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Gene polymorphism of some Immunological parameter in Patient infected with *Cryptosporidium parveum*

A Thesis

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By

Ola Abdallah Mahdi Dahash

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Supervised by

Dr. Prof. Hayam Khalis Al-Masoudi

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﴿وَأَنْزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا﴾

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Certification

I certify that this thesis entitle "Gene polymorphism of some Immunological parameter in Patient infected with *Cryptosporidium parveum*" was prepared by Ola Abdallah Mahdi Dahash under my supervision at the department of microbiology, College of Medicine, University of Babylon, as a Partial Fulfillment of the Requirements for the Degree of Doctorate of philosophy in Medical Microbiology

Supervisor:

Prof. Dr. Hayam Khalis Al-Masoudi

In view of available recommendation, I forward this thesis for debate by the Examination Committee.

Prof. Dr. Hayam Khalis Al-Masoudi

Chairman of Microbiology

College of Medicine

University of Babylon

Date***: / / 2023***

Dedication

To that who has harvested thorns from my way to be the path of knowledge me,…

My Father

To the candle which lights my way all over the years and gives me everything she has …

My mother

To Whom assisted me in life

My brothers and my sister

To my husband, the source of devotion and sincerity

Ahmed suade Al-Amari

To My daughter Rayan

To everyone who wishes me success, I present my consideration and Respect

Ola(2023)

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Summary

This study was designed to diagnose of the *Cryptosporidium Parvum* parasite in Stool sample by used different methods, It was carried During a time span of October 2022 to April 2023.

Diagnosis of parasite were first done by the biological methods. total of (300) stool samples were detected microscopically by modified ziehl-neelsen acid fast staining method in which *Cryptosporidium parvum*, oocyst appear as a pink to red round bodies against blue background.

From (300) stool samples , 75 were found to be positive for diarrhea causing protozoan parasites *C.parvum* ,while 225 samples negative.

In the present study using chromatographic immunoassay method for the identification isolated parasite . The results of the present study showed the presence of three species of parasitic diarrhea agents, these are *E. histolytica* (9.33%), G. *lamblia* (16%) and *C. parvum* (16%).

From (75) Positive samples , The highest infection rate was recorded in patient with age group (1 ≥ month) 27 followed by16 in patient in the age group (3-6 month) , and the lowest rate of infection with *C.parvum* were recorded in patient also which was 2 in the age group (9-10 year) with a Significant value

the distribution of *C.parvum* according to Sex of the highest percentage was found in males (65.3%), and lowest percentage found in females(34.7%). The distribution of *C. parvum* according to Residence showed 39 was found in rural and 36(48%) was found in aruban

To determine if parasite isolates was *Cryptosporidium Parvum* ,the present study characterized the isolates on the basis of the presence of heat

shock protein genes(HSP70) by using PCR technique. the result show of the 75 samples of patient observed carried the heat shock protein genes.

By Using the amplification refractory mutation system- polymerase chainreaction (ARMS-PCR) method, the genotyping of *MBL* polymorphism was determined in patient in both diarrheic and control groups ,In this study, we investigated a possible association between *MBL* (rs 1800450) polymorphisms and *Cryptosporidium parvum* in population of Babylon provinces/Iraq. The results of the present study demonstrate that the polymorphisms in *MBL* gene may affect susceptibility to *Cryptosporidium parvum* and increase risk of developing the disease.

Also , we investigated a possible association between *IL -18* (rs 5744247) polymorphisms and *Cryptosporidium parvum* . The results of the present study demonstrate that the polymorphisms in *MBL* gene may affect susceptibility to *Cryptosporidium parvum.*

By using ELISA technique, the concentrations of IL-18, MBL and INFγ and TGF-β concentrations in serum were measured in diarrhea cases as well as in the control groups, The present study revealed that the mean of IL-18 levels in sera of patients with age (7-9month) was higher (681.56±2.1pg/mL) than the control ones (280.26±0.5 pg/mL). (more susceptible to *Cryptosporidium*) then the other age. according to age groups of patients ,The highest level was in (7-9month) age group (681.56±2.1pg/mL) in male and (673.89±1.31pg/m) in female . While the lowest level was in the age group of (6-8 years) in male (612.43±2.21pg/mL) and in female (623.43±2.3pg/mL).

The mean of the sera levels of IFN-γ in patient suffering from diarrhea was higher in age (7-9 month**)** (593.56±2.3pg/mL) than the control ones

(430.96±0.5pg/mL) in male while in female the higher level in age 6-8 year (559.19±2.45 pg/mL) than the control ones(438.73±1.3 pg/mL).

The mean of the sera levels of TGF-β in patient suffering from diarrhea was higher in age (9-10 years**)** (147.24±2.4pg/mL) than the control ones (84.24±0.4pg/mL) in male while in female the higher level in age 6-8 year (137.24±2.4pg/mL) than the control ones(85.64±0.4pg/mL).

The mean of the sera levels of TGF in patient suffering from diarrhea was higher in age (9-10 years**)** (3692.57±5.1pg/mL) than the control ones (1253.74±2.4 pg/mL) in male while in female the higher level in age 1-2 year (3873.98±5.7 pg/mL) than the control ones(1278.83±4.9 pg/mL).

Also , we investigated The Correlation among the Immunological Parameters, we found confident correlation between IL-18 with TGF , MBL and INF-γ ($R^2 = 0.0043$, $p<0.05$) , ($R^2 = 0.1982$, $p<0.05$) and ($R^2 = 0.3622$, $p<0.05$) respectively .also There was correlation of MBL with TGF-β ($R^2 = 0.0345$, $p<0.05$) and between MBL with INF-γ ($R^2 = 0.2383$, $p<0.05$) and between of INF-γ with TGF ($R^2 = 0.061$, $p<0.05$).

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Chapter One

Introduction and Literature Review

1.1 Introduction:

Diarrhea is defined as passage of watery stool for more than three times in 24 hours . Globally, it is estimated that there are 1.7- 4.6 billion case of diarrhea every year with 2.2 million associated deaths. Diarrhea can be caused by various pathogen such as bacteria, viruses and parasites. Protozoan parasites are important causes of diarrhea and other gastrointestinal infection in humans they include mostly *C. parvum*, (Mafokwane *et al.*,2023).

Cryptosporidium was recognized as an important enteric pathogen in humans for the first time in 1976 . Since then, nearly 20 *Cryptosporidium* species that can infect humans have been identified, with *C. hominis* and *C. parvum* being the most frequently detected (Khan *et al.*,2019).

Even though *C. parvum* is the most common in some countries, such as Lebanon, Egypt, and Tunisia, Over the last 50 years, many studies have caused confusion between this genus and other genera of Apicomplexa, and several new species of cryptosporidia were erroneously named or classified. Recently, the molecular characterization of cryptosporidia has helped to clarify the confusion encountered in the taxonomy of these protozoa (Hijjawi *et al.*,2022).

Cryptosporidiosis of mammals is a major infectious diarrheal disease affecting young livestock and humans of all age groups. Species of the apicomplexan genus. *C. parvum*, is an important zoonotic pathogen worldwide .Parasite reproduction occurs in intestinal epithelial cells (IECs) and culminates in production of oocysts that transmit infection via the fecal-oral route. *Cryptosporidium* is a small protozoan parasite that infects the

microvillous region of epithelial cells in the digestive and respiratory tract of vertebrates(Yaşar.,2023)

It is an obligate intracellular parasite of man and other mammals, birds, reptiles and fish. It requires its host to multiply. Environmentally robust oocysts are shed by infected hosts into the environment. These oocysts can survive the adverse conditions on the environment for months until it is ingested by a new suitable host. In the new host, the life cycle starts again and multiplication occurs, using resources of the host(Dărăbuș *et al.*,2023).

It was first associated with disease in severely immunocompromised individuals. AIDS patients with low CD4-counts, but is now also recognized as widespread, general pathogen of immunocompetent humans.

interferon γ (IFN-γ) and interleukin 12 (IL-12) promote immunity as intestinal expression of these T-helper cell 1 cytokines coincides with recover(Poznanski, *et al.*,2016) .

McDonald and colleagues previously found that IL-18 might reduce *Cryptosporidium* infection via enhancement of secretion of AMPs by IECs (McDonald *et al.,* 2006). More recently, they found that IL-18 confers protection against *C. parvum* infection *in vivo* by coordinating with IL-12 to enhance IFN- γ production by macrophages ((Choudhry *et al.,* 2012).

The genomes of *Cryptosporidium* are containing eight chromosomes, with average size of 0.945-2.2 Mb and a total haploid genome size of about 9.2 Mb (Dąbrowska *et al.,* 2023).

Moreover, *C. parvum* contain two small extrachromosomal cytoplasmic double-stranded RNAs. The RNAs have an open reading frame (ORF), each of them encodes RNA-dependent RNA polymerase and encodes protein that has restricted homology to protein kinases of the mammalian. The Rna gene

of *Cryptosporidium parvum* is composed of small rRNA subunit with a size of 1.7 kb and a large rRNA subunit with size of 3.6 kb, as well as 5.8S rRNA subunit with a size of 151-bp.(Iliyasu *et al.*, 2022).

1.1.1 Aim of the study

The present study aimed to association between MBL and some immunological Parameter by used different methods in patients with *C. parvum* according to objective:

1) Detection of *C. parvum* microscopically by using Modified Ziehl Neelsen technique and Immunochromatography method of stool sample
2) Distribution of *C. parvum* according to Demographic information
3) Estimation the level of some cytokines (INF-γ , IL-18, TGF-β and MBL) in patients and control group by ELISA technique
4) Molecular diagnosis of *C. parvum* by using specific gene.
5) Determination of the *MBL* and IL-18 gene polymorphisms perform by ARMS-PCR .
6) Study the correlation between cytokine and *Cryptosporidiosis.*

1.2. Literature Review:

1.2.1 Historical Perspective of *Cryptosporidium*

In 1907, *Cryptosporidiosis* was first recognized as a commensal protozoan by Edward Tyzzer, who isolated *Cryptosporidium muris* from asymptomatic laboratory mice. Following the initial description of Cryptosporidium, the discovery was elapsed over 50 years, as the parasite was misdiagnosed with other coccidian members particularly Sarcocystis, because the oocyst of Sarcocystis species have thin walls, which rupture and release sporocysts containing sporozoites like sporulated Oocyst of *Cryptosporidium* (Gibson, 2021).

In early 1960s, ultrastructural studies recognized endogenous stages having an attachment organelle of the parasite, which is used as the main character for differentiation between *Cryptosporidium* and *Sarcocystis* and the wrong perception of strict host specificity was used for *Cryptosporidium*. Such recognition led to the new discoveries of various species: *C. garnhami (*humans*), C. bovis (*calves*), C. agni (*sheep*), C. rhesi (*monkeys*), C. anserinum (*geese*), C. cuniculus (*rabbits*) (*RePass 2018).

The veterinary importance was highlighted with high mortality and morbidity in turkeys caused by *C. meleagridis* in 1950s and bovine diarrhea caused by *C. parvum* within the early 1970s , and the diagnosis of *Cryptosporidium* species in the respiratory and gastrointestinal tract of mammals, reptiles, birds and fish (Chen *et al.*, 2023).

Although, broad range of domestic animals were infected with *Cryptosporidium*, but the impact remained ignored until 1980s when cryptosporidiosis was recognized as one of the common primary causes of neonatal diarrhea in calves and lambs (Hatam *et al.*, 2019).

In the twentieth century, several species of *Cryptosporidium* were named based on the host origin, due to cross transmission of the *Cryptosporidium* from one host species to another such as *C. wrairi* in Guinea pigs, *C. meleagridis* in turkeys, *C. felis* in cats, *C. baileyi* in poultry and game birds (Helmy and Hafez, 2022)

Recently, several *Cryptosporidium* spp. are named by using molecular techniques such as *C. andersoni* from cattle, *C. canis* from dogs, *C. hominis* from humans, and *C. molnari* from fish(Blaurock *et al.*, 2022).

1.2.2 *Cryptosporidium*

Cryptosporidium is a diarrhea causing single-celled parasite that infects the gastrointestinal tract of humans, livestock, birds and wildlife populations . Infection of this parasite can be life-threatening and results in chronic illness and mortality especially in immunocompromised patients, such as AIDS patients or in general in individuals with defective immune system as well as in malnourished young children that lack a mature immune system in countries of the developing world . In fact, *Cryptosporidium* is the second most important pathogen associated with diarrhea that causes death in children under five years of age (Ryan and Hijjawi ,2015)

Cryptosporidium is also a major causative agent of waterborne-gastroenteritis outbreaks, such as the outbreak that was documented in Wisconsin in 1993 where more than 400,000 people were infected and developed disease due to the pathogen. The majority of cases of *Cryptosporidium* diarrhea in humans are caused by *C. hominis* and *C. parvum* (Ryan *et al.*,2022)

Cryptosporidiosis is the disease caused by a protozoan parasite of the genus *Cryptosporidium*, Tyzzer noticed that this parasite did not contain sporocysts within the oocysts and it sporulates while still attached to the

host wall. For this reason Tyzzer named the genus Cryptosporidium, from the Greek kruptos meaning ‘hidden’, and the parasite that he identified in the gastric glands, *Cryptosporidium* muris(Makawi *et al.*, 2021)

C. hominis colonizes the gastrointestinal tract in humans while *C. parvum* can infect both animals and humans and is one of the most important agents that can cause waterborne illness in less developed countries(Saraav and Sibley.,2023; Wang *et al.*,2023)

Depending on the parasite and host species other clinical signs may be seen such as vomiting and nausea in some humans infected with *C. hominies*(Ogbuigwe *et al.*,2023)

Despite being identified in many animal species, cryptosporidiosis was not thought to be a very important disease until the early 1980s and the HIV-AIDS epidemic, though it was first reported in humans in 1976 (Nime *et al.*, 1976). Of the first seven reported cases of *Cryptosporidium* infection in humans, six were from immunocompromised patients leading to the belief that if the immune system was not compromised, *Cryptosporidiosis* would not pose any health risk .*Cryptosporidiosis* is now recognized as an important zoonotic disease, which affects many species (Chalmers and Katzer, 2013)

Transmission of the pathogen occurs via the fecal-oral route by coming in contact with water or food that has been contaminated with oocyst-bearing feces coming from diseased humans or animals and ingesting them(Pinto and Vinayak ., 2021)

The difficulty in controlling the transmission of the parasite lies in the properties of *Cryptosporidium* Oocysts that are produced by the parasite and represent the major infective stage of the parasite. These oocysts due to the properties of the Oocyst wall can survive outside the

host for long periods and are resistant to common methods of disinfection, such as chlorination(Khalil *et al.*,2018)

1.2.2.1 Taxonomy of *C. parvum*

According to the classification system proposed by Fayer (2010), *Cryptosporidium* species is classified as:

Phylum: Apicomplexa

Class: Conoidasida

Subclass: Coccidia

Order: Eucoccidiorida

Suborder: Eimeriorina

Family: Cryptosporidiidae

Genus: *Cryptosporidium*

Species*: C. parvum*

The genus *Cryptosporidium* includes protozoan parasites within the Phylum of Apicomplexa, although these parasites have lost the apicoplast which a is unique feature of apicomplexan organisms, they do not possess plastids or mitochondrial genome and there is only evidence of an atypical mitochondrion with no electron transport or oxidative phosphorylation function (Dong *et al.*,2020)

Until recently, parasites of this genus were grouped with coccidian parasites which are obligate intracellular pathogens. However, this parasite possesses unique characteristics that are not found in coccidians, such as the intracellular but extracytoplasmic location of the parasite in the epithelial lining of the host as well as the absence of a sporocyst or polar granules in the oocyst (Osman *et al.*,2017)

Biochemical, molecular and microscopic evidence of the similarities between *Cryptosporidium* and gregarine organisms led to the recharacterization of *Cryptosporidium* species as organisms belonging

to a new subclass, Cryptogregaria within the gregarines group (Piratae , 2018)

One of the key properties that led to the reclassification of *Cryptosporidium* includes the ability of the parasite to produce oocysts that multiply not only epicellularly but also extracellularly after they are excreted in the feces of the host and thus the parasite completes its life cycle outside of the host without the need of attachment to the epithelial cells of the host's intestine (Priest *et al.*,2000)

1.2.2.2 Morphological and life Cycle of *Cryptosporidium*

Cryptosporidium belongs to the Coccidia class of the phylum Apicomplexa. *Cryptosporidium* have some features which differentiate them from all other Coccidia , including (1) intracellular and extra-cytoplasmic localization, (2) forming of a "feeder" organ, (3) presence of morphological (thin- or thick-walled) oocysts as well as functional (auto vs. new-infection) types of oocysts, (4) small size of oocysts, (5) missing some morphological characteristics such as sporocysts or micropyles, and (6) the resistance of Cryptosporidium to all the available anti-coccidial drugs (Lamisere, 2022).

Cryptosporidium has a complex monoxenous life cycle, which is divided into two phases: the asexual phase (sporogony and schizogony/merogony) and the sexual (gamogony) phase. They proliferate and differentiate during the invasion of the free-living stages of Cryptosporidium within the parasitophorous vacuole under the brush border of the host cell located outside the cellular cytoplasm(Shoultz.,2016).

Cryptosporidium parasites can then attach to the cell surface and move along it for a short time using gliding mobility before they start to enter the cell. *Cryptosporidium* does not completely invade the cells actively,

but they provoke the cells to embrace them with a host-cell-derived membrane. Additionally, at the parasite–cell interaction phase, the Cryptosporidium creates an actin rich disk, a feeder organelle responsible for nutrition intake, as well as a channel into the cytoplasm of the host cell (Shrivastava *et al.*,2017).

After *Cryptosporidium* internalization in the host cells, the sporozoite divides inside the parasitophorous vacuole to approximately 4 µm × 4 µm in diameter as a spherical trophozoite with an excentric cell nucleus. After three asexual divisions (merogony/schizogony), the trophozoite is divided into 5 µm × 5 µm large type-1 meront, which contains eight merozoites (Tandel *et al.*,2019).

