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Research Article

Evaluation of interleukin-4 and some serological factors in children infected with cryptosporidiosis at Ramadi Teaching Hospital for women and children

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Abstract
Cryptosporidiosis is a zoonotic disease caused by a protozoan parasite of the genus *Cryptosporidium*. It is widespread worldwide and is among the four main diarrhoea pathogens in children and adults. The present study aimed to investigate infection with the cryptosporidiosis in children under the age of eight years in both sexes who suffered from diarrhoea, and to evaluate the relationship of Interlukin-4 to parasitic infection and the changes in some serological parameters that included lipid profile and total protein. Two hundred fecal samples were collected from Ramadi Teaching Hospital for Women and Children. Microscopic examination of the samples stained with Ziehl–Neelsen stain and ELISA test indicated the presence of egg cysts in 23 samples, with a total percentage of (11.5%). The shape of the parasite was spherical, tending to oval, with a size of (4.8 to 5.7 micrometres). The lipid profile results showed that there was a significant increase in cholesterol (Ch.), triglycerides (TG.), low density lipoprotein (LDL), very low-density lipoprotein (vLDL), and for high-density lipoproteins (HDL) of the patient's group, there was no significant difference between the control group and the infected group at the (P ≤0.05). There was a significant increase in the level of blood proteins (Total Protein, Albumin, Globulin) for the infected group at a significant level (P≤0.05). The study also showed significant differences in interleukin-4 between patients and healthy people, which was 0.0444 ± 0.01141 pg/ml for patients (P≤0.05). Due to the increasing spread, seriousness, and epidemiology of the parasite, considered environmental pollution, and because of its lack of diagnosis in health departments (such as hospitals), and because its entry significantly stimulates the immune system, causes dehydration and death in children with weak immunity, and affects the absorption of fats, proteins, and vitamins significantly, a new factor IL-4 related to the infection was identified. It was also known how significant the effect of injury is on the amount of fats and proteins that are involved in the structure of living cell membranes.

Keywords: *Cryptosporidium* infection, Interleukin-4, Lipid profile, Low Density Lipoprotein, Protiens

INTRODUCTION

Cryptosporidiosis is a zoonotic disease caused by a protozoan parasite of the genus *Cryptosporidium*. It is widespread worldwide, and according to most tests, cryptosporidiosis is among the four main diarrhoea pathogens in children and adults. It causes moderate diarrhoea in many children, but children are more susceptible to infection than adults, as it causes death in them under the age of two (Koyun *et al*., 2023).

These pathogens vary in their effect on the host according to the type, severity of infection, location, and clinical manifestations of the disease, and vary with age and immune status, as they include severe diarrhea for a period of (1-4) days, often accompanied by vomiting and abdominal cramps (Adkins, 2022). *Cryptosporidium* is one of the parasitic diseases shared between animals and humans, as humans can acquire

the infection through direct contact with an infected person or infected animals, or by digesting contaminated food or water (Ryan *et al*., 2018).

Cryptosporidium parvum is a protozoan coccidial parasite in humans and animals and one of the recently discovered parasites that has shown a high rate of intestinal infections in several animal species. It is responsible for infection in humans in developed countries, as the parasite belongs to the phylum Apicomplexa (Li *et al*., 2021). Although cryptosporidiosis causes short-term diarrhoea in people with complete immunity and may cause life-threatening diarrhoea in immune-compromised patients, which may last more than two months and lead to death, infected animals shed the oocysts with the faeces and lead to their transmission to various hosts, including Humans through sources of drinking water or contaminated food (Tamomh *et al*., 2021).

When the parasite enters the human body, Immune response components, such as cytokines (interleukins and interferons), defensive white blood cells (macrophages), immune globulins, particularly IgA, in the mucosal regions, and immune complement C3 and C4, are activated, as a result of the parasite's entry processes of change will begin in some other hematological and serological factors. As a result of the immune response against the parasite, in addition to the parasite's need for some proteins and lipids to form the parasite's vacuole membrane, In addition, membrane receptors must be continuously replaced to maintain resistance and facilitate material exchange between the host cell and the parasite during the parasitic process on the host's intestinal epithelial cells (Pal *et al*., 2016).

