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ASSESSMENT OF SOME BIOMARKER IN PATIENTS INFECTED WITH *CRYPTOSPORIDIUM PARVUM*

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Abstract:

Cryptosporidium parvum, a protozoan parasite of the coccidial class, is a prevalent intestinal parasite which infect both humans and animals. 450 fecal and serum samples from infants aged ≤ 2 years who had acute or chronic diarrhea. The samples were obtained from medical and hospital centers in various districts of the Salah al-Din Government between July 2022 and September 2023. The fecal samples were analyzed to detect the presence of the oocyst *C. parvum* utilizing a modified Ziehl-Neelson staining technique (MZN), ELISA antigen by *Cryptosporidium parvum* antigen in feces, and the company Epitope Diagnostics investigated the qualitative *C. parvum* antigen and immunological markers (CD4, TGF-B, Fecal LF) in serum and fecal samples by ELISA test. From a total 450 stool samples, 40 (8,88%) positive and 410 (91.11%) negative stool samples were detected by MZN, 70 (15.58%) positive fecal samples, and 380 (84.44%) negative fecal samples. This study observed a significant increase in mean concentration of CD4, TGF-B, and Fecal LF in serum and fecal patients compared to the healthy group, respectively (mean *± SE* 5.8*61 ±* 1.116 pg/mL, *mean ± SE* 503.95 *± 104.73 pg/mL,*, *mean ± SE* 368.019 ± 102.025 pg/mL) compared to the healthy group (*mean ± SE* 1.752 ± *0.448* pg/ml, *mean ± SE* 188.12 ± 56.99 pg/ml, *mean ± SE* 154.483 ± 23.268 pg/ml). Cryptosporidiosis is an opportunistic parasite in infants ≤ 2 years with diarrhea. Modified ZN staining is simple and expensive. The ELISA antigen assay is a useful tool in surveys and diagnostic studies. *C. parvum* infection in infants induced some immune marker inflammation.

. **Keywords**: *Cryptosporidium parvum* ; Modified Ziehl-Neelson ; ELISA ; CD4 ; TGF-B ; Fecal LF

INTRODUCTION :

Cryptosporidium parvum is a primary contributor to diarrheal cases in infants and children aged 2 years or younger. This extremely contagious apicomplexan protozoans parasites invades the top layer from enterocytes, which leads to an increase in the contraction of small intestinal tract and a decrease the absorption of nutrients in the intestines[1]. Causing significant diarrhea with watery yellowish or mucus-like stool and a potential for transmitting diseases from animals to people[2]. *Cryptosporidium parvum* is a single-celled protozoan parasite that frequently leads to diarrhea and inadequate nutrition in infants aged 2 years or younger. The global spread of cryptosporidiosis is increasing[3]. *Cryptosporidium parvum*, a parasite smaller than red blood cells measuring approximately 4-5 microns, has garnered significant attention from researchers due to its extensive distribution and ease of transmission to people through contaminated water, food, or insects[4]. Cryptosporidiosis poses a significant risk, with a mortality incidence of approximately 9% of global child fatalities. *Cryptosporidium parvum* is recognised as one of the factors contributing to fatal chronic diarrhea in newborns[4]. Infection of the gastrointestinal epithelia by parasites leads to diarrhea, which is temporary in persons with a healthy immune system but can be life-threatening in those with a weakened immune system. This kind of disease is both zoonotic and anthroponotic, meaning it may be transmitted between animals and humans. It can be spread through the consumption of untreated polluted food or water, or through direct interacting with an infected person or animals[5]. However, it is only *C. hominis* and *C. parvum* that have been identified as the causative agents for human sickness. When comparing the two, the last is more common in undeveloped countries or arises as a result of zoonotic diseases in wealthy countries, whereas the former type has become frequently found in industrialized ones. Consequently, underdeveloped nations pay an unequal burden of the expenses associated with cryptosporidiosis due to insufficient sanitation infrastructure. The life cycles of these parasites are completed exclusively