The merozoites and the sporozoites are similar in shape and size; however, the nucleus of the merozoites is located more centrally to the cell compared to the sporozoites. Upon leaving the parasitophorous vacuole, the merozoites begin their asexual development cycle in the epithelial cells and develop Type-I meronts again, then the trophozoite (Nascimento *et al.*,2020).

Otherwise, the merozoites initiate the sexual development cycle through differentiation to type-II meronts. Inside the meront, four merozoites develop by asexual division and after infection of further enterocytes, they are divided into micro- and macro-gametes (gamogony). The immature micro-gamontes are spherical, 5 µm × 4.5 µm in diameter, contain up to 16 peripherally located compact cell nuclei, and are precursors of the developing micro-gametes Figure(1-1).

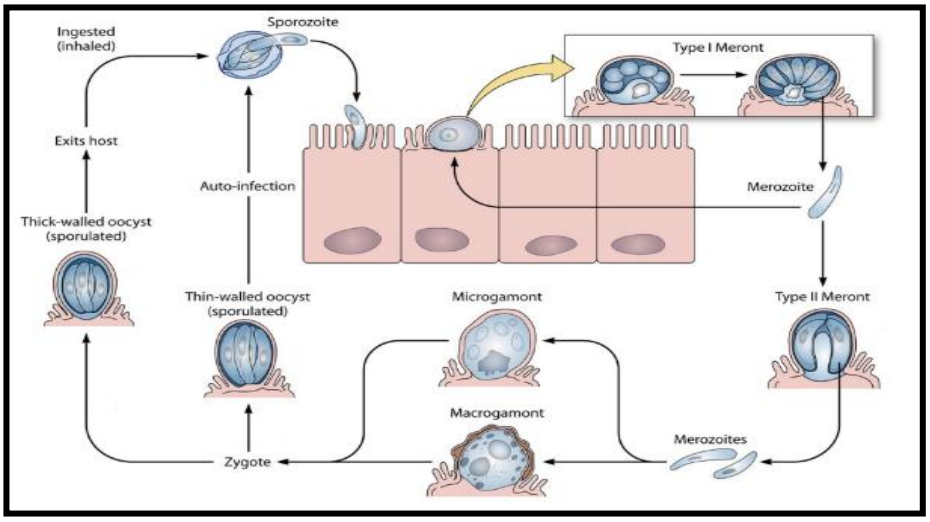


Figure (1-1) Scheme of the life cycle of *C. parvum* (Bouzid *et al* ., 2013)

They also have stubbed front ends and cell nuclei with no flagella. The mature micro-gametes leave their host cell and fertilize the macrogametes. Macrogametes are spherical, 5 µm × 5 µm in diameter and contain granulated cytoplasm and eccentrically positioned wall-forming bodies (Helmy,2014).

The zygote grows by syngamy and then goes through sporogony-a meiosis-like process. The oocysts (thin- or thick-walled) with 4 haploid sporozoites (sporulated oocysts) develop inside the parasitophorous vacuole . Thin-walled oocysts (about 20%) excystate in the host intestinal tract, leading to endogenous autoinfection, and the thick-walled oocysts (about 80%) are extremely resistant to several disinfectants, are excreted with the feces to the environment and can survive outside the host for a long time (Fayer and Xiao,2010).

The thick-walled oocysts represent the exogenous stage of the *Cryptosporidium* parasite. Cryptosporidium oocysts are approximately 4µm× 6µm in diameter, spheric to ovoid shape, have a residual body, and four banana-like or comma-shaped sporozoites with a pointed front end and a stubbed hind end, where the nucleus is localized (Shinn *et al.*,2023)

The residual bodies are 2.4 µm × 2.5 µm in diameter and consist of a spherical to ovoid membrane-bound globule (1.5 µm × 1.6 µm) and are surrounded by small granules (0.2 µm × 1.2 µm). *Cryptosporidium* sporozoites are not encapsulated by a sporocyst and the oocyst wall consists of an outer and an inner layer, and a pre-formed junction that extends from one pole of the oocyst to approximately half of the oocyst(Shinn *et al.*,2023)

Additionally, four sporozoites (5µm× 1µm in diameter) hatch out of the pre-formed joint under the effect of temperature, pH, gall bladder salts, pancreas enzymes, and CO_2 of the host gastrointestinal tract. The free sporozoites adhere to the microvilli of the enterocytes and lead to internalization using their proximal end. The sporozoites' glycoproteins (GP40 and GP900 of 40 kDa and >900 kDa) and the circumsporozoite-like glycoprotein (CSL) play an important role in the adhesion and invasion process of the sporozoites to the host cells(Helmy & Hafez,2022)

The host cell surrounds the sporozoites with membrane protrusions and forms a parasitophorous vacuole in the brush border of the enterocyte. Interestingly, the localization of the parasitophorous vacuole by *Cryptosporidium* spp. is different from that of the other Apicomplexa; thus, Cryptosporidium spp. localization is described as intracellular, but extracytoplasmic (Tandel *et al.*,2019).

The sporulated Oocysts are very resistant to environmental factors and only a few chemical disinfectants show efficacy against the sporulated oocysts due to their thick wall . Therefore, it is difficult to completely remove the *Cryptosporidium* oocysts from contaminated drinking water (Nascimento *et al.*,2020)

The thick wall oocysts are sporulated and are infectious when shedding, which can result in immediate infection of new hosts. The infectious dose of *Cryptosporidium* oocysts for humans is about nine oocysts per Cryptosporidium isolate and about 50 Oocysts for calves . However, it was reported that 1 to 10 Oocysts of *Cryptosporidium* caused infection for some individuals during the Milwaukee outbreak . Although, one infected host can shed up to 10^{10} Oocysts, which results in a huge infection pressure(Nascimento *et al.*,2020).

2.2.3 Pathogenesis of *Cryptosporidium* spp.

The pathogenic mechanisms of *Cryptosporidium* causing diarrhea, malabsorption and wasting are poorly understood, but are clearly multi-factorial and involve both parasite and host immune factors as well as the gut environment (Certad *et al.,* 2017).

Cryptosporidium associated diarrhea is caused by two pathogenic mechanisms: First. Malabsorptive diarrhea occurs due loss of enterocytes and diminishing of villi, which reduces the intestinal surface, leading to decrease nutrient and water absorption . Second. Secretory diarrhea occur due to prostaglandins (mainly PGE2 and PGI2) induce secretion of chloride and carbonate ions into the intestinal lumen and decrease absorption of sodium chloride. This produces an osmotic pressure that forces water into the lumen, resulting in secretory diarrhea (Certad *et al.,* 2017).

After ingestion of the thick-walled oocyst with food or water by the host, many signaling molecules are expressed on the sporozoite surface that mediate their attachment and invasion to the host cells. Calcium-dependent protein kinases (CDPKs) were reported to be involved in the regulation of the invasion process of the sporozoite to the host cell . (Ryan *et al.*, 2016)

Furthermore, *Cryptosporidium* is embraced by the host cell instead of invading the host cells. Therefore, it stays in an epicellular location and this induces tremendous actin rearrangement in the infected cells(Helmy *et al.*,2022)

The initial host–parasite interactions due to attachment and invasion of *Cryptosporidium* in host cells are serious events in the pathogenesis. These connections of attachment, invasion and parasitophorous vacuole formation are complex procedures which involve multiple parasite ligands and host receptors, invasive (zoites) stages of apicomplexans possess specialized secretory organelles (rhoptries, micronemes and dense granules) collectively known as the apical complex (Bouzid *et al.*,2013).

The sporozoites within the parasitophorous vacuole are not directly in contact with the host cell, and occupy a unique intracellular but extracytoplasmic niche, typical of *Cryptosporidium* parasites also named parasitophorous vacules, Intestinal damage caused by massive infection may lead to reduced growth rates. (Certad *et al.*, 2017).

The mucosa of affected areas is hyperemic and edematous and the mesenteric lymph nodes are partially enlarged and also edematous (Ludington and Ward, 2015). Histopathological studies showed mild to moderate villus atrophy may occure, as well as occasional villus fusion. The crypts of affected areas partly are dilated and contain debris and neutrophil granulocytes. Neutrophil granulocytes and a massive mononuclear cell infiltration (among others macrophages) also were proven in the lamina propria mucosa(Genova and Tonelli, 2016).

In the infected host, epithelial cell degeneration, metaplasia of physiological high prismatic to isoprismatic villus epithelial cells, hyperplastic crypt epithelium, displacement of microvilli in the area of the intracellular parasite stages' attachment zone, and long microvilli can

be seen in the vicinity of the parasite stage .These pathological alterations result in the reduction of the intestinal absorption surface and, consequently, malabsorption. Damage to the intestinal epithelium may also have an impact on the activity of brush border membrane enzymes (glucoamylase, alpha-dextrinase, saccharase, lactase), resulting in a reduction in the small intestine's carbohydrate digestion ability (Helmy, and Hafez.,2022)

As a result, osmotically active particles persist in the intestinal lumen, osmotic diarrhea develops, and water resorption is impeded. Several causes can lead to increased chloride secretion into the gut lumen, including immune response to membrane injury, prostaglandins secreted by enterocytes of intra- and sub-epithelial lymphocytes, and plasma cells and macrophages that enhance blood vessel permeability (Ahmed and Karanis.,2020). (Figure 1-2).

1.2.2.5 Transmission:

Infection of *Cryptosporidium* occur as a result of ingestion of parasite oocysts those shed with the feces of infected human and/or animals, or carriers of the disease, as well as autoinfection may happen due to the presence of thin wall oocysts and excyst inside the same host. Transmission of *Cryptosporidium* spp. may occur via different means, direct or indirect contact from animal to animal, animal to human (Zoonotic transmission), or human-tohuman. It can also occur due to ingestion of contaminated food or water with infected oocysts or, via vectors such as rodents, arthropods or even birds may play a role as mechanical agents of transmission (Helmy *et al.*, 2015).

1.2.2.5.1 Direct transmission (animal-to-animal contact)

Direct transmission usually occurs by the fecal oral-route, when animals are resided together in an overcrowded place, it is a common transmission route between the animals. Animals reared indoors are more

expose to acquire infection than grazing cattle and contamination of udders, favors transmission between the dam and their calves (Kuria ,2023). Indirect transmission when feces deposited on the ground are subjected to rain and wind, as well as mechanical transport hosts, liquid waste or sewage discharged into a river orthe sea may play a role as a source of infection (Galvan *et al*., 2014).

1.2.2.5.2 Animal-to-human (Zoonotic transmission)

With a large numbers of animal reservoirs, *Cryptosporidium parvum* is the most etiologic zoonotic agent of cryptosporidiosis, mainly in young animals. Therefore, persons who encounter infected animals, either for occupational or recreational reasons, may be at risk. Outbreaks of cryptosporidiosis have been reported among veterinarians, veterinary students, farmers, livestock owners, slaughterhouse workers and pet owners (Ryan *et al.*, 2017)

Due to the high prevalence of *C. parvum* infection and large numbers of oocysts shed in feces, calves are considered to pose the most significant threat to environmental contamination and transmission to humans (Mahmoudi *et al.*, 2017).

Researches indicated that *C. andersoni* may cause human infection, it was diagnosed in an immunodeficient patients in France, also has been isolated from humans with diarrhea in England, pediatric patients in Malawi and from patients in Australia, which opening the possibility for zoonotic transmission of this species. The cases of human infection with cat, dog, and turkey genotypes mentioned previously also implicate transmission from animals (Hussain *et al.*, 2017).

1.2.2.5.3 Person-to-person transmission

The parasite transmits by the fecal-oral route in households and schools (Bouzid *et al.* 2013). The contact between persons spread the infection that has been implicated in many outbreaks in all parts of the

world (Mohammad *et al.*, 2011). The nosocomial and day care center are also likely related to direct person-to-person spread in institutional settings where sanitation is difficult, further supporting the highly infectious capacity of the parasite (Mahdi and Ali, 2002; Pandak *et al.* 2006). Data showed a higher prevalence of cryptosporidiosis in homosexual men that imply oro-anal contact yield a high risk for exposure to *Cryptosporidium* (Moghaddam, 2007; Al-Warid, 2010). Patient-to-patient or patient-to health care staff transmission may occur in hospitals. The high prevalence of *C. hominis* and of anthroponotic subtypes of *C. parvum*, particularly in children, has also been taken as an indication of the importance of person-to-person transmission (Xiao, 2009).

1.2.2.5.4 Waterborne transmission

Cryptosporidium is one of the most common prevalent waterborne parasitic infections, contaminated water act as one of the major sources of indirect cryptosporidial infections in cattle, other animals and human (Efstratiou *et al.*, 2017; Zahedi *et al.*, 2017). Numerous reports worldwide provide that contaminated water is a highest risk factor for cryptosporidiosis (Castro-Hermida *et al*., 2009; WHO, 2009).

Heavy rains, cattle manure on fields in the watershed, abattoir waste, landfill and sewage overflow were considered potential sources of cryptosporidiosis (Swaffer *et al.*, 2014; Lapen *et al.*, 2016). Recently, cryptosporidiosis outbreaks via related to recreational waters was reported (Xiao *et al.*, 2018).

Nevertheless, routine use of recreational waters by incontinent persons, including diapered children and children, increases the potential for water-borne transmission (Areeshi *et al.*, 2007). Even optimal conditions of pool design, water quality, filtration, and disinfection may not stop fecal accidents (Zafar *et al.*, 2017). Swimming is a very popular

entertaining activity around the world and the majority of recreational water outbreaks have been connected to swimming pools, with others documented at water parks, water slides and fountains (Ryan *et al.*, 2017).

1.2.2.5.5 Food-borne transmission

Cryptosporidium is one of the main food-borne eukaryotes causing a disease of socioeconomic significance worldwide (Putignani and Menichella, 2010). The incidence of food borne disease occurrences caused by contaminated vegetables and fruit juices is increasing. The association of *Cryptosporidium* with fruit juice is an elevated safety concern in food industries (Lynch *et al.*, 2006; Caccio and Putignani, 2014).

Joint Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) considered this parasite as a food borne contaminant. *Cryptosporidium* was ranked the fifth out of 24 potentially food borne parasites in terms of importance as a food borne pathogen (FAO/WHO, 2014).

Contamination of food with *Cryptosporidium* Oocysts has been confirmed in researches from different regions of the world; those studies have mainly focused on fruits and vegetables, because these foods are disposed to contamination and are often consumed raw or after minimal thermal treatment, therefore increasing the possibility of disease transmission (Robertson and Chalmers, 2013; Caccoi and Putignani 2014).

Vegetables can be contaminated from animal or human origin fertilizers by contaminated water used to irrigate or moisten produce by soiled hands of farm workers, produce handlers, or food workers and from contaminated surfaces where vegetables are packed, stored, sold or prepared (Abougrain *et al*., 2010; Ryan *et al*., 2018).

Raw vegetables from the market place may contain *Cryptosporidium* oocysts on the surfaces. In addition, vegetables washing may not totally remove *Cryptosporidium* Oocyst (Robertson & Chalmers 2013). Detection of Oocysts from vegetables washes is usually difficult; therefore, using molecular methods to detect and identify small numbers of oocysts are becoming more significant with increasing international trade of fresh product (Ryan *et al*., 2017).

1.2.2.5.6 Airborne transmission

In 1994, Giang *et al.* was first confirmed the respiratory tract involvement; it occurs frequently in children with enteric cryptosporidiosis in Uganda, with as many as 35% of children with cough and intestinal infection having parasite DNA in respiratory secretions (Mor *et al.*, 2010).

Respiratory tract infection with *Cryptosporidium* spp. was thought to be a rare complication of intestinal disease in people with HIV or other immunosuppressive conditions (Sponseller *et al.,* 2014). Furthermore, if respiratory transmission is confirmed, respiratory precautions, such as using masks, covering the mouth/nose when coughing, environmental surface cleaning, and patient isolation, may be advisable when managing patients infected with *Cryptosporidium* (Mor *et al.,* 2018).

1.2.2.6 Virulence Factor:

Virulence is commonly defined simply as the ability of a microorganism to cause disease . Virulence and pathogenicity are often used interchangeably, but virulence may also be used to indicate the degree of pathogenicity, where pathogenicity is used solely to describe the ability of a pathogen to inflict damage to the host(Bouzid *et al*., 2013).

Virulence, despite being a microbial characteristic, can exist only in a susceptible host and depends on the context and nature of the host-

microbe interaction. In fact, virulence is usually multifactorial, involving a complex interplay between the parasite and the host. *Cryptosporidium* does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium (Okhuysen,2002).

Putative virulence factors for *Cryptosporidium* have been identified as genes involved in the initial interaction processes of *Cryptosporidium* Oocysts and sporozoites with host epithelial cells, including excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance, and host cell damage (Fayer *et al*., 2009) .

Adherence Factors :A critical initial step in establishing infection is parasite attachment to host cells. Two classes of proteins, namely, mucin-like glycoproteins and thrombospondin-related adhesive proteins, have been characterized and shown to mediate adhesion .The characteristics of these proteins are summarized in CSL (circumsporozoite-like glycoprotein), with a molecular mass of_1,300 kDa, was described by Riggs and colleagues and is associated with the apical complex of sporozoites and merozoites ,CSL is released as a soluble glycoprotein and contains a ligand that binds specifically to a receptor on the surface of human and bovine intestinal epithelial cells (Bouzid *et al*., 2013).

1.2.2.8 Immune responses to *Cryptosporidium*

A host can evolve two types of defense mechanisms to ensure its survival when challenged by a pathogen: resistance and tolerance . The human immune system resists the invading pathogens. The host immune response to *Cryptosporidium* involves components of both the innate and adaptive immune systems (Naz *et al*., 2023).

The adaptive immune system consists of T and B lymphocytes, which functions as antigen-specific recognition to identify and eliminate the

pathogen and ensures a long lived immunological memory against reinfections (Chang *et al*., 2023).