The spread of this parasite, its threat, and its epidemiology are all categorized as environmental pollution because hospitals and other healthcare agencies are not able to diagnose it and because the parasite's entry greatly boosts the immune system and may infect or severely become dehydrated children with weakened immune systems (Gerace *et.al*., 2019). The difficulty of diagnosing the parasite in hospitals, the lack of capabilities to detect the parasite except with the presence of some diagnostic dyes or ELISA immunological tests (expensive), and the spread of the parasite in Anbar Governorate prompted us to study its presence and detect it and try to know some of the main physiological and immunological factors that change during infection in an attempt to help. Thus, the study aimed to evaluate interleukin-4 and serological parameters in children with cryptosporidiosis infections at Ramadi Teaching Hospital for Women and Children.

MATERIALS AND METHODS

Samples collection

From October 1, 2022, to March 25, 2023, two hundred fecal samples were randomly collected from children admitted to and hospitalized at Ramadi Teaching Hospital for Women and Children of both sexes under the age of eight and from various locations of Anbar Governorate. Plastic bottles were used to collect the samples. The tubes had a 40 milliliter capacity, were spotless, and were firmly sealed. A questionnaire form was used to record the patient's information. Then, a laboratory examination was conducted for each sample, which included placing a drop of the sample on a glass slide, spreading it and leaving it to dry. This was done after mixing the faeces with distilled water, filtering it through gauze layers and surrounding it with a diabetic solution. Then, it was washed and deposited using a phosphate buffer solution, then fixed with concentrated ethyl alcohol (96. %) for 5 minutes (Sorvillo *et al*., 2001), and then stained with modified Ziehl-Nelson acid-fast dye (Clarke *et al.*, 1996).

The sample was examined and photographed after applying a drop of lens oil and examined at 100x power. The samples were preserved with a 5% potassium dichromate solution. An ocular micrometer was used to measure the dimensions of the *Cryptosporidium* oocytes. The following equation was applied according to the method González-Ruiz and Bendall (1995).

Microscope factor = rate of microscope readings / rate of ocular scale readings ×10 micrometer.

Calculation of the number of oocytes of the *Cryptosporidium parvum*

Due to the unavailability of the modified plastic slide (MC master), the number of parasite oocytes per milliliter of the solution was calculated using the Heamocytometer counting slide according to the method Sonzogni *et al*. (2019). The usual process confirmed the calculation of the number of oocyt, which were placed on a glass slide and 50 microliters of Logals-iodine solution were placed on top of it. The slide was covered with a cover, and the number was calculated. Then the following equation was used to determine the number of oocyte sacs per milliliter.

The No. of oocytes (ml.) = No. of oocytes calculated in 50 µl. × 20 Eq.1

Blood samples collection

Two milliliters of venous blood of the same children from whose feces samples was taken and put in a test tube and allowed to coagulate for 10 minutes at room temperature. After that, the test tubes were put in the centrifuge and spun at a speed of 4000 revolutions per minute for 10 minutes. Following that, the blood serum was extracted and put into Eppendorf tubes. After the sample's details were documented, they were kept at -20°C until they were used in laboratory testing.

ELISA immunoassay for antigenic diagnosis of *Cryptosporidium parvum*

The ELISA Kit manufactured by mybiosource.com in the USA was used.

Determination of serum albumin

The method of Hill (1985) was used to determine the serum albumin, using ready-made kit from the Spanish company Spinreact.

$$\text{S.Albumin(g/dl)} = \frac{\text{Test} \times 5\ (\text{Standard conc.})}{\text{Standard}} \quad \text{Eq.2}$$

Determination of total serum protein

The amount of proteins in the serum was estimated using the Biuret method, which used the ready-made kit from the French company Biolabo. The following equation was applied:

$$\text{T.S.P(g/dl)} = \frac{\text{Test} \times 8\ (\text{Standard conc.})}{\text{Standard}} \quad \text{Eq.3}$$

Calculation of globulin serum

The globulin serum was calculated using the following equation:

$$\text{S. Globulin (g/dl)} = \frac{\text{Test} \times 3\ (\text{Standard conc.})}{\text{Standard}} \quad \text{Eq.4}$$