inside a single host (a monoxenous life cycle). Cryptosporidiosis has the potential to cause death. Oocysts, which are a leading cause of diarrhea, can be present in both food and beverages[6]. The prevalence of Cryptosporidium outbreaks in drinking and recreational water is worsened by the widespread presence of the infectious oocyst, its ability to withstand environmental pressures, and its low infection threshold [7]. The severity of this scenario surpasses that seen in developing countries, when Cryptosporidium-induced diarrhoea is mostly attributed to the scarcity of safe drinking water and restricted access for diagnosis and treatment[8]. When Cryptosporidium oocysts are consumed, it triggers a series of reactions inside the cells lining the intestines. These reactions involve the activation of signaling pathways that allow both cells and molecules for react in response to the invasion and subsequent development of cryptosporidiosis[9, 10]. Furthermore, the immune system triggers both natural and adaptive responses in addition to the localized mucosa immune response against Cryptosporidium oocysts[10]. Recent studies utilizing both in vitro and in vivo methods have indicated that the natural immune response may play a role in counteracting the harmful effects of Cryptosporidium throughout human infection[11, 12]. Nevertheless, it is still unclear if the innate response is indistinguishable from that of the zoonotic C. parvum and the anthroponotic C. hominis. These animals differ in the way their gene alleles are distributed in terms of frequency[13]. It is necessary to determine and assess possible relationships among receptors and ligands that facilitate the invasion epithelial cells in the intestine of human by C. hominis and C. parvum. The enzyme-linked immunosorbent assay (ELISA) technique employed the direct approach to investigate the presence of the C. parvum antigen in a faecal samples. The ELISA kits includes micro titer wells that have been coated with antibodies designed for Cryptosporidium. These Abs are used for detecting the presence of Cryptosporidium antigen in faecal samples. The procedure that was endorsed by the suppliers, was adhered to[14]. The medical diagnosis of cryptosporidiosis is confirmed by detecting the presence of oocysts by concentrating and staining techniques in faecal, duodenal aspirates, and samples tissue [7]. These approaches are both insensitive and laborious serological techniques, such as determining the presence of Cryptosporidium Specific Antigen (CSAg) using an ELISA technique [15, 16]. CD4+ T lymphocytes play a crucial role in determining how susceptible humans are to Cryptosporidium infection[17]. The count is less than 50 cells per microliter[18]. CD4+ T lymphocytes located in the skin layer are one of the primary groups The remission of cryptosporidiosis in treatment with antiretroviral drugs is associated with an increase in CD4+ T cell numbers in the mucosa of the intestine[19]. The β superfamily of transformational growth hormones is a large set of ligands that have survived evolution and play a role in regulating several cellular, physiological in nature, and pathogenic processes[20]. TGF-β receptors are glycoproteins that span the cell membrane and have an area at the beginning that binds to ligands, a single fragment that crosses the membrane, and a region at the end within the cell where the domain for kinase was found[21]. In order to understand the importance of TGF-β in the intestinal epithelial cell compartment, we introduced a modified form of TGF-β RII specifically in these cells. This modification allowed the intestinal epithelial cells to resist the effects of TGF-β, even when it is produced by the cells themselves or by nearby cells and could potentially affect the intestinal epithelial cells[22]. Lactoferrin, a glycoprotein produced by neutrophils, can be quantified in faeces and full guts wash to assess inflammation of the intestines in both inflammatory bowel disease (IBD) and infectious gastritis[23]. Recent research has demonstrated that faecal lactoferrin (FL) is a highly responsive biomarker for paediatric inflammatory bowel disease (IBD)[24]. Lactoferrin is a glycoprotein that binds to iron. It belongs to a group of transferrin families that have existed for approximately 300-500 million years[25]. The protein consists of a single chain comprising approximately 690 amino acids and has an approximate molecular weight of 77 kDa[26]. Lactoferrin is produced by exocrine glands in the digestive and respiratory tracts[27]. Lactoferrin is present in milk, saliva, tears, sperm, and colostrum. In addition, apo Lactoferrin functions as an acute-phase protein to eliminate disease at the location of injection and inhibit the development of pathogens that rely on iron. Neutrophils and their granules have the ability to eliminate this. LF is a highly effective iron scavenger due to its predominantly unsaturated nature (up to 86%)[28]. Multiple studies have demonstrated that lactoferrin is crucial in regulating the body's iron levels, especially in milk. Consequently, breastfeeding infants do not experience iron shortages. Conversely, individuals who consume milk without lactoferrin seem to exhibit a lack of iron and other illnesses[29].