1.2.2.8.1 Innate immune responses

Innate immunity is a first line of the host's defense against infections. It provides robust, immediate and non-specific immune responses to invading pathogens. Innate immunity is further divided into evolutionarily primitive humoral and cellular components (Pantenburg *et al*., 2008)

Innate immune system consists of anatomical barriers, complement system and natural killer cells, phagocytes, pattern recognition receptors (PRRs), toll-like receptors (TLRs) that play a vital role in the protection of the host from pathogenic challenges(Peterson and Artis, 2014).

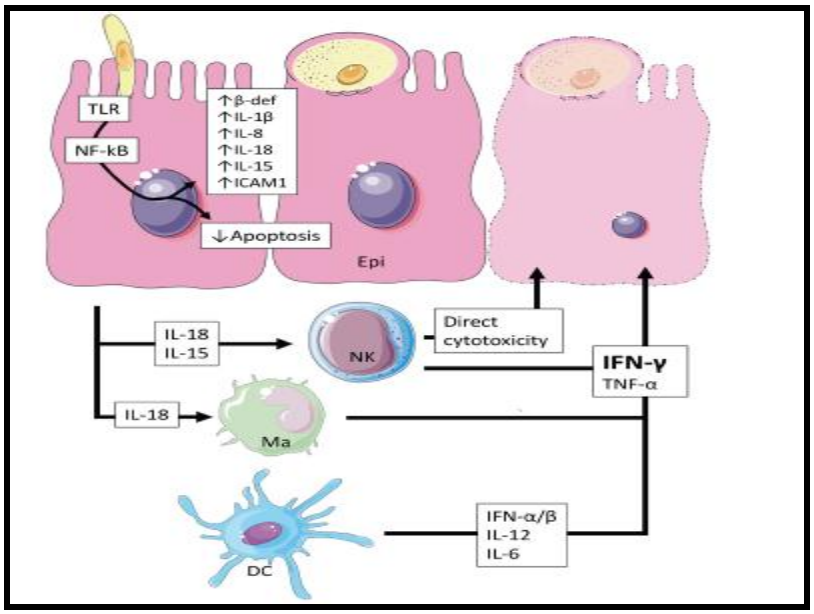


Figure (1-2). Main mechanisms of innate response to Cryptosporidium infection (Stoyanova& Pavlov,2019)

Upon ingestion, oocysts and excysted sporozoites first encounter innate immune components along the gastrointestinal tract. Early mediators of innate immune protection include the thick mucus layer of the small intestine, intestinal epithelial cells (IECs), and chemokines,

cytokines and antimicrobial peptides (AMPs) secreted into the intestinal lumen and/or underlying submucosa and bloodstream (Saez *et al.,* 2023).

Previous studies have elucidated the importance of IECs and soluble mediators during *Cryptosporidium* infection . IECs provide an initial mechanical and functional barrier , and also serve as the primary host cell for *Cryptosporidium* infection. IEC as well as biliary epithelial cells express several toll-like receptors (TLRs), including TLRs 2, 4, 5 and 9, which have been shown to be important in modulation of the host immune response and subsequent parasite clearance (Perez *et al.,* 2014).

In response to infection, IECs secrete chemokines and cytokines such as IL-8, CXCL10, and CCL2 responsible for the recruitment of inflammatory cells and activation of adaptive immune cells, prostaglandins that enhance intestinal fluid secretion and AMPs like β-defensins, which are capable of directly killing sporozoites *in vitro* (Guesdon *et al.,* 2015).

Recently, it was shown that CCL20, a chemokine and AMP secreted in part by IECs in the intestine, was down regulated during *Cryptosporidium* infection of neonatal mice . Furthermore, oral administration of recombinant CCL20 reduced parasite burden in a manner independent of immune cell recruitment, but rather via direct cytolytic activity on extracellular infective stages of the parasite (Zhou *et al.,* 2013).

CX3CL1 is another chemokine recently shown to be important during *Cryptosporidium* infection . Its soluble form acts as a potent recruiter of leukocytes, while its membrane-bound form functions as an adhesion molecule for CX3CR1+ T lymphocytes, NK cells and monocytes (Stievano *et al.,* 2004).

In addition to chemokines, proinflammatory cytokines secreted by IECs and phagocytes have been shown to play a crucial role in the innate

immune response to *Cryptosporidium* . Lastly, mannose-binding lectin (MBL), asoluble innate immune mediator secreted by hepatocytes, has been shown to be important in the protection against cryptosporidiosis(Ludington & Ward, 2015).

Several studies have shown that low serum MBL in children and AIDS patients is associated with increased susceptibility to recurrent *Cryptosporidium* infection. The mechanism by which MBL protects against infection is not fully understood, but likely involves complement activation on extracellular stages of the parasite (Lemieux *et al.*, 2017).

Early in infection, IFN-γ secreted by NK cells, macrophages and dendritic cells is thought to be the major cytokine involved in orchestrating both the innate and adaptive immune responses, but recent evidence suggests that IL-18 is important in the control of *Cryptosporidium* infection as well (Bedi and Mead 2012).

1.2.2.8.2 Adaptive immune responses

The importance of the adaptive immune response during *Cryptosporidium* infection is highlighted by the susceptibility of AIDS patients to cryptosporidiosis, as well as the resolution of infection observed following CD4+ T cell reconstitution in patients given antiretroviral therapy (Singh *et al.*, 2011). Though disease severity in humans is often viewed as being inversely proportional to absolute CD4+ T cell numbers, Tzipori and colleagues found that persistent cryptosporidiosis in macaques was more dependent on SIV load, viral damage to gut lymphoid tissue, and rapid depletion of mucosal CD4+ T cells during the acute phase of viral infection than on declining circulating CD4+ T cell levels during chronic SIV infection (O'Connor *et al.*, 2011), suggesting that depletion of local CD4+ T cells may be more predictive of disease severity than absolute CD4+ T cell numbers.

Much of our understanding of the cell-mediated immune response to *Cryptosporidium* infection is limited to CD4+ T cells; the role and importance of CD8+ T cells is less clear . Previous studies found that CD8+ T cell numbers increased during *Cryptosporidium* infection of macaques , and both CD4+ and CD8+ T cells isolated from humans with previous *Cryptosporidium* infection could produce IFN-γ in response to stimulation by *C. hominis* antigens (Borad and Ward,2010).

Furthermore, *in vitro* studies found that CD8+ T cells isolated from donors with prior exposure to *Cryptosporidium* were able to lyse *C. parvum*-infected intestinal epithelial cells in a manner dependent on the release of cytotoxic granules (Pantenburg *et al.*, 2020). The importance of CD8+ T cells *in vivo* was recently studied by Salát and colleagues (Kvac *et al.*, 2011), who found that reconstitution of immunocompromised mice with activated CD8+ T cells significantly reduced the length and severity of *C. muris* infection, albeit to a lesser extent than reconstitution with CD4+ T cells. Regardless, these studies suggest that CD8+ T cells contribute to the cell-mediated immune response to *Cryptosporidium*, likely via direct cytolysis of infected intestinal epithelial cells, and through IFN-γ-mediated protection and clearance.

The role of humoral immunity during *Cryptosporidium* infection is incompletely understood. Passive immunization studies in animal models have shown a correlation between anti-*Cryptosporidium* antibody administration and reductions in oocyst shedding and disease severity (Ajjampur *et al.*, 2011). The use of hyperimmune bovine colostrum for passive immunotherapy of cryptosporidiosis in humans has also been evaluated, with variable results. Numerous studies in humans have found an association between levels of anti-*Cryptosporidium* antibodies and history of infection (Heimburg *et al.*, 2013).

Other studies found significantly increased IgG, IgM and IgA responses to *C. parvum* p23, and increased IgG and IgA responses to *C. parvum* and *C. hominis* gp15 in Bangladeshi children infected primarily with *C. hominis* , suggesting a cross-reactive humoral response to gp15.they found a similar phenomenon in anti-gp15 antibody responses in South Indian children infected with *Cryptosporidium.(* Borad *et al.*, 2012)

Interestingly, several studies found that acute and asymptomatic cryptosporidiosis were associated with higher IgG, IgM and/or IgA responses to *Cryptosporidium* spp. antigens compared to persistent cryptosporidiosis (Allison *et al.*, 2011), suggesting that humoral immunity may play a role in limiting the length and severity of infection.

However, it is also likely that the association between anti-*Cryptosporidium* antibody responses and disease may reflect underlying cell-mediated immune responses. they found that HIV-infected patients with asymptomatic cryptosporidiosis not only had higher circulating IgG and fecal IgA levels to *Cryptosporidium* antigens compared to patients with diarrhea, but these patients, on average, also had significantly higher CD4+ T cell counts , which is known to be more predictive of disease severity. Future studies should aim to distinguish between the correlative and causative effects of the humoral response during *Cryptosporidium* infection(Wanyiri *et al.*, 2014).

1.2.2.9 Interleukins-18

IL-18 is secreted by IECs, macrophages and dendritic cells at sites of infection . Its effects are pleiotropic, and include stimulation of IFN-γ and TNF-α production by immune cells, chemotaxis of inflammatory cells, maintenance of epithelial integrity, and stimulation of AMP secretion by IECs (Choudhry *et al.*, 2012).

McDonald and colleagues previously found that IL-18 might reduce *Cryptosporidium* infection via enhancement of secretion of AMPs by IECs (McDonald *et al.,* 2006). More recently, they found that IL-18 confers protection against *C. parvum* infection *in vivo* by coordinating with IL-12 to enhance IFN- γ production by macrophages ((Choudhry *et al.,* 2012). These results were supported by studies done by Mead and colleagues, who found that IL-18 protected against *C. parvum* infection *in vivo* via stimulation of IFN-γ production and AMP expression (Bedi *et al.,* 2015). They also found that mouse dendritic cells were able to produce IL-18 upon stimulation with *C. parvum* antigens . It is unclear whether the effects of IL-18 on IFN-γ and AMP production are mutually exclusive, or whether IL-18-dependent stimulation of AMP production by IECs is due to enhanced IFN-γ secretion.

Upon their interaction with *Cryptosporidium*, IECs relay signals to innate immune effector cells . These include dendritic cells, NK cells, macrophages, neutrophils mast cells and eosinophils. Dendritic cells migrate toward areas of *C. parvum* infection in an IFN- γ-dependent manner , but until recently, their role in clearance of the parasite was unclear. *In vitro* studies found that bone marrow-derived dendritic cells challenged with *C. parvum* sporozoites or antigens secreted a number of cytokines, including type I INF, TNF-α, IL-6, IL-1β, IL-12, and IL-18 (Bedi *et al.,* 2014).

Furthermore, parasite burden was reduced upon adoptive transfer of dendritic cells, with longer protection observed in mice receiving dendritic cells stimulated with live parasite *in vitro* prior to transfer. Laurent and colleagues extended this finding by evaluating the role of dendritic cells during *C. parvum* infection in neonatal mice (Lantier *et al.,* 2013), which are more susceptible to infection compared to adults. They found that neonatal mice had significantly lower numbers of intestinal

CD103+ dendritic cells during the first weeks of life, when the mice were most susceptible to infection. Stimulation of dendritic cell production during this period led to increased resistance to infection through a mechanism dependent on IL-12 and IFN-γ, and independent of adaptive responses. Recently, dendritic cells were shown to transport *Cryptosporidium* parasites and antigens to mesenteric lymph nodes in mice (Perez *et al.*, 2014).

Mast cells play a pivotal role in bacterial and parasitic infections . They secrete inflammatory mediators (histamine, cytokines, prostagalandins, leukotrienes) locally and systemically; mobilize and recruit innate and adaptive immune components; and generally influence an overall Th2-type immune response . In mice infected with *C. muris*, accumulation of gastric mucosal mast cells was shown to correlate with oocyst shedding, suggesting a role in parasite clearance (Abraham and John,2010).

Future studies should aim to elucidate whether *Cryptosporidium* can trigger mast cell degranulation, and whether mast cell depletion or inhibition of degranulation affects parasite clearance. It would also be interesting to evaluate whether mast cells influence the Th1/Th2 balance during infection, as *Cryptosporidium* generally induces a Th1-type response (Codices *et al.*, 2013).

1.2.2.10 IFN-γ

Previous studies found that mice lacking functional NK cells were more susceptible to *Cryptosporidium* infection (Wang *et al.*, 2017), while treatment of immunocompetent and immunodeficient mice with IL-12, a potent NK cell activator, enhanced protection (Korbel *et al.*, 2011).

Both studies found that NK cell-dependent protection was primarily mediated through IFN-γ. Drouet and colleagues recently found an

increase in early recruitment of activated, perforin+ NK cells to areas of infection in neonatal lambs infected with *C. parvum* (Olsen *et al.,* 2015).

These studies suggest that the role of NK cells during *Cryptosporidium* infection may involve both IFN-γ-mediated protection and direct cytolysis. In addition to dendritic and NK cells, mast cells may be necessary for parasite clearance , but their role during *Cryptosporidium* infection remains poorly understood.

1.2.2.11 Transforming growth factor- β (TGF-β)

Transforming growth factor b (TGF-β) is an anti-inflammatory cytokine that stimulates repair of damaged mucosal epithelial integrity following injury .

Lymphocytes secreting TGF-β down regulate host immune and inflammatory responses, especially in the intestinal mucosa . Absence of these regulatory lymphocytes is thought to play an important role in the pathogenesis of inflammatory bowel diseases(Zhou *et al.,* 2014)

TGF-b is also a key signal for epithelial repair in vitro . TGF-β stimulates restitution by causing migration of epithelial cells into denuded areas, deposition of extracellular matrix, and restoration of epithelial barrier integrity .

TGF-β has the striking ability to repair the permeability defect of intestinal monolayers induced in vitro by C. parvum infection or gamma interferon (IFN-g). We reasoned that in cryptosporidiosis, following epithelial cell damage and barrier integrity disruption by C. parvum and inflammatory cytokines, TGF-b may be needed to restore epithelial integrity and promote healing (Hu *et al.,* 2013).

1.2.2.12 Mannose-Binding Lectin (MBL):

Mannose-Binding Lectin (MBL) is a part of the humeral innate immune system. It is a pattem recognition molecule able to detect a wide range of microbial and altered-self targets and recruit a number of host immune effector systems to clear those targets(Liakath *et al.*,2021).

Mannose-binding lectin (MBL) is a pattern recognition molecule of the innate immune system. It belongs to a family of proteins called the collectins, in which lectin (carbohydrate recognition) domains are found in association with collagenous structures (Alhayali *et al.,* 2018)

MBL was the first lectin protein described to initiate the lectin pathway. It is synthesized in the liver and acts as an acute-phase plasma protein (APP) during infections. MBL is an oligomer consisting of three polypeptide chains, each containing a collagen-like domain and a carbohydrate recognition domain (CRD) similar to C1q to recognize pathogens(Stravalaci *et al.*,2022).

1.2.2.12.1 MBL Function:

The complement system consists of 30-40 plasma and cell membrane proteins and functions between the innate and adaptive immune systems. It plays a vital role in host defenses against infections. Activation of the complement leads to robust and efficient proteolytic cascades to eliminate pathogens by inducing chemotaxis (attraction of leukocytes), opsonophagocytosis, and direct destruction of the microorganisms by enabling membrane attack complexes (MAC) in the cell wall.

The lectin complement pathway plays a key role in innate immunity by recognizing invading pathogens .The lectin pathway is initiated by the binding of mannosebinding lectin (MBL) and other c-type lectins to sugar moieties exhibited on cell surfaces of several microbes and activates the

esterase activity of MBL-associated serine proteases (MASP-1,2 and 3). Once activated, MASPs cleave and activate C4 and C2 thus generating the C3 convertase (C4bC2a). C3 convertase binds and cleave C3, generating C3b and C3a. The C3a fragment has anaphylatoxic and pro inflammatory activity whereas C3b has opsonic activity(Wallis,2007).

The activation of complement by MBL represents a pathway which is independent of both the classical and alternative pathways, but which has similarities to the classical pathway. In the circulation MBL is found in association with four structurally related pro-enzymes. These are the MBL associated serine proteases (MASPs) and a truncated version of MASP-2 called MAp (Kulkarni *et al.*,2020).

In serum there is a 20-fold excess of MASP-1 over MBL and some evidence that the enzyme cleaves C3 directly. MASP-2, which is present at much lower concentrations than MASP-1, appears to be the more important in complement activation. The available data suggest that MBL – MASP-2 complexes become activated when bound to appropriate sugar arrays on microbial surfaces. The enzyme specificity of MASP-2 is apparently identical to that of Cl esterase and results in the sequential cleavage of C4 and C2 (Kulkarni *et al.*,2020)

The C4b fragments generated bind covalently either to the MBL itself or to the nearby microbial surface and act as a focus for C2 binding/activation. The resultant C4b2a complex has C3 convertase activity and cleaves C3 in a similar manner to the C3 convertases of both the classical and alternative pathways of complement activation figure (1-4).

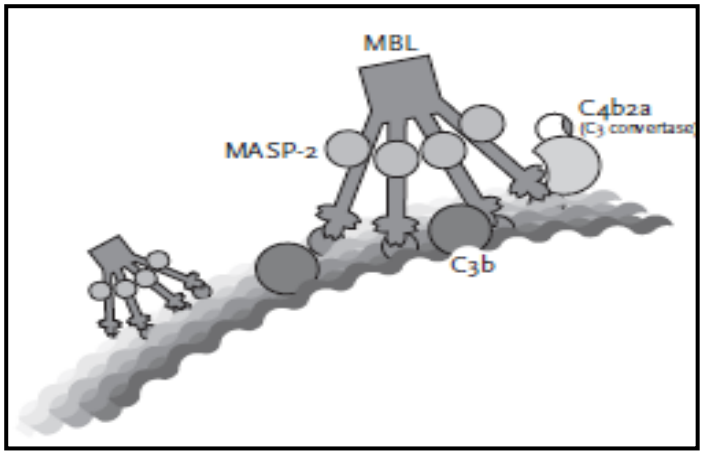


Figure (1-3): Mannose-binding lectin (MBL) complexed with MASP-2

The activated lectin pathway directs many immune effector functions, that ultimately result in destruction of invading parasites via effector molecules, such as the anaphylatoxins (C3a and C5a), opsonins (C3b/iC3b) and the lytic terminal complement complex (TCC) .