Determination of triglycerides level

The concentration of triglycerides in blood serum was measured using the enzymatic method in an analysis kit prepared by the Spanish company Spinreact. The equation below was used to calculate the concentration of triglycerides:

$$\text{Triglycerides ml/dl} = \frac{\text{Test} \times 200\ (\text{Standard conc.})}{\text{Standard}} \quad \text{Eq.5}$$

Determination of cholesterol level

The cholesterol level in blood serum was estimated using the enzymatic method, using an analysis kit prepared by the Spanish company Spinreact. The serum cholesterol concentration was calculated by applying the following equation:

$$\text{Cholesterol ml/dl} = \frac{\text{Test} \times 200\ (\text{Standard conc.})}{\text{Standard}} \quad \text{Eq.6}$$

Estimation of high-density lipoprotein cholesterol levels (HDL-C)

The concentration of HDL in the subjects' blood serum was measured by following the steps of the instructions accompanying the test kit imported from the Spanish company Spinreact**.**

HDL concentration (mg/dL) = O.D. of test x 320 (conversion factor) Eq.7

Estimating of low density lipoprotein- cholesterol LDL-C

Low-density lipoprotein levels were estimated using the following formula:

LDL (ml/dl) = Cholesterol – (HDL+vLDL) Eq.8

Estimating of very low density lipoprotein-cholesterol VLDL-C

Very low-density lipoprotein levels were estimated according to the following formula

$$\text{vLDL (ml/dl)} = \frac{\text{T.G.}}{5} \quad \text{Eq.9}$$

Measurement of interleukin-4 by ELISA test

Human IL-4 High Sensitivity ELISA Kit from Diaclone / France was used to estimate the level of IL-4.

Ethical approval

The patient's companions were informed of the nature of the research and verbal consent was obtained from them prior to sample collection. The research protocol was reviewed and approved by the Research Ethics Committee of Biotechnology and Environmental Center - University of Fallujah under document number 2311 on 9-11-2022.

Statistical analysis

Statistical analysis was conducted using the ready-made program SPSS (2009) and using a t-test to compare infected and healthy people at the probability level ($P < 0.05$).

RESULTS AND DISCUSSION

Sample collection (for *Cryptosporidium* parasite)

Out of 200 faecal samples, 23 individuals had an infection rate of 11.5%, according to the results (Table 1).

Diagnosing infected samples

The results of the current study confirmed the existence of significant differences in the accuracy of the methods for diagnosing the *Cryptosporidium* and detecting the parasite in feces, as the result of the ELISA test examination was 23 positive samples (infected with the parasite). In comparison, 28 samples were positive in the staining method with the modified Ziehl-Nelson stain, indicating the diagnostic techniques' efficiency. The most accurate way to express the results,

Table 1. Number and percentages of samples tested for the infection

No. of samples	Test methods	Positive samples	percentage	Negative samples	percentage
200 samples Male and female	ELISA	23 samples	11.5 %	177 samples	88.5 %
	Ziehl-Nelson	28 samples	14.0 %	172 samples	86.0 %

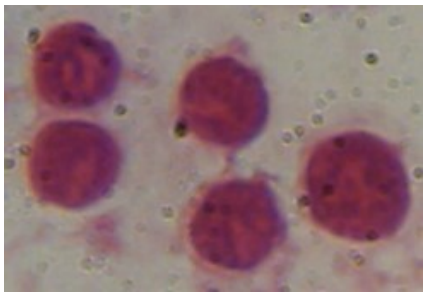

Fig. 1. *Showing the Cryptosporidium parvum after staining with Ziehl-Nelson stain (5.3 micrometres) (40x)*

was the ELISA method, which gave a percentage of (11.5) %, followed by the staining method with modified Ziehl-Nelson stain, with a rate of 14.0% (Fig. 1).

The ELISA test had great sensitivity identifying 10 oocytes in the feces, making it a very effective diagnostic tool. Because it recognizes the antigens of the parasite in the solution, it is also easy to use. This outcome is similar with the findings of Razakandrainibe *et al*. (2021), who reported that the ELISA test is one of the most reliable methods for identifying parasites. The percentage of accuracy of the test was 98.86%, and this was attributed to the fact that the ELISA test is sensitive to even a small number of parasite oocytes and because the interaction between the antibody and the antigen occurs quickly with the help of some interaction factors.