AIM OF THE STUDY

This study aimed to diagnose *Cryptosporidium parvium* through microscopic examination of fecal samples and ELISA antigen by Cryptosporidium parvum antigen in feces and to determine CD4, TGF-B, and fecal LF levels in patient's serum.

MATERIALS AND METHODS

i-Time and location :-

The research lasted from July 2022 to September 2023. A grand overall of 450 stool samples were collected from infants ≤ 2 years with diarrhea who received medical assistance in public hospitals and private medical clinics in some region in the government of Salah al-Din.

ii-collection of fecal and blood samples:-

450 feces and serum samples were collected from patients and control (infants ≤ 2 years of both sex sick and the controlling) from lying and coming to Tikrit Educational Hospital and Tikrit Emergency Hospital after the patient's consent was taken and control over special laboratory tests to detect the parasite, which are part of the researcher & apos;s work ethic for research. 450 feces of approximately 20 grams were collected and placed in sterile plastic packaging with a strict cover to preserve the moisture of the samples and prevent their drying, without the name of the patient, age, or sex, and transferred to the Parasite Laboratory at Tikrit College of Sciences .In addition, a questionnaire form containing some information was adopted from patients in terms of residential area, sex, age groups, and months,

after which the sample was sampled in terms of observation of color, strength, smell of shit, presence or absence of blood, and snot within a period of not more than half an hour in preparation for micro-tests. The rest of the shit was placed in sterile and well-preserved tubes and kept at -20 degrees for the remaining immune tests. Each patient provided five milliliters of venous blood. Blood samples were placed in gel tubes and centrifuged at 4000 rpm for 10 minutes to collect serum. The tubes were left at room temperature for 15–20 minutes to let the blood clot. To maintain sample quality, serum was transferred to Eppendorf tubes (200 µL) for tests and maintained at -20°C until immunological testing. To minimize repeated freezing and thawing, which could compromise the quality of the findings, all samples were evaluated at the same time after sampling was complete.

1-Dignosis of *Cryptosporidium parvum* by Microscopic examination.

450 fecal samples were analyzed utilizing the adapted Ziehl-Neelson staining procedure under a microscope. Cryptosporidium oocysts appear circular, spherical, small, and pink-red, and the rest of the stool is blue or green.

2-Dignosis of *Cryptosporidium parvum* by *ELISA antigen* assay

90 fecal samples were examined using Cryptosporidium parvum antigen in feces by the company Epitope Diagnostics to detect the qualitative C. parvum antigen.

Human CD4, TGF-B , Fecal LF ELISA test:-

Serum samples were used to assess CD4, TGF-B, and fetal LF using the ELISA kits (China-Sunlong). The ELISA plates were precoated with human CD4, TGF-B, and fetal LF antibodies. The samples were added to the plates, and the color development in the substrate solution correlated with the levels of CD4, TGF-B, and fetal LF. The process had be stopped by introducing a stopping solution, and the level of absorption was quantified at a wavelength of 450 nm.

Approval granted based on ethical considerations

Permission for performing the study was given on September 21, 2022 by the Scientific Committee of the Salah al-Din Department of Health and the Scientific Committee of the College of Science at Tikrit University, in compliance with administrative order 13896**.** The questionnaire was completed to collect samples from the children after their parents were told to study, and the information of each child was taken with the consent of their parents. (the name, address, and typical symptoms) was completed with the consent of the parents.

Statistical analysis

Statistics were analyzed using IBM SPSS 26.0. We displayed categorized data using frequencies and percentages. Continuous variables were presented as the mean ± SE. A T-test was performed to determine significant values ($P \leq 0.05$).

RESULTS

Microscopic results using the *modified Ziehl-Neelson:* -

From a total of 450 fecal samples, 40 (8.88%) were positive stool samples, and 410 (91.11%) were negative stool samples. The oocysts were observed to have a spherical morphology and an a reddish colour, whilst the remainder of the faecal exhibits a blue coloration. As depicted in figure [1].