The C3b generated by the MBL – MASP pathway is fed into the positive feedback amplification loop of complement activation and results in the deposition of large amounts of opsonic C3b on the microbial surface. There is some evidence to suggest that MBL is able to interact directly with cell surface receptors and promote opsonophagocytosis and other immune processes (Woehl *et al.*,2014).

Several putative MBL-binding proteins/receptors have been proposed, including cClqR/calreticulin , ClqRp and CR1. However, it is unclear whether MBL acts as a direct opsonin for micro-organisms or simply enhances other well-established pathways of complement and/or immunoglobulin receptor-mediated phagocytosis. (Woehl *et al.*,2014).

1.2.2.12.2 Mannose Binding Lectin Genetics and Polymorphisms

Within the human collecting gene cluster mapped to 10q 21-24 there is a single functional MBL gene comprising four exons. Exon 1 encodes the

signal peptide, a cysteine rich region and part of the glycine-rich collagen-like region. Exon 2 encodes the remainder of the collagenous region, whilst exon 3 encodes for the helical coiled-coil neck region (Luo *et al.*,2014).

The fourth exon encodes the C-terminal carbohydrate recognition domain. Upstream of the MBL gene are a number of regulatory, promoter elements which are believed to enhance MBL transcription approximately threefold during acute-phase responses (Garred *et al.*,2006).

MBL deficiency results predominantly from three single point gene mutations in codons 52, 54 and 57 of exon 1 of the MBL gene. These are commonly referred to as the D, B and C variants, with A indicating wild type. The B variant mutation occurs in approximately 26% of Caucasians, whereas the C variant mutation is characteristic of sub-Saharan African populations in whom it may reach frequencies of 50 to 60%. Both the B and C mutations result in the substitution of a dicarboxylic acid for an axial glycine, and this impairs correct oligomerisation (Jahan *et al.*,2022).

In addition to the above structural gene mutations, several polymorphisms exist within the promoter region of the MBL gene. These polymorphisms are the H/L, X/Y and P/Q loci at positions -550, -221 and +4 of the MBL gene. The alleles expressed at these loci are in linkage disequilibrium and four promoter haplotypes (LXP, LYP, LYQ and HYP) are commonly found. Of these the HYP haplotype is associated with high MBL levels, whereas the LXP haplotype is found in association with low levels of the protein (Oudshoorn *et al.*,2008).

The HXP haplotype has never been unequivocally identified. The three structural gene mutations are also in linkage disequilibrium with the promoter polymorphisms and every individual expresses two of the

following seven possible haplotypes – HYPA, LYQA, LYPA, LXPA, LYPB, LYQC and HYPD.

1.2.2.12.3The Role of MBL in Health and Disease

MBL deficiency has been genetically defined as possession of haplotype associated with decreased levels, which applies to ~ 40% of the population. Profound MBL deficiency (<400 ng/ml) occurs in -7-10 % of the Caucasian population . MBL deficiency is thought to be one of the most common immunodeficiencies. The fact that most MBL deficient people do not get infectious has led to speculation that a second immune defect needs to be present to render the individual susceptible to infection (Cedzyński *et al*, 2019).

In support of an earlier suggestion , MBL deficiency only presented clinically when there was an associated immunodeficiency such as IgG subclass deficit .There is evidence that severe infections were more common in patients with combined deficiencies, either IgA or any of the IgG subclasses or IgA and MBL deficiency. (Leppäniemi, S. 2023).

The high frequency of MBL deficiency in the general population suggests that defective MBL mediated immunity can be compensated by alternative defense strategies. This is not true of deficiencies of some immune components. For examples, patients with X linked agammaglobulinaemia suffer from severe and repeated bacterial infections, particularly those caused by encapsulated organisms. It would appear that MBL deficiency may not be as important as some other immune components, because alternative means of eradicating pathogens have evolved. For example there are many ways to kill bacteria. Immunoglobulin, independently and in association with the complement system, can effectively destroy bacteria in the absence of MBL. While MBL may enhance opsonisation, is not absolutely required.

The role of MBL in disease was first appreciated within paediatric population and the most rigorous MBL clinical research has been conducted in this group. Low serum MBL and *MBL-2* mutations have been implicated in childhood infection. This association has been demonstrated in children with mild respiratory tract infections managed within community, in more severe infections requiring hospital admission, as well as in the paediatric intensive care unit setting where MBL deficiency was associated with a greater risk of developing the Systemic Inflammatory Response Syndrome (SIRS) (Fidler *et al,* 2004).

1.2.2.12.3.1 The Role of MBL in opsonization and phagocytosis

MBL may also interact directly with cell surface receptors and thereby promote opsonophagocytosis and modify cellular activation. This property was first reported by Kuhlman *et al,* (1989) in a study of MBL coated *Salmonella enterica* serovar *Montevideo* organisms. MBL opsonized *Salmonella enterica* directly without the involvement of the complement system.

In another studies, the phagocytosis of *C. neoformans* or *Mycobacteruim avium* was significantly enhanced by recombinant MBL . Subsequently, a number of putative MBL binding proteins/ receptors have been proposed including cClqR/ calreticulin , ClqRp/ CD93 and CRl/ CD35 (Dzwonek , 2008).

The recent study by Neth and co-workers (2002) showed that MBL-MASP complexes can promote opsonophagocytosis of *Staphylococcus aureus* independently of complement activation (Neth *et al,* 2002). However, it is unclear whether MBL is acting as a direct opsonin for microorganisms or is enhancing other pathways such as complement - (CRl) or immunoglobulin - (Ig) receptor mediated phagocytosis. Indeed,

it is still unclear whether MBL receptors are distinct from receptors for the structurally similar Clq molecule

1.2.2.12.3.2 MBL and inflammation

MBL has also been proposed to display anti-inflammatory properties by modulating the cytokine production by monocytes. Addition of low levels of MBL to whole blood resulted in increases of TNF, IL-1 p and IL-6 production, whereas higher doses of MBL suppressed the production of all three inflammatory cytokines . It has also been found that MBL binds to apoptotic cells and stimulates their ingestion by phagocytes (Kany *et al*, 2019).

1.2.2.12.3.3 The common opsonic defect and MBL deficiency

The common opsonic defect and eventually MBL deficiency was first reported in 1968 when Miller *et al* described the case of a child, who had suffered from recurrent upper respiratory tract infections, diarrhoea and failure to thrive in the first two years of life (Jacobs and Miller, 1972).

The patient was treated with plasma infusions, which ameliorated the condition. Further studies showed the abnormality was present in approximately 5-8% of the general population. The frequency of this defect was higher in recurrent unexplained infections, but the defect was also present in healthy adults with no history of persistent infection.

In the early 1980's, it was known that the complement system is activated by two or more immunoglobulin G molecules or one IgM molecule bound to a target surface (classical pathway) or by the binding of hydrolysed C3 to a permissive surface such as a bacterium (alternative pathway). Activation in this manner would lead to opsonisation of the target by the amplified deposition of multiple opsonic C3 fragments or

the direct lysis of Gram negative bacteria by formation of macromolecular complex, MAC (Adrian , 2019).

Since a gross immunoglobulin defect did not appear to be cause of the opsonic defect, and in any case would be unlikely in such a large proportion of the population, a study of complement deposition was begun. Biochemical assays using the D-mannose polymer zymosan confirmed that the absence of a serum factor led to the poor deposition of C3b and iC3b on the yeast surface without deficiency of any known component of either the alternative or classical pathways of complement activation (Langereis *et al.*, 2018).

MBL appeared to be an ideal a candidate molecule for the serum factor deficient in patients with the common opsonic defect. It was demonstrated these individuals were deficient in MBL and that the addition of purified MBL to the serum of these patients resulted in increased deposition of C3b, Factor B and C4 on zymosan (Dobó *et al.*, 2018).

When mannan, a predominant polysaccharide in zymosan, was used in complement binding assays to study a population of blood donors, defective opsonisation was associated with low levels of MBL and the role of the novel lectin pathway of complement activation was established (Dobó *et al*, 2018).

1.2.2.13 Treatment and vaccination of *Cryptosporidium*

Individuals infected with cryptosporidiosis are treated based on each case's unique symptoms. While treatment methods are still under various studies, the US Food and Drug Administration (FDA) approved only one medication, nitazoxanide (NTZ), to treat diarrhea related to Cryptosporidium . Clofazimine (CFZ) is a new potential medication as studies show its effectiveness in treating Cryptosporidium in vitro

Since this parasite localized to the intestinal tract, a vaccine that stimulates mucosal immune responses will likely be necessary. The few commercial mucosal vaccines that exist are typically "live" and attenuated by serial passage, chemical mutagenesis, deletion of virulence genes (Pasetti *et al*., 2011).

It not known whether differences between the main two *Cryptosporidium* species that infect humans, *C. parvum* and *C. hominis*, will be problematic when developing a vaccine. The homo logy between the two species exceeds 95–97% DNA sequence identity (Luo *et al*., 2014).

Suggesting high protein conservation. Two studies examined antibody responses in Bangladeshi children to 2 *C. parvum* immune dominant antigens, the Cp23 and Cp15/17, in order to determine differences in immune response to *C. hominis* and *C. parvum* (Pasetti *et al*., 2011). While most children infected with *C. hominis*, there were cross-reactive antibody responses to the *C. parvum* antigen, Cp23 (Borad *et al*., 2012).

1.2.2.4 The Genome of *Cryptosporidium* Species in humans

The genomes of *Cryptosporidium* are containing eight chromosomes, with average size of 0.945-2.2 Mb and a total haploid genome size of about 9.2 Mb (Dąbrowska *et al.,* 2023).

Moreover, *C. parvum* contain two small extrachromosomal cytoplasmic double-stranded RNAs. The RNAs have an open reading frame (ORF), each of them encodes RNA-dependent RNA polymerase and encodes protein that has restricted homology to protein kinases of the mammalian. The Rrna gene of *Cryptosporidium parvum* is composed of small rRNA subunit with a size of 1.7 kb and a large rRNA subunit with size of 3.6 kb, as well as 5.8S rRNA subunit with a size of 151-bp(Iliyasu *et al.,* 2022).

The analysis of the genome revealed the presence of metabolic pathway and the lack of cellular structures found in other apicomplexans . Although, the main energy source for parasite is glycolysis metabolism, but both aerobic and anaerobic metabolisms are presented, hence reflecting the flexibility of the environment. The limitation of biosynthesis and metabolism suggesting the major requirement on nutrient acquisition from the host (Rider and Zhu 2010.).

The analysis of genome encoded many transporters which are necessary for the importance of the critical nutrients from the host. Genomic analysis presented that *Cryptosporidium* spp. and Plasmodium spp. shared over 150 ancestral "apicomplexan" proteins, involved mainly in interactions with host cells of the eukaryotic and the biogenesis of the apical complex (Dhal *et al.,* 2022).

In humans there are two species which are routinely diagnosed in clinical cases of cryptosporidiosis; these are the zoonotic species *C. parvum* and the human adapted species *C. hominis*. These species account for over 90% of human infections worldwide and 96% of clinical cases are attributed to these two species (Hadfield *et al.,* 2015).

Cryptosporidium parvum infections peak in the spring months while *C. hominis* is more common in the autumn months , this is thought to be related to springtime calving and lambing and an increase in people participating in outdoor activities at this time of year .Other species which commonly infect humans include; *C. meleagridis* in the UK and *C. felis, C. canis and C. meleagridis* in other parts of the world (Colston, 2018).

1.2.2.7 Laboratory diagnosis:

For many years, microscopic examination has been the only tool available for the detection of gastrointestinal parasites in stool specimens and it remains the cornerstone of the diagnosis of parasitic infections in

routine diagnostic laboratories (McHardy *et al*., 2014;Verweij & Stensvold, 2014).

1.2.2.7.1 Microscopic Examination:

Romanowsky stains were first to be used for the identification of the oocysts that appear semi-translucent with a narrow clear halo around it and stains blue to azure with four to six red or purple eosinophilic granules appearing as dots. such as Giemsa and Jenner's stain (Brar *et al* ., 2017; Ryan *et al* ., 2017).

The modified Ziehl-Neelsen stain was depended upon to test and define the form and measurements of the oocysts isolated from the infected children with the *Cryptosporidium parvum* . The microscopic examination of the stool containing the oocysts, after staining by the modified Ziehl-Neelsen stain exhibit that the oocysts are spherical , shining red (Khurana & Chaudhary 2018, Al-Dulaimi *et al* ., 2021).

In 1981, Henriksen and Pohlenz used acid-fast Ziehl–Neelsen (ZN) stain to identify the oocyst, which was modified by Casemore with better results, and subsequently, it became the widely used method for oocyst detection (Khurana & Chaudhary,2018).

Fluorogenic stain auramine-phenol stain, Various studies have recommended it as an alternative to the MZN stain. Many laboratories consider it as a gold standard method as it provides the highest combination of sensitivity and specificity (Hadfield *et al* .,2015)

Others methods used for the concentration are centrifugation, Sheather's sucrose flotation method, saturated salt flotation (Smith *et al*., 2010), Allen and Ridley's formol-ether method (Allen *et al* . ,1970) , and Electron microscopy

1.2.2.7.2 Serological Diagnosis

Serological diagnosis These methods have reported to yield good sensitivity and specificity in the range of 93%–100%. Based on either antigen detection or antibody detection. antibody detection tests are useful in sero-epidemiological surveys ;but, antigen detection are good for diagnosis of acute infection (Chan *et al* ., 2000).

Antigen detecting methods: Using antibodies labeled with fluorescent reporter *Cryptosporidium* oocysts can be using mAbs against Oocyst wall antigen (C-mAbs). The variant in species and genotypes of genus *Cryptosporidium* which different in the Oocyst epitope expression will fluoresce less intensely. That is why the negative samples should always be confirmed by either conventional methods or polymerase chain reaction (PCR) methods (fayar *et al* ., 2007).

Antibodies labeled with enzyme reporters Kits for antigen detection based on the antibodies which are labeled with enzyme are commercially available for enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) formats depending on the antibody used in the kit, it can have low sensitivity in some cases (Khurana *et al* ., 2012).

Immunochromatographic (IC): its popular procedure practiced in various laboratories; it is known for its rapid results Antibody detection methods**:** it is an indirect diagnostic methods for the evidence of infection or exposure by demonstration of the presence of antibodies to *Cryptosporidium*-specific antigens in serum, saliva, or fecal samples (fayar *et al*., 2007).

Detection of specific circulating antibody is of benefit only in case of seroconversion, demonstrating elevation in titer, or antibody isotype switching . Some commercially available tests that employ finger prick

blood or oral fluid for detection of these antibodies (Lammie *et al* .,2012; Griffin & Lawson, 2011)

Chapter Two

Materials and Methods

2 Materials and Methods :

2.1 Materials:

2.1.1 Laboratory Instruments and Equipment:

The main scientific apparatus, and technical instruments with disposable materials, those were employed during the course of this study are listed in Tables (2-1) and Table (2-2) respectively.

Table (2-1): Laboratory Apparatus

Item	Company	Country
Autoclave	Herayama	Japan
Bacteriological cabinet	Labogene	Denmark
Benson burner	Dolphin	Syria
Centrifuge	Gemmy	Taiwan
Deep Freezer	Aucma	China
Digital camera	Samsung	Japan
Distillator	GFL	Germany
ELISA instrument system	Solarbio	China
Exispin vortex centrifuge	Bioneer	Korea
Gel electrophoresis	BioRad	USA
Light microscope	Stermite Olympus A &D	Japan
Mechanical Vortex mixer	Gemmy	Taiwan
Micro centrifuge	Eppendorf	Germany
Micropipettes 5-50 µl , 0.5-10 µl ,100-1000 µl	Top Dragon	Europe
Microwave	Argose	Germany
Nano drop 2000	THERMO	USA
Oven	GS	Taiwan
Platinum wire loop	Himedia	India
Refrigerator	Concord	Italy
Sensitive electric balance	Kern	Germany
Spectrophotometer	Perkin Elmer	Germany
T100 Thermal cycler	BioRad	USA
UV-trans illuminator	BioRad	USA
Water bath	GFL	Germany

Table (2-2) Technical Instruments and Disposable Materials

Item	Company	Country
EDTA-tubes, Gel tubes 5mL.	Hebei Xingle	China
Eppendorf tubes 1.5 ml, 200mL	Biobasic	Canada
Glass slides, flasks and beakers	Hirschman	Germany
Microscopic Cover slide	Gitoglas	China
Para film	Bemis	USA
Pettri dishes	Blastilab	Lebanon
Plastic test tubes 10mL	Dolphin	Syria
Sterile gloves	Broche	PRC
Sterile cotton	Medicare Hygiene	India
Sterile injection Syringes	MEDECO	UAE
Sterile swabs	Sigem	Spain
Tips	Dolphin	Syria

2.1.2 Chemical and Biological Material:

The main chemical and biological materials used in the present study as shown in Table (2-3).

Table (2-3) Chemical Materials and Reagents

Chloral Phenol red, Methylene blue	Searle	England
Chloroform	Riedel-de Haen	Germany
Crystal violet	Sigma	USA
Ethanol (95%), (70%)	Scharlau	Spain
Glacial acetic acid CH3COOH	Panreac	Spain
Glycerol, Barium chloride	B.D.H	England
Methanol	Flukachemika	Switzerland
Normal saline	Pharmaline	Egypt
Phosphate buffer	Himedia	India
Ziehl Neelsen stain	Crescent	KSA

2.1.3 Molecular Materials:

The essential molecular materials has been used in the current study show in Table (2-4).

