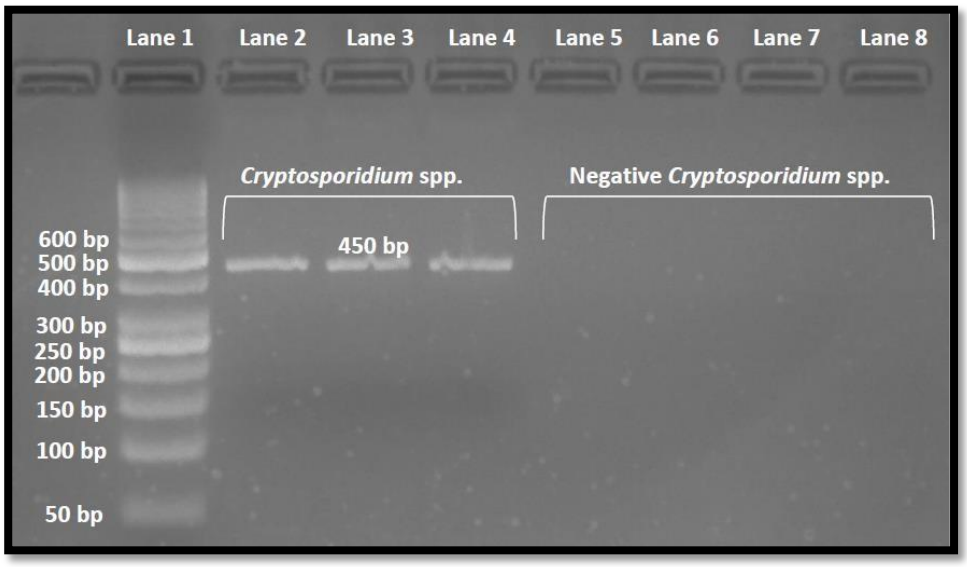

Fig. 6 Molecular-based Identification of Cryptosporidium spp. Isolates. Lane 1: Ladder (1kb), Lane 2, 3, and 4 were Positive PCR Sample Products for Cryptosporidium spp., Lane 5, 6, 7, and 8 were Negative PCR Sample Products for Cryptosporidium spp.

None of common diagnostic laboratory techniques, such as acid fast staining, direct or indirect immunofluorescence microscopy allows species and genotype discrimination of the

oocysts (Tzipori and Ward, 2002). The new tools of molecular genetics allow the characterization of *Cryptosporidium* at the species level. The genetic techniques including PCR and sequence analysis can help to determine genotypes of the parasite and the possible source and harmful effect to human health (Xiao *et al*., 2004).

The PCR assay developed for detection of different intestinal protozoan parasites can be easily used for species diagnosis of *Cryptosporidium* and other parasites without any loss of sensitivity and specificity. Detection of parasite-specific DNA by PCR is more sensitive than microscopy (Guy *et al*., 2003; Schuurman *et al*., 2007).

Molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica*, *G. lamblia* and *C. parvum / C. hominis* infections (Blessmann *et al*., 2002; Verweij *et al*., 2003). Their use in routine diagnostic laboratories is still very limited. The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from faecal specimens and the presence of inhibitory substances in such samples. Furthermore, amplification of DNA was previously laborious and expensive, and cross contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles (Verweij *et al*., 2000).

The expected amplicon size for CPB-DIAG (18S rDNA) of *Cryptosporidium* spp. supposed to be 450bp (Fig. 6), applying molecular procedure based PCR sequencing tool results got from sequencing were only 400bp (missing 50 bp due to the quality of nucleotide sequence). BLAST program from Genbank (http://blast.ncbi.nlm.nih.gov/) was used to compare the results of amplified sequences of the present study with other stored species of *Cryptosporidium* sequences. The results got from the BLAST indicated that the query sequence was 100% identical to *Cryptosporidiumparvum* (Figure 7, Fig. 8).

The present study confirmed and detected the *Cryptosporidium* spp. by molecular assay, the species was diagnosed as a *Cryptosporidium parvum,* which were genotyped on the basis of the 18S rRNA gene, this result was in agreement with Mallah and Jomah (2015) in Al-Muthanna-Iraq.

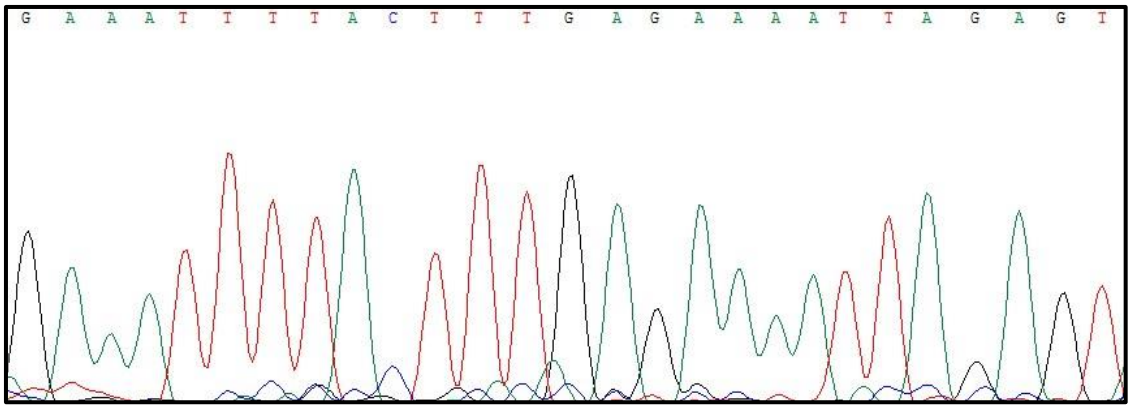

Fig. 7 The Partial Sequencing Result of CPB-DIAG (18S rDNA) of C. parvum.