Shape and size of *Cryptosporidium*

The results showed that the shape of the parasite was spherical or tended to be oval. Its dimensions ranged from 4.8 to 5.7 micrometres, as measured by the Ocular - Stage Micrometer, after taking 23 infected samples, and the general average of these measurements was 5.3 micrometres.

This is in line with observations from Johnson *et al*. (2008) and Bones *et al*. (2019), which indicated the shape of the parasite is oval and that the parasite's dimensions are unequal when detected and colored, and that its size ranged from 4.3 to 6 micrometer. Sharma and Busang (2016) and Borowski *et al*., (2010) also provided information that the parasite has an oval shape and a size range of 5-7 micrometers. They explained that the parasite is stained with the modified Ziehl-Nelson dye so that its color tends to be red or pink due to it taking the red carbol fuchsin dye, and that the medium around it will take the blue dye methylene to be a light blue color. Thus the parasite appears in its oval shape (Ahmed *et al*., 2023).

lipid profile and its relationship to the *Cryptosporidium parvum* infection

According to the current study's findings, there were notable variations (significant increases) in a number of serological markers between individuals with a *Cryptosporidium* infection and the healthy control group. There was a significant increase in the group of blood lipids (Ch., TG., LDL, VLDL) in those infected, while High-density lipoproteins (HDL), as there was no significant difference between the control group and the infected group at the (P≤0.05), as shown in Table 2.

The cause of elevated cholesterol, LDL and VLDL during cryptosporidiosis infection was attributed to abnormalities in lipoprotein metabolism that play an important role in lipid peroxidation, which increases the generation of free radicals and is evidence of oxidative damage. The process of lipid peroxidation breaks down the cell membrane and releases cellular contents, including cholesterol, into the blood, leading to increased levels of cholesterol, LDL and VLDL in the blood, and decreased levels of HDL due to the parasite's ability to eliminate LDL-derived cholesterol.

The high level of triglycerides during parasite infection is due to their splitting into monoglycerol and free fatty acids due to the secretion of the enzyme lipase. Triglycerides are rebuilt in the intestine cells and stored with cholesterol. They are secreted from the cells, collected by the lymphatic system, and then transported to the blood vessels. The level of triglycerides rises due to a decrease in the effectiveness of the enzyme lipoprotein lipase, which is responsible for breaking down triglycerides, and a decrease in the level of the enzyme leads to an imbalance in the level of triglycerides and thus an increase in their concentration in the blood (Al-Ani and Al-Warid, 2023) .

This study confirms the findings of Nelson *et al*. (2006), as he explained that the *Cryptosporidium*, when it enters the host's body, begins to attack the epithelial cells and release the lipids present in the cell membranes with the help of a type of transporter present on the surfaces of the epithelial cells of the intestine called (NPC1L1 transporter). So that it can create the para-

Table 2. Blood lipids profile levels upon infection with the *Cryptosporidium parvum*

Samples	Ch. mg/dl	TG. mg/dl	HDL mg/dl	LDL mg/dl	VLDL mg/dl
Patients	*25.47±123.09	*24.42±87.35	5.32±42.93	*27.39±62.60	*4.88±17.47
Control	10.0±104.31	6.08±67.12	5.97±47.03	11.80±44.09	1.21±13.42
Sig.	0.0003	0.0002	0.42	0.0003	0.0003

•Above values represent the mean of the sum of values ± the SD at a significance level of (P≤0.05)

sitophorus vacuole that houses it and helps it in the process of parasitism without the knowledge of the host's immune system, the level of lipids in the body rises temporarily, leading to steatorrhea and loss and wastage of the amount of lipids leaving the body, the parasite will eventually lose its activity and start to cyst if the host does not receive good nutrition rich in lipids (Bertuccini *et al.*, 2024; Pardy *et al*., 2024).

The finding of this study somewhat agrees with Mahmoud *et al*. (2018) findings that discovered that in young calves experiencing diarrhea, the disease group's level of HDL of all kinds significantly decreased, but the liver enzyme Alkaline phosphatase (ALP) and fats TG, Ch., VLDL, and LDL significantly increased.