Table (2-4) Molecular Materials

Materials	Company	Country
AccuPower PCR PreMix Kit, consist of: Taq DNA polymerase, dNTPs 400μM for each,Tris-HCl (pH 8.5-9.0)10 mM, KCl 30 mM,$MgCl_2$3mM,2eppendroffs of Nuclease free water ,Stabilizer and tracking dye	Bioneer	Korea
Agarose.	Promega	USA
gel Loading Dye, 6X	Bioline	USA
Ethidium Bromide Solution, (10mg/ml).	Kappa Biosystem,	South Africa
Free nuclease water.	Kappa Biosystem,	South Africa
Genomic DNA extraction Kit	Kappa Biosystem,	South Africa
Ladder 100bp, 3000bp	Kappa Biosystem,	South Africa
Primers of gene polymorphism	IDT	Canada
TBE Buffer, 10X (pH 8.3)	Kappa Biosystem,	South Africa

2.1.4 Commercial kits:

The commercial kits used in this study are shown in Tables (2-5)

Table (2-5): Commercial kits

Kits and their materials	Company	Country
INF-γ ELISA kits 1) user manual ×1 2) closure plate membrane×1 3) stock solution 1000pg/mL 4) biotin-conjugated antibody 5) Twenty mL of wash solution 6) Substrate solution 7) Stop solution **IL-18 ELISA kits** 1) user manual ×1 2) closure plate membrane×1 3) stock solution 1500pg/mL 4) biotin-conjugated antibody 5) Twenty mL of wash solution 6) Substrate solution 7) Stop solution **Humen (MBL)ELISA kits** 1) user manual ×1 2) closure plate membrane×1	Solarbio company	China

Kits and their materials	Company	Country
3) stock solution 1000pg/mL 4) biotin-conjugated anti human MBL 5) Twenty mL of wash solution 6) Substrate solution 7) Stop solution **TGF-β ELISA kits** 1) user manual ×1 2) closure plate membrane×1 3) stock solution 2000pg/mL 4) biotin-conjugated antibody 5) Twenty mL of wash solution 6) Substrate solution 7) Stop solution **DNA Extraction Kit** 1) RNase A 1 ml 1 2) Proteinase K 1 ml 3) Solution SA 40 ml 4) Solution SB 20 ml 5) Solution SC 30 ml 6) Washing Buffer 15 ml 7) Elution Buffer 15 ml 8) Filter Column 50 Units 9) Adsorption Column 50 Units	Solarbio company	China

2.1.5 Primers

PCR primer for heat shock protein **(HSP70)** showed as following table (2-6):

Table(2-6):The PCR Primers with their Sequence and Amplicon Size:

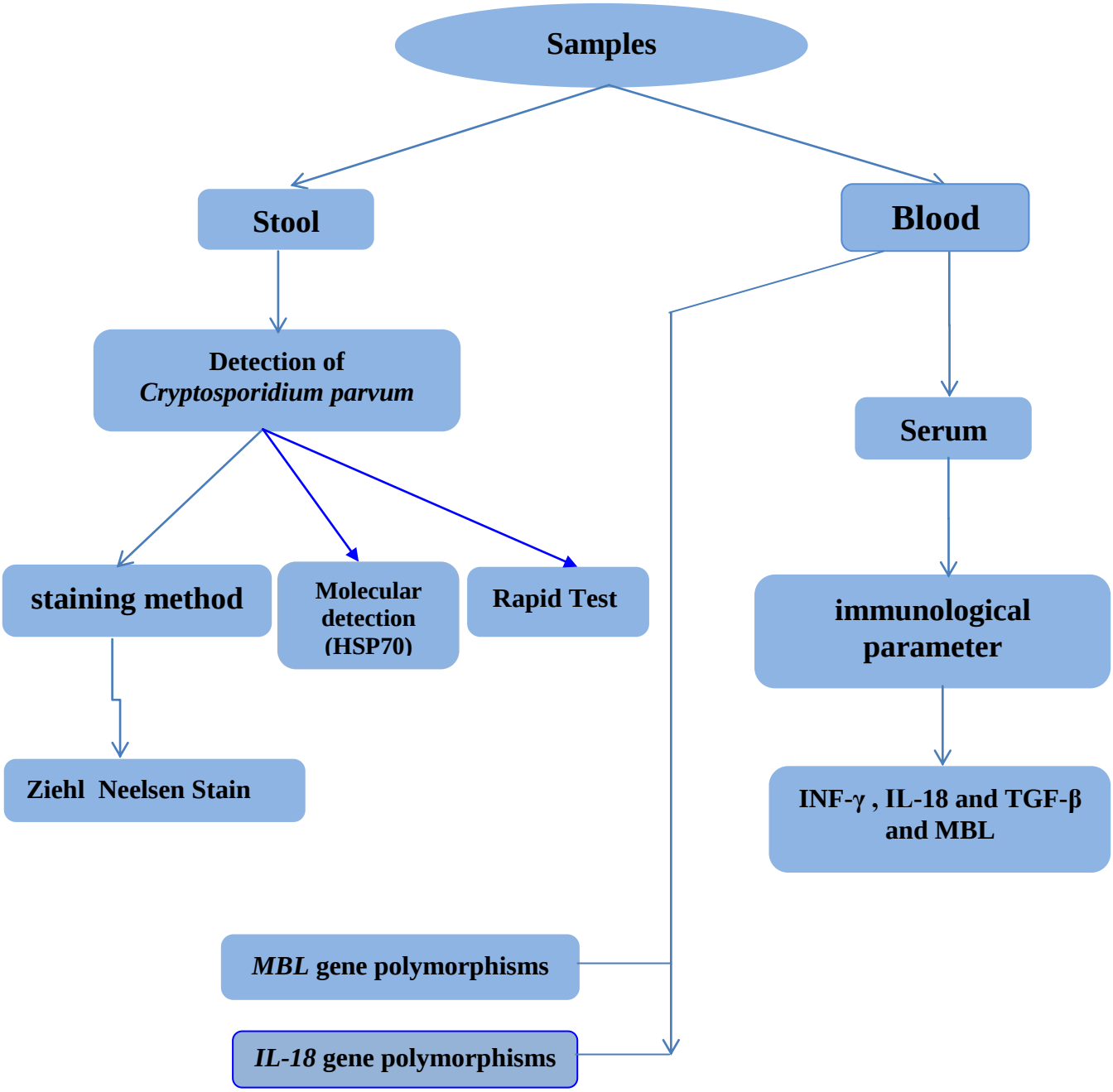
Primer	Sequence (5'-3')		Product Size
heat shock protein (***HSP70***)	F '	GGGTCGCCAAATTAAGAACG	1800bp
	R'	GGAACTGGCGAGAGAAATGT	

2.1.6 SNP

Table (2-7):Primer sequence polymorphism

SNP	Polymorphism	Company/Country
MBL rs 1800450	T/C	New design
IL-18 rs5744247	G/C	

2.2 Experimental plan:



Figure(2-1):Design of Current Study

2.3 Methods:

2.3.1 Preparation of Reagents and Solutions:

2.3.1.1 Ziehl-Neelsen Stain: according to Shyamasundari and Rao, (2019).

1) Solution1 : carbol fuchsin ,Basic fuchsin (4g) ,Ethanol 95% (20mL) Phenol (8mL) Distilled water (100mL).
2) Basic fuchsin was dissolved in alcohol and then water added slowly while shaking, first melt phenol crystals at 56 ◦C and then 8ml was added to the stain.
3) Solution2 : declorizer Ethanol 95% 9.7mL , HCL 3mL (HCL was added into Alcohol slowly)
4) Solution3: counter stain
5) Methylene blue (300mg)
6) Distilled water (100mL)

2.3.1.2 Normal Saline Solution:

(8.5) grams of NaCl was dissolved in a small volume of distilled water, then completed to (1000mL), pH was fixed at (7.2) and sterilized in autoclave at (121˚C) for (15) minutes, then kept at (4˚C) (McFadden, 2000).

2.3.1.3 Phosphate Buffer Solution:

The solution was prepared according to a company (Himedia/ India) by dissolving one tablet in (100mL) of distilled water (D.W.).

2.3.1.4 Ethidium Bromide Solution:

This Solution has been provided from manufacturing company, which used as dye in gel electrophoresis

2.3.1.5 Tris-borate-EDTA Buffer (TBE):

Tris-OH (0.08M), Boric acid (0.08M), and EDTA (0.02M), were dissolved in (800ml) of distilled water, pH was adjusted to (8), then the

volume was completed to (1000ml) by distilled water, then sterilization by autoclave at 121˚C for 15 minutes, and then kept at 4˚C until using

2.3.1.6 Ecodye Solution:

This solution was equipped by manufacturing company, as a dye in gel electrophoresis.

2.3.2 Molecular Study

2.3.2.1 Preparation of Molecular Materials:

2.3.2.1.1 Preparation of 1X TBE Buffer:

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:10 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus each 100ml of 10X TBE was added to 900ml of sterile distilled water to produce final concentration,1X TBE (Sambrook and Russel, 2001).

2.3.2.1.2 Preparation of Agarose Gel:

This gel was prepared by adding agarose powder in 1X TBE buffer to be dissolved by boiling, and then it was left to cool to 50ºC. For DNA profile (visualization of the DNA after extraction), 1% agarose is used. While for visualization of PCR product (amplicon), 1.5%-2% of agarose was employed and for single nucleotide polymorphism detection,3% agarose is used. Ethidium Bromide stock solution with a concentration of 10mg/ml was used. Only 5µl of this stock solution were supplemented to 100ml of melted agarose gel to get final concentration 0.5µg/mL Then after the addition of ethidium bromide, the materials were mixed well and dispensed to the tray of gel electrophoresis (Sambrook and Russel, 2001).

2.3.3 Collection of Specimens:

Out of 300 clinical specimen from blood and Stool were collected from male and female were suspected to have parasite after showing clinical manifestations, Whole blood and stool samples were collected from 180 male, and 120 female suffering from diarrhea in Babylon Teaching Hospital for Maternity and Children, with ages ranged from (less than one month -10 years), during the period from October 2022 to April 2023, as shown in Table (1) in Appendix

The specimen were transported quickly by sterile transport cup to the department of bacteriology laboratory and each sample was tested by using Ziehl Neelsen stain.

2.3.3.1 Collection of Blood

Up to Five mL of freshly blood was drawn according to (Forbes *et al*., , in which 3ml used for serum separation and 2mL used for DNA extraction. The whole blood were obtained by vein puncture from all study subjects after cleaning the skin with 70% alcohol, then, the blood samples were divided into EDTA tubes and plain tubes. To separate the serum for serological studies, 3ml of the blood samples allowed to clot at room temperature, then the samples were centrifuged for 3 min at 3000 round per minute (rpm) and finally the serum transferred to other tubes for storage at liquid nitrogen. For the DNA purification and genetic studies, 2ml of the whole blood samples were put in EDTA tubes and mixed well, then stored at (4°C) in the refrigerator(Forbes *et al*., 2007).

2.3.3.2 Collection of Stool

The stool samples were collected in suitable clean plastic container, and each container was labeled by a special number. Samples were stored in clean Ependrof tube at deep freeze -20 ◦C for molecular study. The samples should be examined within (30 min to1 hour) from the time its

gained from the patient, because delaying the examination for a longer period of time leads to disintegrate of parasite.

2.3.3.3 Demographic information

The demographic information of *cryptosporidium* infected patients and controls, including age, sex , residence.

2.3.3.4 Inclusion and Exclusion criteria

The inclusion criteria was all patients with any diarrhea and the exclusion criteria was auto-immune diseases (e.g., rheumatoid arthritis), steroid therapy and, antibiotics, or anti-inflammatory drugs or other agents known to affect cytokine production

2.3.3.5 Ethical Approval:

The necessary ethical approval was obtained by verbal consent from patients and control . This study was approved by the committee of publication ethics at college of medicine, Babylon university, Iraq.

2.3.4 Diagnosis of Parasite :

2.3.4.1 Primary diagnosis:

The diagnosis of parasite involves two steps, macroscopic and microscopic examinations, the macroscopic examination of stool sample was done visually. For consistency (formed, unformed 'soft', or liquid), color (white, yellow, brown, or black), smell and presence of any abnormal components (mucus or blood). While the Microscopic examination of stool sample was done to demonstrate, intestinal protozoa,

2.3.4.2 Identification of Isolates:

Identification of isolates was carried out according to (Garcia, 2003).

2.3.4.2.1 Ziehl-Neelsen Stain

1) Modified ziehl neelsen-acid fast stain has been used in the current study for identification of *cryptosporidium* oocytes.
2) Briefly, a small drop of the concentrated stool sample was used to prepare thin smear on a clear microscope glass slide.

3) The slides were allowed to air dry and then fixed for 3-5 min in absolute methanol.
4) Fixed slides were placed on staining racks and flooded with ZN carbol fuchsin for 20-25 min and the slides were rinsed under slow ran tap water.
5) Five percent acid Alcohol was added approximately for 20-30 s. for de-colorization and the slides were rinsed under slow ran tap water.
6) The slides were then flooded with methylene blue for 2-3 min. for counter staining and then slides were rinsed under slow ran tap water and allowed to air dry.
7) After drying, slides were examined microscopically with a drop of oil under high power (100x oil immersion) lens.

Positive *cryptosporidium* oocytes slides stain bright red with a blue background

2.3.4.2.2 Certest Crypto Combo Card test Immuno-Chromatography (IC)

A. Assay Principle :

This technique is called A lateral flow assay (LFA) and is composed of a chromatographic system (separation of components of a mixture based on differences in their movement through reaction membrane) and immunochemical reaction (between antibody-antigen It is based on the movement of the sample across the membrane via capillary force.is composed of four parts: a sample pad, which is the area on which the sample is dropped; a conjugate pad, on which labeled tags are combined with biorecognition elements; a reaction membrane (usually nitrocellulose membrane pre-coated with monoclonal antibodies on the test line T) containing test line and control line for target antigen-antibody interaction; and absorbent pad, which reserves waste (Singh *et*

al., 2015). For the construction of LFAs gold nanoparticles, colored latex beads, carbon nanoparticles, quantum dots, and enzymes are used as a label for increasing the sensitivity.

B. Specimen preparation :

• the stick was Use to pick up a sufficient sample quantity.

• Add into stool collection tube (for liquid samples, add 125 μL in the stool).

• The tube was Close with the diluent and stool sample. Shake the tube to assure good sample dispersion

C. Test Procedure:

1- the stool collection tube was shake to assure good sample dispersion.

2- the sealed bag was Remove before using it.

3- the stool collection tube wasTake, cut the end of the cap, and pour three drops into the circular window with the letter A, three drops into the window with the letter B, and three drops into the window with the letter C. Avoid mixing solids with the liquid.

4-Read the result for 10 minutes.

D. Interpretation of the results:

Strip A consist of a nitrocellulose membrane pre-coated with monoclonal antibodies against *Cryptosporidium*.

2.3.5. ELISA Assay

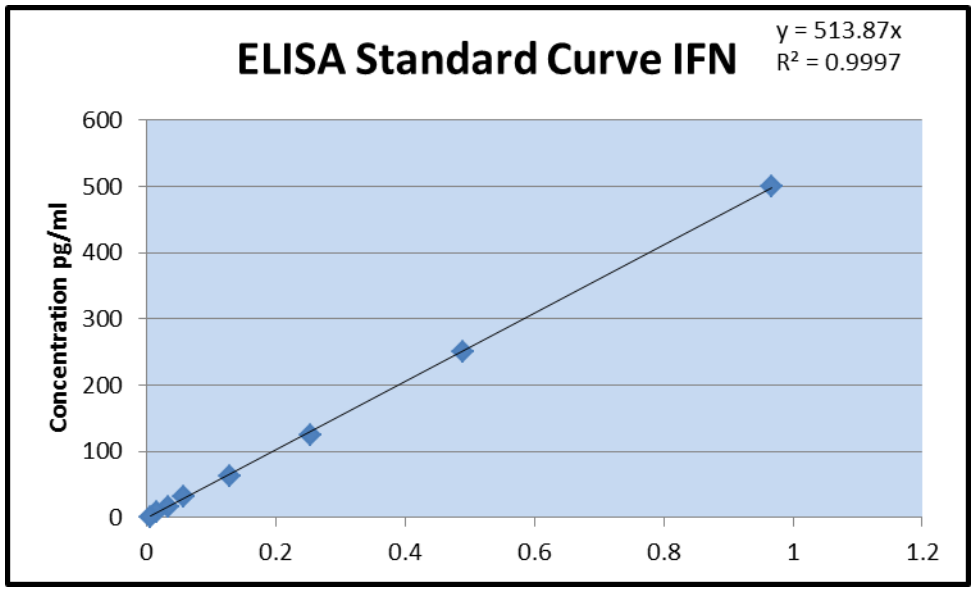
2.3.5.1 ELISA Assay for Determination of Human (INF-γ) Concentration:

ELISA assay was achieved according to the method described by the manufacturing company (Solarbio /China):

2.3.5.1.1 Standard Preparations:

The concentration of the standard in the stock solution is 1000pg/mL. The stock solution had been diluted to (500 pg/mL).Then, 7 tubes containing (500μL) standard diluent was prepared, then pipette (500μL)

of stock solution and produce a double dilution series. Each tube were mixed thoroughly before the next transfer. 7 points of diluted standard was sated up such as (500pg/mL, 250 pg/mL, 125pg/mL, 62,5pg/mL, 31,2pg/mL, 15,6pg/mL,7,8pg/mL), and the last EP tubes with Standard Diluent is the blank as (0pg/mL).



Figure(2-2): Standard Curve INF-γ

2.3.5.1. 2 Assay Diluent A and Assay Diluent B Preparations:

Six ml of Assay Diluent A or B Concentrate (2×) had been diluted with 6 mL of deionized or distilled water to prepare (12 mL) of Assay Diluent A or B.

2.3.5.1.3 Working detector Preparations:

The stock detection biotin-conjugated antibody and detection SR2 had been centrifuged before use. Then, the reagents were diluted with working assay diluent to the working concentration 100-fold.

2.3.5.1.4 Wash Solution Preparations:

Twenty mL of wash solution concentrate (30×) had been diluted with 580mL of deionized or distilled water to prepare (600mL) of Wash Solution (1×).