Table (1) Dignosis of Cryptosporidium parvum by microscope examination

Assay	***The number of sample***	***Positive +ve***	***Percentag %***	***Negative -ve***	***Percentag %***
modified Ziehl-Neelson	*450*	*40*	*8.88%*	*410*	*91.11%*

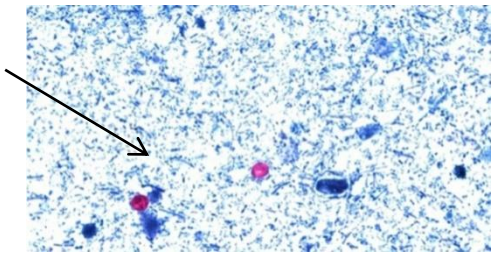


Figure1 :- It showed that the parasite oocysts appeared in the infected feces, and I colored the dark red oil lens with acid fast stain (100X).

***Dignosis of C. parvum by ELISA antigen* assay**

From a total 90 fecal samples, 70 (15.58%) were positive fecal samples and 380 (84.44%) were negative fecal samples. The samples were diagnosed, and we noticed that he was highly sensitive to the parasite infection of Cryptosporidium parvum.

Table (2) Dignosis of Cryptosporidium parvum by immunochromatographic assay

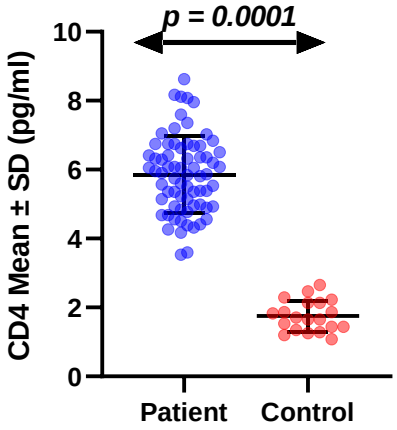
Assay	***The number of sample examined***	***Positive +ve***	***Percentag %***	***Negative -ve***	***Percentag %***
ELISA – antigen	*450*	*70*	*15.58 %*	*380*	*84.44 %*

Serum *CD4 levels* in patients and control groups

The study observed a significant increase in the average concentration of CD4 in infected infants (*mean ± SE* 5.8*61* ± 1.116 pg/mL) compared to the control group (*mean ± SE* 1.752 *± 0.448 pg/mL*).

Table (3) Serum *CD4* level in patients and control groups

CD4 pg/mL	***Patients N = 70***	***Control N = 20***	***P Value***
Mean ± SE	5.8*61* ± 1.116	1.752 *± 0.448*	*0.0001*
P Value is high significant at P ≤ 0.05			

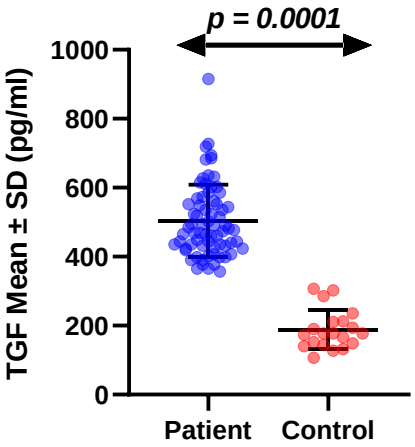


Serum *TGF-B* level in patients and control groups

The study observed a significant increase in the average concentration of *TGF-B1* in infected infants (*mean ± SE* 503.95 ± 104.73 pg/mL) compared to the control group (*mean ± SE* 188.12 ± 56.99 pg/mL).

Table (4) Serum *TGF-B* level in patients and control groups

TGF-B Pg/mL	***Patients N = 70***	***Control N = 20***	***P Value***
Mean ± SE	503.95 ± 104.73	188.12 ± 56.99	*0.0001*
P Value is high significant at P ≤ 0.05			



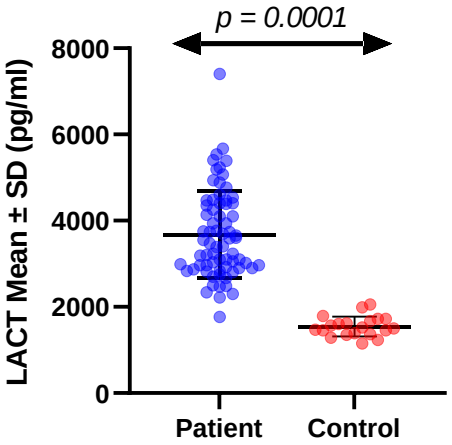
Fecal LF level in patients and control groups

The study observed a significant increase in the average concentration of ***fecal LF*** in infected infants (368.019 ± 102.025 pg/mL) compared to the control group (154.483 ± 23.268 pg/mL).