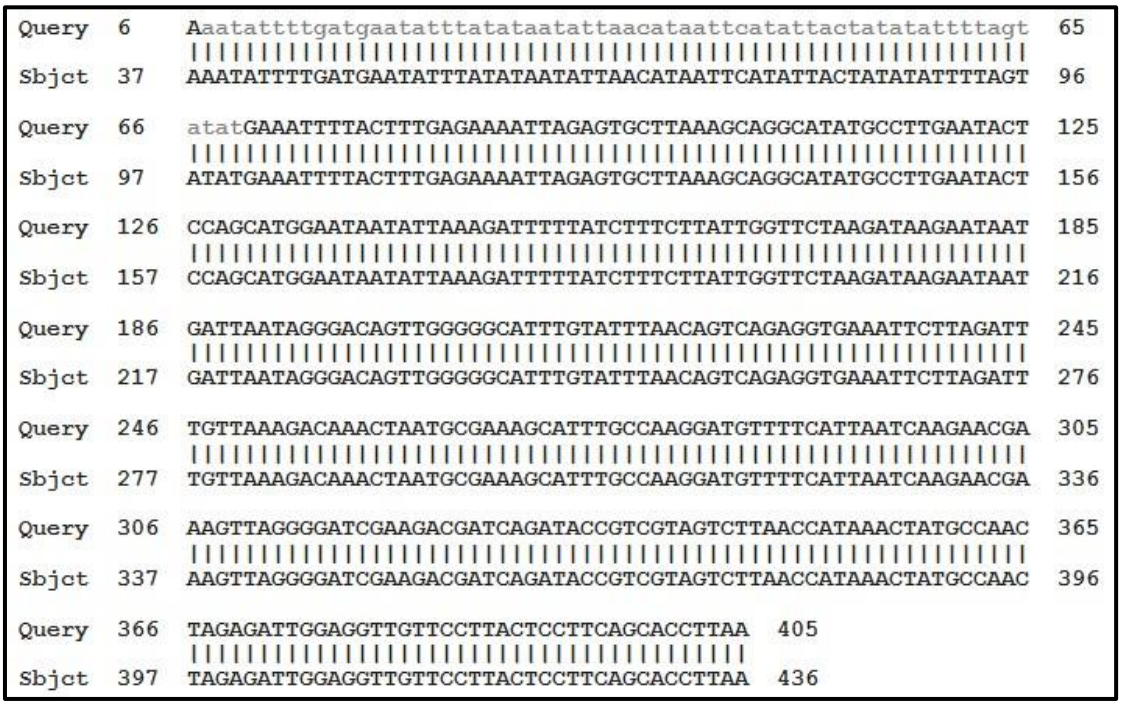

Fig. 8 Pairwise Alignment of CPB-DIAG (18S rDNA) sequence of C.parvum. Query is the Study or Sample Sequence and Sbjct is the Gen Bank Sequence.

Efforts are being made to develop diagnostic methods based on molecular techniques; such methods may help in cases in which cysts are passed in very low numbers. PCR based techniques can detect a single cyst and also distinguish between different species and strains of parasites (Roberts and Janovy, 2009). PCR has primary been used for identification of different species and genotypes of *Cryptosporidium* spp. for taxonomical research, although there is potential for diagnostic use (Caccio *et al.*, 2005).

In the past decade, few studies reported molecular characterization of *Cryptosporidium* from immunocompetent and immunocompromised patients in the Arab world. Al-Brikan *et al.* (2008) examined 35 *Cryptosporidium* positive fecal samples (by microscopy and ELISA) from children in Jeddah, Saudi Arabia, using molecular techniques. Of the samples, they identified *C. parvum* in 15 (42.9%), which was lower than that reported in the present study (100%). Essid *et al.* (2008) analyzing the eight *Cryptosporidium* positive cases of immunocompromised children by PCR to determine the species, showed that four were *C. hominis*, three were *C. parvum*, and one was C. *meleagridis*.

Recently, Poor *et al.* (2015) identified 1.76% of *C. parvum* oocysts from the total of 113 fecal samples which were collected from diarrheic children hospitalized in Tabriz Pediatric Hospital. Iqbal *et al.* (2011) determined the species of 83 *Cryptosporidium*-positive specimens from diarrheic Kuwaiti children by PCR. They identified *C. parvum* in 73.5% (61/83) and *C. hominis* in 26.5% (22/83).

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