2.3.5.1.5 Assay Procedure:

1. Wells for diluted standard, blank and sample had been determined. A7 wells for standard . (100µL) each of dilutions of standard and samples were added into the appropriate wells. Then, plate incubated for 120 min at (25^{o}C).
2. One hundred µL of prepared working detector was added to each well, the plate were incubated for 1 hour at (25^{o}C).
3. The solution was aspirated and then, (350µL) of 1× Wash Solution were added to each well using a multi-channel pipette and let it sit for 2 minutes. Totally wash 3 times.
4. The aspiration/wash process were repeated for total 5 times as conducted in step 3. A(100µL) of substrate solution had been added to each well. Incubated for 10 - 20 minutes at (25^{o}C).
5. Fifty µL of stop solution were added to each well. Then, the microplate reader had been runes and measurement were conducted at 450 nm immediately.

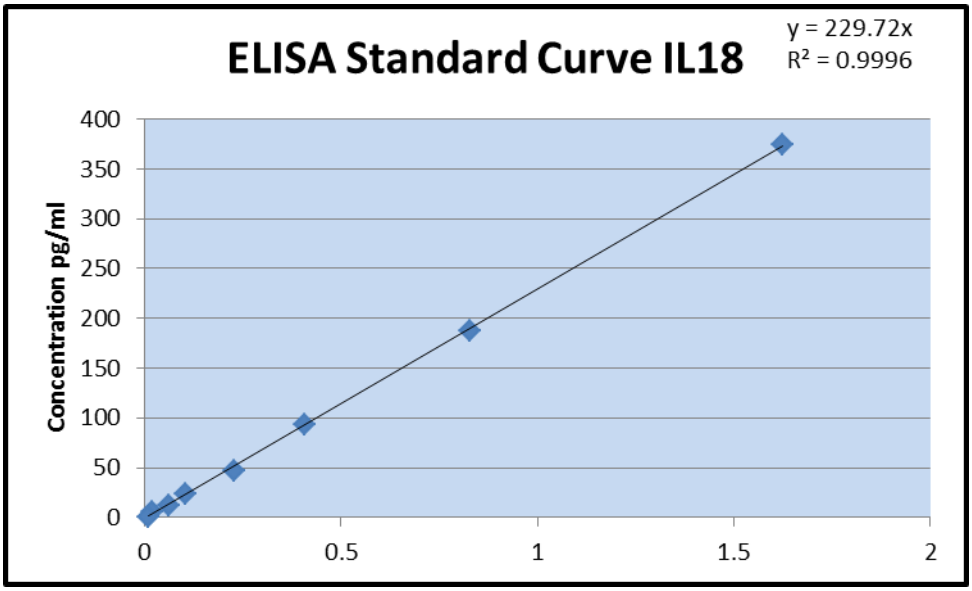
2.3.5.2 ELISA Assay for Determination of Human (IL-18) Concentration:

ELISA assay was achieved according to the method described by the manufacturing company (Solarbio /China):

2.3.5.2.1 Standard Preparations:

The concentration of the standard in the stock solution is 1500pg/mL. The stock solution had been diluted to (250 pg/mL).Then, 7 tubes containing (375µL) standard diluent was prepared, then pipette (500µL) of stock solution and produce a double dilution series. Each tube were mixed thoroughly before the next transfer. 7 points of diluted standard was sated up such as (375pg/mL, 187 pg/mL, 93.7pg/mL, 46,8pg/mL,

23,4pg/mL, 11,7pg/mL,5,8pg/mL), and the last EP tubes with Standard Diluent is the blank as (0pg/mL).



Figure(2-3): Standard Curve IL18

2.3.5.2.2 Working detector Preparations:

The stock detection biotin-conjugated antibody and detection SR2 had been centrifuged before use. Then, the reagents were diluted with working assay diluent to the working concentration 100-fold.

2.3.5.2.3 Wash Solution Preparations:

Twenty mL of wash solution concentrate (30×) had been diluted with 580mL of deionized or distilled water to prepare (600mL) of Wash Solution (1×).

2.3.5.2.4 Assay Procedure:

1. Wells for diluted standard, blank and sample had been determined. A7 wells for standard . (100µL) each of dilutions of standard and samples were added into the appropriate wells. Then, plate incubated for 90 min at (37^{o}C).

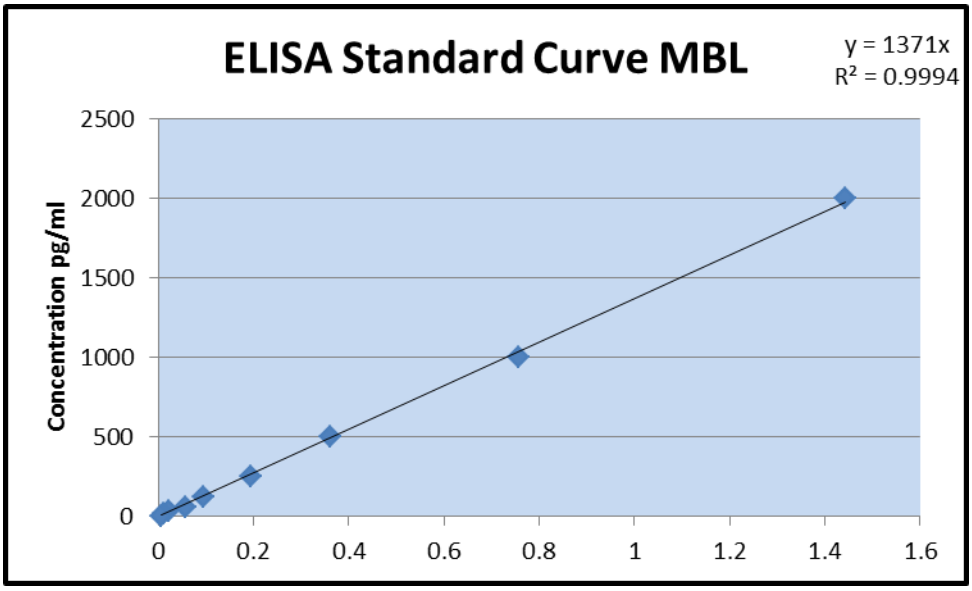
2. The solution was aspirated and then, (350µL) of 1× Wash Solution were added to each well using a multi-channel pipette and let it sit for 2 minutes. Totally wash 4 times.
3. One hundred µL of prepared working detector was added to each well, the plate were incubated for 1 hour at (25^{o}C).
4. The aspiration/wash process were repeated for total 4 times as conducted in step 3.
5. One hundred µL of prepared working detector (streptavidin-HRP) was added to each well, the plate were incubated for 30 MIN at (25^{o}C).
6. The aspiration/wash process were repeated for total 5 times .
7. A(100µL) of substrate solution had been added to each well. Incubated for 15 minutes at (25^{o}C).
8. Fifty µL of stop solution were added to each well. Then, the microplate reader had been runes and measurement were conducted at 450 nm immediately.

2.3.5.3 ELISA Assay for Determination of Human (MBL) Concentration:

ELISA assay was achieved according to the method described by the manufacturing company (Solarbio /China):

2.3.5.3.1 Standard Preparations:

The concentration of the standard in the stock solution is 1500pg/mL. The stock solution had been diluted to (250 pg/mL).Then, 7 tubes containing (375µL) standard diluent was prepared, then pipette (500µL) of stock solution and produce a double dilution series. Each tube were mixed thoroughly before the next transfer. 7 points of diluted standard was sated up such as (375pg/mL, 187 pg/mL, 93.7pg/mL, 46,8pg/mL, 23,4pg/mL, 11,7pg/mL,5,8pg/mL), and the last EP tubes with Standard Diluent is the blank as (0pg/mL).



Figure(2-4): Standard Curve MBL

2.3.5.3.2 Working detector Preparations:

The stock detection biotin-conjugated anti-humen MBL and detection SR2 had been centrifuged before use. Then, the reagents were diluted with working assay diluent to the working concentration 100-fold.

2.3.5.3.3 Wash Solution Preparations:

Twenty mL of wash solution concentrate (30×) had been diluted with 580mL of deionized or distilled water to prepare (600mL) of Wash Solution (1×).

2.3.5.3.4 Assay Procedure:

1. Wells for diluted standard, blank and sample had been determined. A7 wells for standard . (100µL) each of dilutions of standard and samples were added into the appropriate wells. Then, plate incubated for 90 min at (37^{o}C).
2. The solution was aspirated and then, (350µL) of 1× Wash Solution were added to each well using a multi-channel pipette and let it sit for 2 minutes. Totally wash 4 times.
3. One hundred µL of prepared working detector was added to each well, the plate were incubated for 1 hour at (25^{o}C).

4. The aspiration/wash process were repeated for total 4 times as conducted in step 3.
5. One hundred μL of prepared working detector (streptavidin-HRP) was added to each well, the plate were incubated for 30 MIN at (25^{o}C).
6. The aspiration/wash process were repeated for total 5 times .
7. A(100μL) of substrate solution had been added to each well. Incubated for 15 minutes at (25^{o}C).
8. Fifty μL of stop solution were added to each well. Then, the microplate reader had been runes and measurement were conducted at 450 nm immediately.

2.3.5.4 ELISA Assay for Determination of Human (TGf) Concentration:

ELISA assay was achieved according to the method described by the manufacturing company (Solarbio /China):

2.3.5.4.1 Standard Preparations:

The concentration of the standard in the stock solution is 2000pg/mL. The stock solution had been diluted to (500 pg/mL).Then, 7 tubes containing (500μL) standard diluent was prepared, then pipette (500μL) of stock solution and produce a double dilution series. Each tube were mixed thoroughly before the next transfer. 7 points of diluted standard was sated up such as (1000pg/mL, 500 pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.2 pg/mL), and the last EP tubes with Standard Diluent is the blank as (0pg/mL).

2.3.5.4.2 Working detector Preparations:

The stock detection biotin-conjugated antibody and detection SR2 had been centrifuged before use. Then, the reagents were diluted with working assay diluent to the working concentration 100-fold.

2.3.5.4.3 Wash Solution Preparations:

Twenty mL of wash solution concentrate (30×) had been diluted with 580mL of deionized or distilled water to prepare (600mL) of Wash Solution (1×).

2.3.5.4.4 Assay Procedure:

1. Wells for diluted standard, blank and sample had been determined. A7 wells for standard . (100µL) each of dilutions of standard and samples were added into the appropriate wells. Then, plate incubated for 90 min at (37^{o}C).
2. The solution was aspirated and then, (350µL) of 1× Wash Solution were added to each well using a multi-channel pipette and let it sit for 2 minutes. Totally wash 4 times.
3. One hundred µL of prepared working detector was added to each well, the plate were incubated for 1 hour at (25^{o}C).
4. The aspiration/wash process were repeated for total 4 times as conducted in step 3.
5. One hundred µL of prepared working detector (streptavidin-HRP) was added to each well, the plate were incubated for 30 MIN at (25^{o}C).
6. The aspiration/wash process were repeated for total 5 times .
7. A(100µL) of substrate solution had been added to each well. Incubated for 15 minutes at (25^{o}C).
8. Fifty µL of stop solution were added to each well. Then, the microplate reader had been runes and measurement were conducted at 450 nm immediately.

2.3.6 Polymerase Chain Reaction

2.3.6.1 Product Description

Stool Genomic DNA Extraction Kit is suitable for extracting of microbial DNA from stool. This kit has a good lysis effect on various bacteria and fungi in stool to preserve Micro-organism DNA diversity to the utmost. The extracted DNA is large yield and good integrity, it can be directly used for a variety of routine operations, including enzyme digestion, PCR, library construction, Southern blot, etc.

2.3.6.2 Protocol

Add fresh opened absolute ethanol in Washing Buffer before use, volume is based on the label of bottle as a reference. Put cap back on bottle and shake well. All centrifuge steps are performed at 2-8℃.

1. Take 50-200mg stool sample, add 400-800μL Solution SA and place on ice for 10min. Centrifuge at 12000rpm for 1 min.
2. Discard supernatant. Add 400μL Solution SB, 20μL RNase A(10mg/ml), 20μL Proteinase K(10mg/ml) to precipitation, whirl for 30s. Incubate in 65℃ water bath for 30-60min. Invert tube several times during incubating. Centrifuge at 12000rpm for 2 min.
3. Supernatant was Transfer to a 1.5 ml centrifuge tube, add 300-600μL of Solution SC. Centrifuge at12000rpm for 1-2min. Take one Filter Column in Collection Tube, add supernatant to Filter Column. Centrifuge at 12000rpm for 1-2min.
4. The filtrate was Take to Adsorption Column, place at room temperature for 1-2min. Centrifuge at 12000rpm for 0.5-1min. This step can be repeatable once.
5. Pour waste liquid out of Collection Tube, add 500μl Washing Buffer to Adsorption Column. Centrifuge at 12000rpm for 1 min. This step can be repeatable once.

6. Pour waste liquid out of Collection Tube, put Adsorption Column back into Collection Tube. Centrifuge at 12000rpm for 0.5-1min.

7. Take Adsorption Column out, make it dry at room temperature for a few minutes (time is different because of season, climate and other factors).

8. Adsorption Column was Put in a new centrifuge tube, add 50-100μl Washing Buffer(preheated at 65℃). Centrifuge at 12000rpm for 1 min.

9. liquid was Add in centrifuge tube in step 8 to Adsorption Column, centrifuge at 12000rpm for 1min. Liquid in centrifuge tube is stool microbial DNA solution.

2.3.6.3 Estimation of DNA extracts

The extracted DNA was checked by using Nanodrop (THERMO. USA) that measured DNA concentration (ng/μL) and checked the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1) After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2) A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2μl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3) The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1μl of DNA was added to measurement.

2.3.6.4 PCR master mix preparation

PCR master mix for each gene was prepared by using (Maxime PCR PreMix kit) and this master mix done according to company instructions as following table(2-8):

Table (2-8) PCR Master Mix preparation

PCR Master mix	Volume
DNA template 5-50ng	5µL
Forward primer (10pmol)	1µL
Reveres primer (10pmol)	1µL
PCR water	13µL
Total volume-	**20 µL**

After that, these PCR master mix components that mentioned in table above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at3000rpm for 3 minutes, and then placed in PCR Thermocycler (BioRad- USA).

2.3.6.5 PCR Thermocycler Conditions

PCR thermocycler conditions were done by using convential PCR thermocycler system is same for each gene as following table(2-10):

Table (2-9):PCR Thermocycler Conditions

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C^o	5min	1
Denaturation	95C^o	30sec.	30 cycle
Annealing	58C^o	30sec	
Extension	72C^o	1 min	
Final extension	72C^o	5min	1
Hold	4C^o	∞	-

2.3.6.6 PCR product analysis

1) The PCR products were analyzed by agarose gel electrophoresis following steps:
2) 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

3) Then 5µL of ethidium bromide stain were added into agarose gel solution.
4) Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.
5) The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.
6) PCR products were visualized by using UV Transilluminator.

2.3.7 PCR Technique for Detection Human MBL Gene Polymorphism:

ARMS- PCR technique was performed for detection and genotyping Human-MBL and IL-18 polymorphism in patients and in healthy control blood samples.

2.3.7.1 Amplification Refractory Mutation System - PCR

The allele-specific PCR also called as an amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) or PCR amplification of specific alleles (AS-PCR) used to detect the SNPs . Tetra ARMs – PCR protocol was used for SNPs detection MBL rs**1800450** and IL-18**rs5744247** genetic polymorphism.

2.3.7.2 Principal of allele specific PCR

The ARMS-PCR is one of the molecular techniques which was designed for creating thousands to millions copies of demanding DNA fragment**.** Some reagents and components are essential for ARMS-PCR, these components comprise of DNA target (DNA template) that includes the region of DNA to be amplified, primers (forward and reverse primer)

which are complementary to the DNA template, *Thermus aquaticus* DNA polymerase (*Taq polymerase*) enzyme, deoxy nucleotide tri- phosphates (dNTPs) and buffer solution which make an appropriate chemical environment for maximum favorable stability and activity of the DNA polymerase.

The ARMS PCR is mostly done to identify a mutation or a polymorphism which is simply a difference in DNA sequence between two related organisms, e.g. two individual humans. Polymorphisms may be divided into those consisting of base changes and those where there is a difference in the length of the corresponding region of DNA , it is also important that it should be able to identify whether the change in DNA is heterozygous or homozygous. A heterozygote or homozygote is differentiated by using ARMS primers for the mutant/polymorphic and the normal (wild type) alleles. The reactions for the mutant and the normal alleles are usually carried out in separate tubes **.**

2.3.7.3. Primer Design

Two SNPs rs**1800450** and **rs5744247** were amplified by means of specific primers to study the SNPs, the present result used primer oligonucleotide designing by using gene, PCR reaction was performed by using specific primers designed based on NCBI database, all genes information, sequence and SNPS details, were collected. Using specific software, primers were designed. Primers were taken in a lyophilized state, the units of a lyophilized primer are known as a mass in picomoles. The subsequent steps were done for the reconstitution and dilution of the primers:

-The tube was centrifuged at 10000 for 5-10 min before decamping.

-The chosen volume from nuclease free water was added according to the manufacturer to give a primary concentration of 100 pmoles /µL so

lyophilized primers were dissolved in a nuclease free water to give a (stock solution).