Table (5) Fecal LF level in patients and control groups

FLF pg/mL	***Patients N = 70***	***Control N = 20***	***P Value***

Mean ± SE	368.019 ± 102.025	154.483 ± 23.268	*0.0001*
P Value is high significant at P ≤ 0.05			



DISCUSSION

Cryptosporidium spp. are parasitic protozoa that opportunistically infected the epithelial cells of the small intestinal tract, resulting in diarrhoea in patients. These diseases have become common among children under 2 years old in impoverished countries. Cryptosporidium parvum had a high prevalence because it was the smallest of the oocysts, necessitating a specialized detection procedure[30]. The reason for the different incidences of Cryptosporidiosis in cases of diarrhea and natural feces is the geographical environment, direct contact between citizens, especially children, with domestic animals and livestock leading to animal-to-human transmission, or perhaps the water sources used from pollis, as well as failure to comply with proper prevention to reduce parasitic infection, as well as eating foods and drinks from street vendors with no sanitary conditions, which are one of the means of transmitting the parasite to children[4] . Cryptosporidiosis is widespread in all countries of the world and affects all age groups, especially children. Studies and research carried out to investigate the epidemic of this parasite around the world have shown that the prevalence rates vary across different countries and regions, depending on the geographical differences of those countries, where the parasite prevalence was 1.4% on the continent In Europeans and North American continent, the prevalence ranged from 3% to 20%, whereas in Asian countries, Africa, Australia as a whole and Latin America with Caribbean [34]. The presence of this parasite has been recorded in countries neighboring Iraq, including Iran, where the incidence was 1.7%[6]. In Turkey, the infection rate was 17%, while in the United States of America, it was 1.1%[31]. Some studies and research carried out in the governorates of Iraq also recorded a discrepancy in the incidence of cryptosporidiosis, with the incidence in the city of Ramadi and its suburbs being 39.13%. In the governorate of Diyala, the incidence was 2.3%. The incidence of this parasite was also recorded in the city of Mosul, where it was 14.3%, while in the north of Baghdad, it was 14.78%. [32] Oocysts were seen in the feces dyed with the modified Zilnsen dye (MZN). Parasite oocysts appeared spherically or ovally, painted red or pink, and surrounded by a distinctly transparent aura .

Table 1. The results of the 450 samples of feces dyed in the MZN dye showed that in 40 children of less than two years of age, of both sexes, and by 8.88%, the infection rate was close and morally different, as shown in Table 1 of the diagnostic results. Our current study showed a marked increase from the [33] 6.6% in the city of Al-Hila and the 6.6% in the city of Diwaniyah[34]. The current study recorded a remarkable 8.2% match on the study carried out by [35] at Samarra Hospital in Salah al-Din. While the findings of the present investigation were differed from[36], the total injury rate was 34% in Baghdad hospitals and also different from that in Kirkuk[37], and the incidence was 16.28% in the parasite. The *modified Ziehl-Neelson* stain was defined by being simplified, economical, and ability to clearly emphasize the internal complexities of the oocysts. However, this is a laborious process that necessitates expertise. The shape of the oocyste is round and has an a reddish colour, contrasting with the blue color of the rest of the stool.

Table 2. The results of the Elisa-antigen test showed a slight increase from the microscopic examination, where out of a total of 450 samples, 70 cases of parasitic disease showed a 15.55% increase. The Elisa-antigen test might be attributed to a strong link between immobilized specific Cryptosporidium antigen and the added stool specimen containing the parasite. and the study in Mosul City, which was 40%[31]. The findings of the present investigation demonstrated an increase in 15.55% from the results of the [6] study in Kirkuk, which was 19.11%. These results of this study (MZN and Elisa-antigen) have varied from previous studies and may be attributed to such causes as lifestyle, hygiene, immune treatments, food and environmental pollution within and outside the home, a healthy culture about infectious diseases, clean drinking water, and the economic situation. , until cryptosporidiosis becomes severe and chronic[30].