Proteins and their relationship to the *Cryptosporidium parvum*

The results of the study showed that there are significant differences in the level of blood proteins (TP, Alb., Glo.) at the (P≤0.05) between those infected with the *Cryptosporidium* and healthy people (control group) (Table 3).

The results of the current study are consistent with what was observed by Guerin *et al.* (2023) who indicated that infection with *Cryptosporidium* causes an increase in the level of total proteins and a decrease in the level of calcium. He explained the immunological response against the parasite, saying that since proteins form the majority of antibodies and other immune response components against the parasite, the percentage of total proteins—including globulins—increases.

Zhang *et al*. (2015), in the event of a *Cryptosporidium* infection, the proportion of total proteins remained within the normal range and was unchanged. The body may be losing many nutrients needed to survive because of the infection and severe diarrhea, which prevents food from entering the body and may cause a decline in proteins. A large amount of the body's protein reserves will be used up throughout metabolism, body-building and injury recovery.

Interleukin-4 (IL-4) and its relationship to the *Cryptosporidium parvum*

The present study showed significant differences in interleukin-4 between the patient treatment, 0.0444 ± 0.01141 pg/ml, and the control treatment, 0.0265 ± 0.00196 pg/ml at P≤0.05. (Table 4).

An increase in IL-4 is necessary when infected with cryptosporidiosis. Treatment with Anti-IL-4 mAb in laboratory mice has been proven to eliminate infection with *C. parvum*. The release of IL-4 during infection will stimulate a large group of immune cells that contribute to Getting rid of the infection, including CD4+αβ+IFN-γ+ and CD4+αβ+IL-4+ lymphocytes in Peyer's patches and intraepithelial of adult C57BL/6 mice during resolution of *C. parvum* infection, and this proves the increase of interleukin-4 upon infection with the parasite (Aguirre *et al*., 1998).Shakir and Hussein (2014) found that when interleukin-4 (IL-4) is tested using ELISA technology, there is a considerable rise in the level of IL_4 secreted in the host's body following cryptosporidiosis infection. Mead (2023) came to the further conclusion that injecting IL-4 along with certain antibiotics improves lymphocyte efficacy and aids in the removal of *Cryptosporidium* and other opportunistic infections. The present study on variables related to the infection, including changes in IL-4, lipid profile, and abnormalities in the general level of proteins, will help to treat parasitic infection.

Conclusion

The present study showed a relationship between cryp-

Table 3. Changes in blood proteins upon infection with the *Cryptosporidium parvum*

Samples	Protein g/dl	St. Error	Albumin g/dl	St. Error	Globulin g/dl	St. Error
Patients	0.81±7.59	0.129	0.59±2.25	0.09	0.54±5.34	0.08
Control	*0.45±7.11	0.072	*0.98±1.96	0.15	*0.99±5.15	0.15
Sig.	0.003		0.001		0.0001	

• Above values represent the arithmetic mean of the sum of values ± the standard deviation at a significance level of P≤0.05

Table 4. Changes in interleukin_4 upon infection with the *Cryptosporidium parvum*

Samples	Interleukin-4 pg/ml	St. Error
Patients	*0.01141±0.0444	0.00054
Control	0.00196±0.0265	0.00033
Sig.	0.02	

• Above values represent the arithmetic mean of the sum of values ± the standard deviation at a significance level of P≤0.05

tosporidiosis and interleukin-4, as the level of interleukin- the results indicated that Cho, TG, VLDL and LDL increased in patients with a significant difference from healthy people, HDL level decreased in patients with a non-significant difference, and proteins (TP, Alb and Glo) increased with a significant difference in patients. When the *Cryptosporidium* parasite enters the intestine, it will attack the host's epithelial cells, which contain protein-bound fats in their outer coverings and excrete them in the blood and intestinal cavity, which leads to their increase. In addition, it was found that the parasite's vacuole and the oocysts produced by the parasite and the stages that the parasite passes through contain an average it is high in fats in its membranes, especially HDL, so the level of HDL decreases due to its consumption by the parasite.

Conflict of interest

The authors declare that they have no conflict of interest.

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