-For working solution: The Primers were re- mixed by suitable vortexing, then 10 Pmol of stock solution were diluted with 90 µL of nuclease free) water in a 0.5 mL eppendorf tube in microcenterfuge to obtain (10 pmol) as a final concentration (working solution)

Table (2-10) Primer sequence of rs 1800450 (T/C)**polymorphism**

SNP and Primer sequence	PCR recipe	PCR conditions	PCR product
rs 1800450 (T/C) Forward outer primer (5' - 3') 250GATGGCCTGACCTGTGGGCCACTTTCCT Reverse outer primer (5' - 3') ACCAAGGTGAGGACCATGTCCCTGTTTCCA Forward inner primer (C allele): CTGGTTCCCCCTTTTCTCCCTTGGGGC Reverse inner primer (T allele): TTCCCAGGCAAAGATGGGCGTGAGGA	Genomic DNA: 3.0µL Fw-Oprimer: 1.0µL Rv-Oprimer: 1.0 µL Fw-primer: 1.0 µL Rv-primer: 1.0 µL 2XPCR master mix: 12.5 µL NFW[c]: 5.5 µL Tot volume: 25 µL	1 cycle: Initial denaturation: 95 °C, 5 min 30 cycles: each cycle Denaturation: 94 °C,1 min Annealing : 62°C, 1 min Extension :72 °C, 1 min 1cycle Final extension: 72 °C, 10 min	Product size for C allele: 203 Product size for T allele: 277 Product size of two outer primers: 427

Table: (2-11) Primer sequence of rs5744247 (G/C)**polymorphism**

SNP and Primer sequence	PCR recipe	PCR conditions	PCR product
rs5744247(G/C) Forward outer primer TGCCAGAAGTTTCAGCTTATGAATACCCA Reverse outer primer AATCGAGACTCCAGATCAAAAATCACCG Forward inner primer (C allele): CATCTAATCGTGCCCTTTAGGAAGGAGTC Reverse inner primer (G allele): CCACTGGTTGTAAGAGAAGGATGAACCC	Genomic DNA: 3.0µL Fw-Oprimer: 1.0µL Rv-Oprimer: 1.0 µL Fw-primer: 1.0 µL Rv-primer: 1.0 µL 2XPCR master mix: 12.5 µL NFW[c]: 5.5 µL Tot volume: 25 µL	1 cycle: Initial denaturation: 95 °C, 5 min 30 cycles: each cycle Denaturation: 94 °C,1 min Annealing : 60°C, 1 min Extension :72 °C, 1 min 1cycle Final extension: 72 °C, 10 min	Product size for C allele: 240 Product size for G allele: 269 Product size of two outer primers: 452

Table:(2-12): A representative example for catching up results of allele specific PCR(T/C)

rs 1800450	DNA sample	PCR product	Allele	Possible Genotype
Forward inner primer (C allele CTGGTTCCCCCTTTTCTCCCTTGGGGC	Allele C is present	positive	C	CT heterozygous
Reverse inner primer (T allele): TTCCCAGGCAAAGATGGGCGTGAGGA -3’	Allele T is present	positive	T	
Forward inner primer (C allele CTGGTTCCCCCTTTTCTCCCTTGGGGC	Allele C is absent	negative	-	TT homozygous
Reverse inner primer (T allele): TTCCCAGGCAAAGATGGGCGTGAGGA -3’	Allele T is present	positive	T	
Forward inner primer (C allele CTGGTTCCCCCTTTTCTCCCTTGGGGC	Allele C is present	positive	C	CC homozygous
Reverse inner primer (T allele): TTCCCAGGCAAAGATGGGCGTGAGGA -3’	Allele T is absent	negative	-	

Table: (2-13) A representative example for catching up results of allele specific PCR (G/C)

rs5744247	DNA sample	PCR product	Allele	Possible Genotype
Forward inner primer (C allele): CATCTAATCGTGCCCTTTAGGAAGGAGTC	Allele C is present	positive	C	CG Heterozygous
Reverse inner primer (G allele): CCACTGGTTGTAAGAGAAGGATGAACCC	Allele T is present	positive	G	
Forward inner primer (C allele CATCTAATCGTGCCCTTTAGGAAGGAGTC	Allele C is absent	negative	-	GG Homozygous
Reverse inner primer (G allele): CCACTGGTTGTAAGAGAAGGATGAACCC	Allele T is present	positive	G	
Forward inner primer (C allele CATCTAATCGTGCCCTTTAGGAAGGAGTC	Allele C is present	positive	C	CC Homozygous
Reverse inner primer (G allele): CCACTGGTTGTAAGAGAAGGATGAACCC	Allele T is absent	negative	-	

2.3.8 Statistical Analysis

Statistical analysis of this study was performed by using a commercially available software program Statistical package for the social science (SPSS), (Version 23.0, SPSS, Inc., Chicago, Illinois, USA).

To determine whether any significant differences in polymorphisms frequencies occurred between the case and the control populations the allele and genotype frequencies in cases and controls group were compared using the Chi- squared test.

Associations between the disease and genotypes were assessed by calculating odds ratios (OR) and (95%) confidence intervals (CI). (Joda ,2008)

(P value less than 0.05 was considered statistically significant).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Diagnosis of *C.parvum*

3.1.1 Microscopic diagnosis of stool Specimen:

A total of (300) stool Specimen were detected microscopically by modified ziehl-neelsen acid fast staining method in which *C. parvum*, the oocyst appear as a pink to red round bodies against blue background.

From (300) stool Specimen ,75 (25%) were found to be positive for diarrhea causing protozoan parasites *C.parvum* ,while 225 (75%) Specimen negative This results were shown in Figure (3-1) and (3-2).

Microscopy is one of the most widely used methods for the detection of *Cryptosporidium* Oocysts in water, food, and fecal samples . However, the identification of the different species of *Cryptosporidium* based on light microscopy alone is unreliable and not specific enough, because many species of *Cryptosporidium* share similar morphological characteristics (Vohra *et al*., 2012).

Several staining techniques have been employed to help in the differentiation (on glass slides) of *Cryptosporidium* oocysts from other environmental or fecal debris. The Ziehl–Neelsen stain, also known as the acid fast stain, is the most popular direct stain used in clinical microbiology laboratories to stain *Cryptosporidium* Oocysts

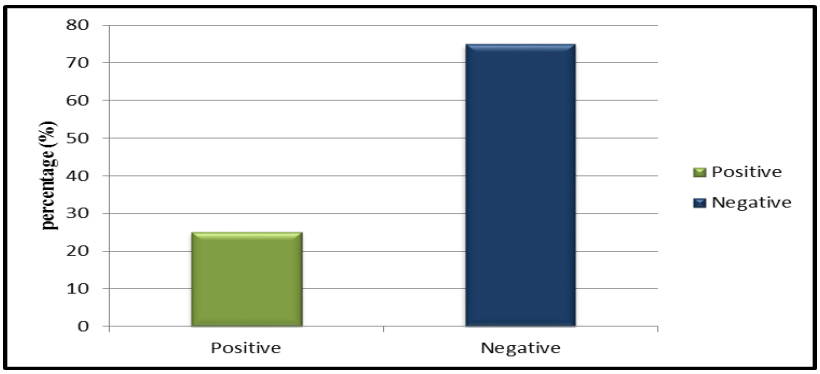


Figure (3-1):Total percentage of infected with *C.parvum*

Omoruyi *et al*., (2014) ; Elsawey *et al*., (2020) reported prevalence rate of *C.parvum* associated with diarrhea in patient which accounted for 34 %.

The result were in accordance with Abdulsada and Safa (2021) who found that the positive result of *C.parvum* associated with diarrhea in patient was (50%)

The percentage of infection with the *C.parvum* recorded in the current study is lower than that recorded in many previous studies in Iraq such as Al-Mosa, (2002) in the Babylon province (44.1%), Al-Musawi (2004) in the Babylon province (45.1%), Jaeffer (2011) in Baghdad (22%). Moreover, the percentage of infection in current study is lower than that recorded in other studies in countries around the world such as; Tinuade *et al*. (2006) in Nigeria (23%) and Nagosso *et al*. (2015) in Tanzania (29.6%).

While the percentage of infection with diarrhea parasites recorded in the current study is higher than the percentage recorded in some studies, in either Iraq or other countries such as Al-Taie (1997) in Diyala province (2.8%), Alousi *et al*. (2003) in Mosul city and Uppal *et al*.(2014) in India (4.7%).

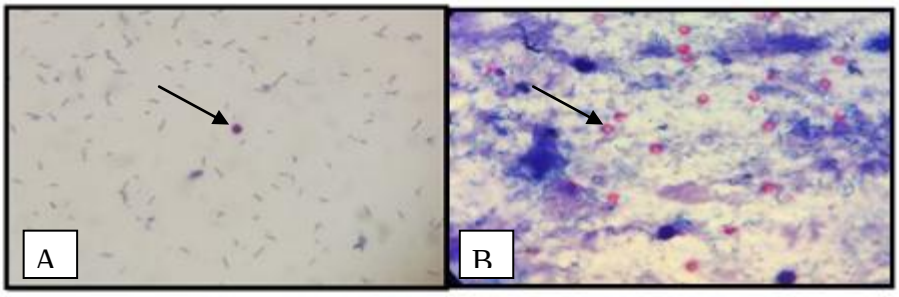


Figure (3-2): Microscopic image of *C.parvum* by use MZN

Al-Barakat *et al.*, (2015) found that the positive results in AL-Muthanna was (21.09%) also Al-yasary *et al*.,(2021) found that positive results was (26%) in Karbala .

in Saudi Arabia , the positive rate was (70%) which agreed with the present results and by Sanad *et al* .,(2015), *C. parvum* was in southwest of Iran recorded (70.38%) this percentage agreed with our results by Ghafari *et al*.,(2018) .

Also the mismatch was showed in Najaf (10.9%) by Hussein *et al*. (2013), and in Kirkuk city (7.60%) by Salman *et al*.,(2015) . while Hijjawi *et al* .,(2017)in Jordan found the result were (14.4%).

This disagreement may be retain to difference in sample or the period of sample collection.

3.1.2 Diagnosis of *C. parvum* by ImmunoChromatography method

Table (3-1) shows the method of detection of *C. parvum* parasites by used chromatographic immunoassay method. Where the appearance of the red line is the positive result and the absence of the red line indicates the negative result also show co infection with *C. parvum*.

Table (3-1): Total percentage of infected with diarrheal agents by ImmunoChromatography method

Examined Number	Positive	%	Negative	%
300	40	13.33	240	80

The present study recorded three parasite species that infected patients with diarrhea in Babylon province (Oleiwi, 2020). who recorded the highest percentages of infection were 54.2%, 36.2 and 7.9% for *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp., respectively.

In Ramadi province, Al-joudi, and Ghazal.,(2005) found two species of parasites, are *G. lamblia* (34.5%) and *E. histolytica* (8.08%) where mixed with *C. parvum*

Also, Al-Quraishi *et al*.,(2014) in Baghdad province found two parasites species; the *G. lamblia* most prevalent parasite with percentage

of infection 45.54 % and *E. histolytica* (33.44%) . Hijjawi *et al.*,2017) mentioned that in Kirkuk the rate of *Cryptosporidium* infection to be lower than the present study which was 16.28%.

In other countries around the world, several studies conducted on parasite-causing diarrhea, such as Arani *et al*. (2008) reordered two parasites in Iranian peoples (in north Tehran) were *E. histolytica* (71%) and *G. lamblia* (17.5%). In Makkah Almukarramah, Al-Harthi and Jamjoom (2007) showed three protozoan parasites are *E. histolytica* (64%), *G. lamblia* (1.9%) and *C. parvum* (1.7%).

Yacoub (2014) showed the Palestinian patients infected by three parasites *E. histolytica* (69%), *G. lamblia* (17.2%) and *Cryptosporidium* (13.8%). In north-west Ethiopia, Sitotaow *et al*. (2019) recorded the highest percentages of infection were 19.95%, 5.9% and 4.2% for *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp., respectively.

3.2 Distribution of *C. parvum*

3.2.1 The total percentage of infection with *C. parvum* According to Age:

From (75) Positive samples , The highest infection rate was recorded in patient with age group (1 ≥ month) 27(36%) followed by 16(21.3%) in patient in the age group (3-6 month) , and the lowest rate of infection with *C.parvum* were recorded in patient also which was 2(2.7%) in the age group (9-10 year). These results were shown in figure and table (3-3) and Figure (3-3)

Table (3-2): Total percentage of *C.parvum* According to Age

Age	Total exam	Total infected
1 ≥ month	98(32.7%)	27(36%)
3-6 month	61(20.3%)	16(21.3%)
7-9 month	52(17.3%)	11(14.7%)
1-2 year	36(12%)	9(12%)
3-5 year	25(8.3%)	7(9.3%)
6-8 year	17(5.7%)	3(4%)
9-10 year	11(3.72%)	2(2.7%)
Total	300(100%)	75(100%)
X^2	2.3	1.8
P-value	0.05	0.04

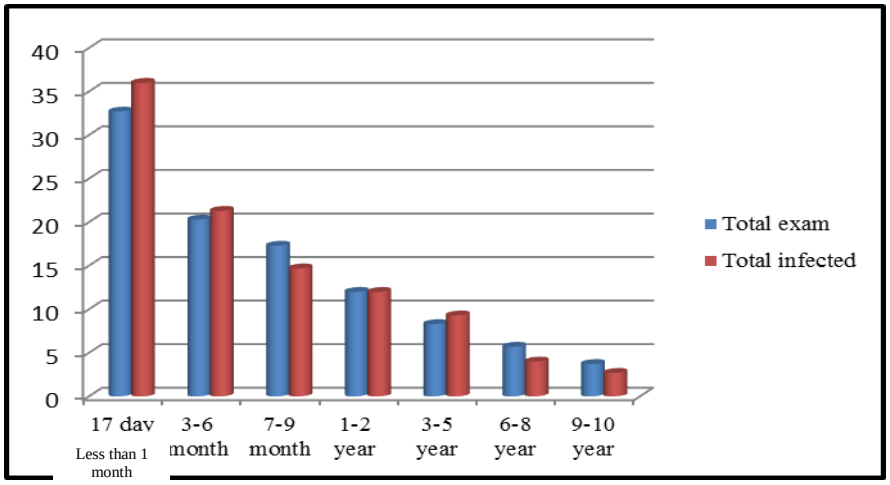


Figure (3-3): Distribution of infection with *C.parvum* According to Age

Adler *et al*., (2017) and Kotloff *et al*., (2013) found that, the *Cryptosporidium* spp. the second leading pathogen associated with moderate-to-severe diarrhea (MSD) in children under two years old and the leading pathogen associated with death in toddlers (ages 12 to 23 months).

The higher infection in children occurs due to their immune system functions which were undeveloped so intake small number of oocysts may result in cryptosporidiosis and repeated low dose infections may stimulate the immunity to *Cryptosporidium* which may protect children

tend to have relatively more symptomatic disease than older with(Najafi *et al.*,2020)

The Study of Darogha's & Kanabe (2021) In Erbil city, recorded (10.77%) in the age group (1-5 months) which were agreed with our study and this study agree with Al-Saeed *et al* .,(2020) study In Duhok ,(11.11%) , (3.13%) and (16%) in the age group(1-5 months) ,(15-20 months) and (<20 months) which agreed with our result, respectively.

There are some many factors affecting the prevalence competing Interests: location of sampling, The high incidence of cryptosporidiosis in children probably reflects a lack of immunity due to few prior exposures and the immaturity of the gut mucosa, season, climatic condition, volume of sample, the population of the animals in the areas and rainy seasons etc.

3.2.2 The total percentage of infection with *C. parvum* According to Gender

Table(3-3) explained the distribution of *C. parvum* according to Sex of the highest percentage was found in males (65.3%), and lowest percentage found in females(34.7%).

Table (3-3): The percentage of infection with *C.parvum* According to Gender

Gender	Infected sample	%
Male	49	65.3
Female	26	34.7
Total	75	100

The results in microscopic examination showed the highest infection in male and lowest then infection in female, this result was agreed with study AL-Kubaisy *et al.*, (2014) in Baghdad, where They scored the highest rate of infection in males (58.5%) and lower than in females (41.5%).

Also agree with the study in the Al- Diwaniya province mentioned the highest rate of infection for males (6.12%), while the lowest infection for females (5.11%) but disagree with the study in Baghdad, where he scored the highest rate of infection (15.35%) in females and (12.28%) in males (Salim *et al* ., 2019)

The difference in the rate of infection between males and females may be due to the fact that males are the more movement and active and their contact with the external environment factors at play and of being working group in the communities, this is what makes them more relevant pathogens sick of females as males eat well and drink in public places or from street vendors, in addition to the nature of anarchism and a lack of attention to personal hygiene and wash hands which increases the chances of being infection either for *C. parvum* and the absence of significant differences between male and females could be due to portability and having the same opportunity to infection both sexes intestinal parasites(AL-Mamouri., 2000).

3.2.3 The total percentage of *C.parvum* According to Residence

The present study, The distribution of *C. parvum* according to Residence showed 39 (52%) was found in Rural and 36(48%) was found in urban . as showing in Table (3-4).

Table(3-4): The total percentage of *C. parvum* According to Residence

Residence area	No. of sample	%
Rural	39	52
urban	36	48
Total	75	100

In this study found there was highest infection in rural areas which was (52%) while lowest infection urban areas was (48%) in Babylon city in case of infection by the parasites microscopically, The result rural area more than urban area was agreed with AL-Kubaisy *et al.*, (2014). Where

they scored the highest rate of infection in the rural area, reaching 50.9% in Baghdad.

Derso *et al.*,(2019) found that infection in rural areas was (34.1%) while infection in Urban areas was (31.1%), also Al-Mussaw., (2012) confirm the rate of infection in the rural area amounted to higher than in the cities area .

The reason for the high incidence of infection in rural areas due to several factors, including the absence of clean drinking water availability, and rely on river water directly as a source of water, and the absence of guidance and counseling by the authorities concerned as well as lower health and cultural level of the rural population as well as the lack of hospitals and health centers in those areas, as well as use of animal waste and human feces and sometimes as an organic fertilizer for the growth and plants and vegetables, socio environmental factor such as dejection level sanitation infrastructure used and water sources so that the difference in the protozoan infection in patients was insignificant with regards to the education level so, infection was less common in family with private sanitation as compared community sanitation(Faria *et al.*, 2023)

3.3 Immunological Study:-

3.3.1 Measurement the IL-18 Concentration in patient with cryptosporidiosis :

The mean of IL-18 levels in sera of patient with diarrhea was higher (985.86±62.71 pg/ml) than the control ones (290.74±13.04 pg/ml). Statistical analysis revealed a significant difference between the sera levels of IL-18for diarrhea cases and controls ($p<0.03$).