Table 3: The results of these tests showed a high increase, indicating that they were rising in the case of Cryptosporidiosis, and we noticed a significant increase in the moral differences of the infected samples where they were ≤ 0.0001. Experimental findings have demonstrated that CD4+ T lymphocytes serve as a deterrent against

cryptosporidium infections. CD4 cells regulate the length of the disease. The relationship between cryptosporidium infection and CD4 levels. The study revealed that individuals with cryptosporidiosis and CD4 counts below 100 cells/μL had a susceptibility to cryptosporidium infections that was 6.09 times higher than patients with higher CD4 counts. This association was statistically significant, with a p value of 0.002. The findings were in line with previous research that identified cryptosporidium as an opportunistic infection in patients with CD4 cell counts below 200/μL[14].There is compelling evidence that the likelihood of carrying faecal matter, the seriousness of the illness, and the prevalence of uncommon problems of cryptosporidiosis have a correlation with CD4 counts. The elevated occurrence of Cryptosporidium spp. in patients is likely due to the increased likelihood of contracting the infection from infected individuals and longer excretion. which increases the likelihood of further transmissions. The impact on cryptosporidiosis results in the restoration of CD4 levels[38].

Table 4 :- The maintenance of intestinal balance relies on the intestine mucosal capacity to endure harm caused by luminal substances and to subsequently repair and restructure itself. A variety of chemokines, cytokines, growth variables factors, and other mediators which suggested to play a role in controlling and preserving the balance of the intestines in laboratory settings[39]. TGF-B has been shown to have a pivotal function in this process due to its diverse effects on both epithelial and immunological cells[40]. Recovery trials were used to indirectly evaluate the healing of intestinal wounds. These experiments found that both the TGFB and wild-type control mice showed identical levels of damage when they were continuously exposed to 2.5% DSS. When the amount of DSS was elevated to 7.5%. The significance of TGF-B's role in these processes is indicated by changes in TGF-B levels or responsiveness observed in humans with intestinal inflammation disease and animal models of intestinal inflammation disease, in addition to in inflammatory bowel disease-associated with malignancies. TGF-B has been suggested to have a crucial function in controlling the migratory process of intestinal epithelial cells and promoting wound healing[22]

Table 5: The results of these tests showed a high increase, indicating that they were rising in the case of Cryptosporidiosis , and we noticed a significant increase in the moral differences of the infected samples where they were ≤ 0.0001. not agreed with study. The mean levels of FL for the IBD patients were 314 μg/g CD (SD 212.8) and 371 μg/g UC (SD 181.5) whilst mean FL in controls was only 1.3 (SD 2.4). . Patients with normal FL had no evidence of microscopic or histologic intestinal inflammation. This is in accordance with other pediatric and adult studies[24]. The results of this study detected a great deal of excellence when compared to the previous one. A weakness of the study with respect to parameters such as sensitivity or specificity is the fact that the study group is selected for suspicion of IBD in a tertiary center. This means that those parameters might be lower in a less restricted patient group. But we have clearly shown in a relatively large control group with symptoms severe enough to justify endoscopy that normal FL excludes intestinal inflammation caused by IBD with high probability ($P < 0.0001$). Clinically ,in the pediatric setting, f many patients have functional abdominal pain or irritable bowel syndrome[41]

The difference in the results between the various studies that have been examined in the epidural the hidden of parasite is due to a number of reasons, including: the difference in the number of samples examined in each study; the different geographical areas in which studies and research have been carried out, whether urban or rural; the level of health services provided; the efficiency of sanitation systems; the educational level of the inhabitants of those areas; the density of the population; and the extent to which animal husbandry overlaps with family members in one place. The deteriorating security situation in the country in 2014 may play a role in the incidence of parasitic disease, which has led to population movements from one region to another that have increased the population's overlap with different groups and consequently led to a decline in the economic situation of the country in general and in the study area in particular, which has adversely affected the financial allocations to the municipal and health sectors, resulting in the incidence of boating disease among persons in general and children in particular.

CONCLUSIONS: Cryptosporidiosis is an opportunistic parasite in infants ≤ 2 years with diarrhea. Modified ZN staining is simple and expensive. The ELISA-antigen detected is a useful tool in surveys and diagnostic studies. C. parvum infection in infants induced some immune marker inflammation.

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