Table(3-5):The Concentration of Human IL18 in patient infected with *C.parvum*

Groups	No.	Mean	SD	P value
Control	25	290.74	13.04 pg/ml	0.03
Case	75	985.86	62.71 pg/ml	

The present study revealed that the mean of IL-18 levels in sera of patients with age (7-9month) was higher (681.56±2.1pg/ml) than the control ones (280.26±0.5 pg/ml). (more susceptible to *Cryptosporidium*) then the other age

Table (3-6) shows the distribution of percentage of *C. parvum* infection according to age groups of patients. The highest level was in (7-9month) age group (681.56±2.1pg/m) in male and (673.89±1.31pg/m) in female . While the lowest level was in the age group of (6-8 years) in male (612.43±2.21pg/m) and in female (623.43±2.3pg/m).

Statistical analysis revealed a significant difference between the sera levels of IL-18 for *Cryptosporidium* and controls ($p<0.003$).

Table (3-6):IL18 Concentration in patient with *C. parvum* compare with control according to age group

Sex / Age	Male				Female			
	Mean± SE			P-value	Mean± SE			P-value
	No	Case	Control		No.	Case	Control	
17 day	5	489.07±1.7 pg/m	290.83±0.7 pg/m	0.03	5	459.67±2.1 pg/m	283.01±0.8 pg/m	0.02
3-6 month	5	613.58±2.3 pg/m	317.89±0.9 pg/m	0.02	5	637.24±2.3 pg/m	310.87±0.9 pg/m	0.03
7-9 month	5	681.56±2.1 pg/m	280.26±0.5 pg/m	0.003	5	673.89±1.3 pg/m	280.56±0.3 pg/m	0.001
1-2 years	7	677.90±2.6 pg/m	302.83±1.5 pg/m	0.01	6	648.78±2.7 pg/m	308.80±0.9 pg/m	0.01
3-5 years	7	650.54±1.8	296.11±2.4	0.003	5	656.22±2.4 pg/m	288.30±1.6 pg/m	0.02
6-8 years	5	612.43±2.2	307.23±1.3	0.02	5	623.43±2.3 pg/m	309.67±2.3 pg/m	0.01
9-10 years	5	632.17±3.1	313.34±2.4	0.001	5	634.45±3.3 pg/m	317.34±2.4	0.01

The IL-18 (Interleukin-18) concentration is a measure of the level of this particular cytokine in the body. IL-18 is involved in various immune responses and plays a role in regulating inflammation. It is produced by cells such as macrophages and plays a role in both innate and adaptive immunity (Ihim *et al.*,2022)

Early in infection, IFN-γ secreted by NK cells, macrophages and dendritic cells is thought to be the major cytokine involved in orchestrating both the innate and adaptive immune responses, but recent evidence suggests that IL-18 is important in the control of *Cryptosporidium* infection as well (Gullicksrud *et al.*,2022).

McDonald and colleagues previously found that IL-18 might reduce *Cryptosporidium* infection via enhancement of secretion of AMPs by IECs . More recently, they found that IL-18 confers protection against *C. parvum* infection *in vivo* by coordinating with IL-18 to enhance IFN- γ production by macrophages (Choudhry *et al.*,2012)

Study of Bedi *et al.,(*2015) confirm that IL-18 play a key role limiting *C.parvum* infection ,they show the treatment with recombinant IL-18 significanlty decreases the parasite load.

Other study reported that ,in vitro *C.parvum* can directly activate the inflammasome and downstream caspase-1 in DC resulting in IL-18 secretion (Gibson *et al.,* 2021).

In contract ,Study of Parthasarathy *et al.,* (2020) found that enterocyte intrinsic NLRP6 inflammosome activation was required for IL-18 mediated resistance to *C. parvum*

3.3.2 Measurement the IFN-γ Concentration in patient with cryptosporidiosis:

The mean of the sera levels of IFN-γ in patient with *C. parvum* infection was higher (963.51±34.91pg/ml) than the control groups

(425.23±25.32pg/ml). Table (3-7) shows the result of sera IFN-γ concentrations in cases and in control groups There was significant increase in IFN-γ in diarrheal cases than control ones

Table(3-7): Concentration of Human IFN-γ with *C. parvum*

Groups	NO.	Mean	SD	P value
Control	25	425.23±25.32 pg/ml		0.002
Case	75	963.51±34.91 pg/ml		

The mean of the sera levels of IFN-γ in patient suffering from diarrhea was higher in age (7-9 month**)** (593.56±2.3pg/ml) than the control ones (430.96±0.5pg/ml) in male while in female the higher level in age 6-8 year (559.19±2.45 pg/ml) than the control ones(438.73±1.3 pg/ml).

Table (3-8):The Concentration in patient with *C. parvum* compare with control according to age group

Sex / Age	Male				Female			
		Mean± SE		P-value		Mean± SE		P-value
	NO.	Case	Control		NO.	Case	Control	
17 day	5	525.69±1.6	433.08±0.7	0.04	5	535.29±1.9	423.08±0.7	0.05
3-6 month	5	539.58±2.1	437.59±0.9	0.05	5	536.38±2.4	427.59±0.9	0.04
7-9 month	5	593.56±2.3	430.96±0.5	0.05	5	553.36±2.1	433.96±0.5	0.01
1-2 years	7	577.93±2.4	443.43±1.5	0.03	6	547.23±2.0	435.43±1.5	0.03
3-5 years	7	568.59±1.9	446.31±2.4	0.03	5	548.69±1.6	436.31±2.4	0.02
6-8 years	5	579.49±2.1	443.73±1.3	0.05	5	559.19±2.4	438.73±1.3	0.03
9-10 years	5	567.57±2.5	447.24±2.4	0.02	5	557.67±2.2	437.24±2.4	0.04

IFN-γ (Interferon-gamma) is a cytokine, which is a type of signaling molecule involved in immune responses. It plays a crucial role in the immune system's defense against various pathogens, including intracellular parasites like *Cryptosporidium*. (Bedi *et al.*,2015)

Zaalouk(2020)finding that was found to have a marked inhibiting effect of *C. parvum* infection the effect of IFN-γ was partially reversed by TGF- β which preduce asignificant dose dependent antagonist of IFN-γ activity Lacroix *et al.*, (2001) found, IFN-γ is one of the key components of this immune response. It is primarily produced by T cells, particularly CD4+ T helper 1 (Th1) cells and CD8+ cytotoxic T cells, in response to an infection and serves several important functions in the context of *Cryptosporidium* infection as Activation of Macrophages and Inducing Antimicrobial Pathways and Regulation of Inflammation

Mead (2023) suggested that TNF-α expression in the intestinal mucosa of susceptible mice correlates strongly with the presence of IFN-γ. So that an important element in the central of *Cryptosporidium* infection may be the activation of TNF-α expression via up-regulation of transcription factor, NF-KB by IFN-γ .

3.3.3 Evaluation the TGF Concentration in patient with cryptosporidiosis

The mean of the sera levels of TGF-β in patient suffering from diarrhea was higher (444.48±17.82pg/ml) than the control groups (96.05±9.63pg/ml). Table (3-9) shows the result of sera TGF-β concentrations in cases and in control groups There was significant increase in TGF-β in patient than control group p>0.02

Table(3-9): Concentration of Human TGF-β in patient with *C. parvum*

Groups	No.	Mean	SD	P value
Control	25	96.05	9.63	0.02
Case	75	444.48	17.82	

According to age, The mean of the sera levels of TGF in patient suffering from diarrhea was higher in age (9-10 years**)** (147.24±2.4pg/ml) than the control ones (84.24±0.4pg/ml) in male while in female the higher

level in age 9-10 years (137.24±2.4pg/ml) than the control ones(85.64±0.4pg/ml).

Table (3-10): The Concentration TGF in patient with *C. parvum* compare with control according to age group

Sex / Age	Male				Female			
	Mean± SE			P-value	Mean± SE			P-value
	No.	Case	Control		NO.	Case	Control	
17 day	5	137.08±0.7	88.12±0.7	0.04	5	133.08±0.7	84.32±0.7	0.05
3-6 month	5	136.59±0.9	89.59±0.9	0.05	5	134.59±0.9	83.59±0.9	0.04
7-9 month	5	138.96±0.5	80.66±0.5	0.05	5	136.96±0.5	85.66±0.5	0.01
1-2 years	7	142.43±1.5	84.43±0.5	0.03	6	135.43±1.5	80.73±0.5	0.03
3-5 years	7	144.31±2.4	91.31±0.4	0.03	5	132.31±2.4	81.81±0.4	0.02
6-8 years	5	143.73±1.3	87.73±0.3	0.05	5	130.73±1.3	80.73±0.3	0.03
9-10 years	5	147.24±2.4	84.24±0.4	0.02	5	137.24±2.4	85.64±0.4	0.04

TGF-β (Transforming Growth Factor-beta) is a multifunctional cytokine involved in various biological processes, including cell growth, differentiation, immune response regulation, and tissue repair. It plays a crucial role in modulating immune responses during infections and inflammation (Zaalouk,2020).

When *Cryptosporidium* infects the gastrointestinal tract, it can trigger an immune response. TGF-β is one of the cytokines that are involved in regulating the immune response to *Cryptosporidium* infection. Studies have shown that TGF-β plays a dual role in the immune response to *Cryptosporidium*(El-Kholy *et al* .,2021).

On other hand, TGF-β can contribute to the control of *Cryptosporidium* infection by promoting the development and activation of immune cells such as T cells and macrophages. It helps in the production of specific antibodies and enhances the activity of certain immune cells to eliminate the parasite (Yang *et al* .,2020)

On the other hand, TGF-β can also have immunosuppressive effects during *Cryptosporidium* infection. It can inhibit the function of certain

immune cells, suppress the production of pro-inflammatory cytokines, and promote the development of regulatory T cells. These immunosuppressive effects can limit excessive inflammation and prevent tissue damage but may also hinder the clearance of the parasite (Rusyati,2020)

The exact role of TGF-β in *Cryptosporidium* infection is complex and can vary depending on the specific host and parasite factors, as well as the stage of infection.

Study of Zaalouk,(2020) confirm that the up regulation of TGF-β expression in the human intestine during cryptosporidiosis has been proved and a potential source of the cytokine is the IEC, TGF-β may have a healing role ,as it has been shown to hinder the disruption of the epithelial barrier by *Cryptosporidium* infection therefore ,that the presence of TGF-β at the infection site can have important antagonistic effects on the microbic activity of intestinal epithelial cells.

3.3.4 Measurement the MBL Concentration

The mean of MBL levels in sera of patient with diarrhea was higher (6621.15±278.3pg/ml) than the control ones (1203.33±51.36 pg/ml). Statistical analysis revealed a significant difference between the sera levels of MBL for diarrhea cases and controls ($p<0.05$).

Table(3-11): Concentration of Human MBL in patient with *C. parvum*

Groups	No.	Mean	P value
Control	25	1203.33±51.36 pg/ml	0.04
Case	75	6621.15±278.3pg/ml	

C.parvum can activate both classical and lactin complement pathway ,leading to deposition of C3b on the parasite, so the increase

susceptibility to the *c.parvum* could only be demonstrated in adult locking mannose binding lectin(Mead,2023)

According to age, the mean of the sera levels of TGF in patient suffering from diarrhea was higher in age (9-10 years**)** (3692.57±5.1pg/ml) than the control ones (1253.74±2.4 pg/ml) in male while in female the higher level in age 1-2 year (3873.98±5.7 pg/ml) than the control ones(1278.83±4.9 pg/ml). Figure(4-7)shows the result of sera MBL concentrations in cases and in control groups.

Table(3-12):MBL Concentration in patient with *C. parvum* compare with control according to age group

Sex / age	Male				Female			
	Mean± SE			P-value	Mean± SE			P-value
	No.	Case	Control		NO	Case	Control	
17 day	5	3940.25±11.7	1276.32±3.7	0.002	5	3935.68±12.1	1272.59±3.2	0.001
3-6 month	5	3923.44±10.3	1265.89±2.9	0.01	5	3919.56±8.13	1260.44±4.9	0.02
7-9 month	5	3891.56±7.1	1280.36±4.5	0.03	5	3883.79±6.3	1275.26±5.3	0.01
1-2 years	7	3877.93±8.6	1272.86±7.5	0.01	6	3873.98±5.7	1278.83±4.9	0.001
3-5 years	7	3750.84±5.8	1266.41±3.4	0.01	5	3766.12±3.4	1268.37±3.6	0.01
6-8 years	5	3742.73±5.2	1257.63±1.3	0.01	5	3733.93±4.2	1259.47±4.1	0.01
9-10 years	5	3692.57±5.1	1253.74±2.4	0.01	5	3687.95±3.6	1250.94±3.2	0.01

Mannose-binding lectin (MBL) is a type of protein that plays a significant role in the innate immune system. It is part of a group of proteins called collectins, which are involved in recognizing and binding to certain types of carbohydrates on the surface of pathogens like bacteria, viruses, and fungi. MBL acts as an opsonin, which means it enhances the immune response by facilitating the engulfment and destruction of these pathogens by immune cells such as macrophages and neutrophils. MBL deficiency can lead to increased susceptibility to various infections (Yilmaz *et al.*,2023).

MBL is a serum protein that mainly has a role in the innate immune response by binding the surface of the microorganisms and activating the complement system . While MBL distinguishes the ligands of the

microorganisms, MASP-2 can be activated and starts an antibody-dependent pathway of the complement system(Mead.,2023)

Lanz *et al.*,(2023) report 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.

MBL is a conserved protein that binds to microbial surfaces and promotes opsonophagocytosis. Studies have suggested the protective role of MBL may be most important in young children and individuals with immunodeficiencies (Yang *et al* .,2020)

3.4 The Correlation among the Immunological Parameters:

3.4.1 correlation of IL-18 with TGF- β :

There was confident correlation between IL-18 with TGF ($R^2 = 0.0043$, $p<0.05$) as shown in Figure (3-4)

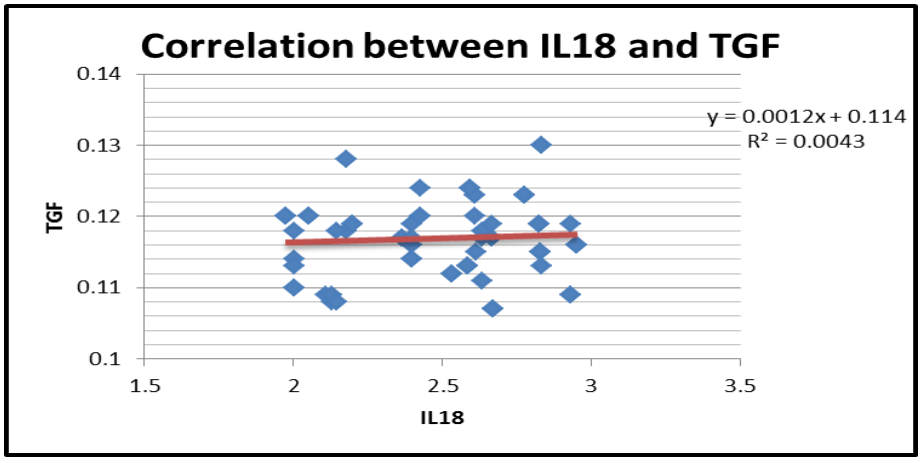


Figure (3-4): the correlation between IL-8with TGF- β

From the result of this study ,there was positive correlation between IL-8with TGF- β,this finding is agree with other study such as finding of Ehigiator *et al* (2007) who confirm that the IL-18 plays regulatory role in the Th1/Th2 balance during *c.parvum* infection and demonstrated the establishment of robust acute infection and consequent strong cellular

immune response as indicated by mRNA expression of IL-18 and TGF-βin the intestine as well as parasite specific proliferation.

3.4.2 The correlation of IL-18 with MBL :

There was correlation between IL-8 with MBL (R^2 = 0.1982 , $p<0.05$) and this correlation was negative. Study of (ward and Borad, 2011) finding low MBL level may be related to maturation or protein losses in the gut and children with MBL deficiency were more likely to be infected with *c.parvum* as shown in figure (3-5)

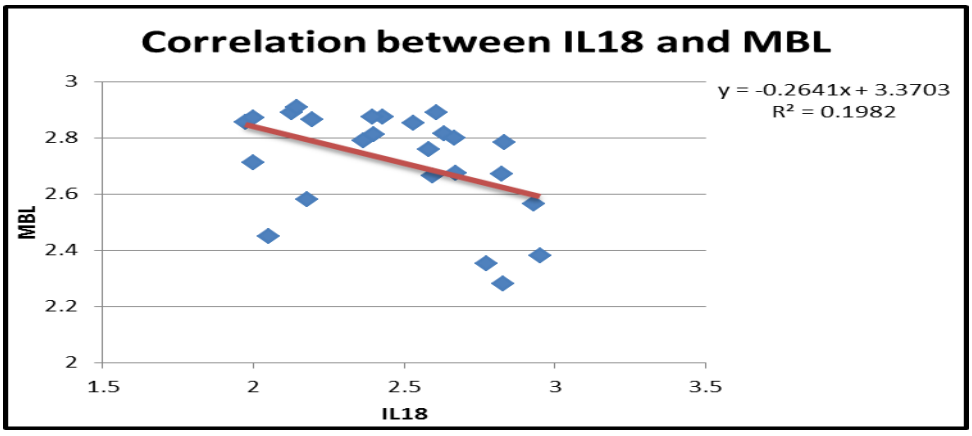


Figure (3-5): the correlation between IL-8with MBL

3.4.3 The correlation of IL-18with INF-γ :

There was correlation between IL-8 with INF-γ (R^2 = 0.3622 , $p<0.05$) and this correlation was strong positively as shown in Figure (3-6)

Two key inducer of INF-γ ,IL-18,IL-12 are important in primary immune response to infection ,both cytokines work synergistically to produce INF-γ, so lack of this cytokines lead to greater susceptibility and severity of cryptosporidiosis,IL-18 synergizes with IL-12 to stimulate effector cell to produce INF-γ(Gullicksrud *et al.*,2022)

Study of Bedi *et al.*(2015) suggest IL-18 can mediate its protective effects via different routes as INF-γ induction or directly stimulating intestinal epithelial cells to increase antimicrobial activity.

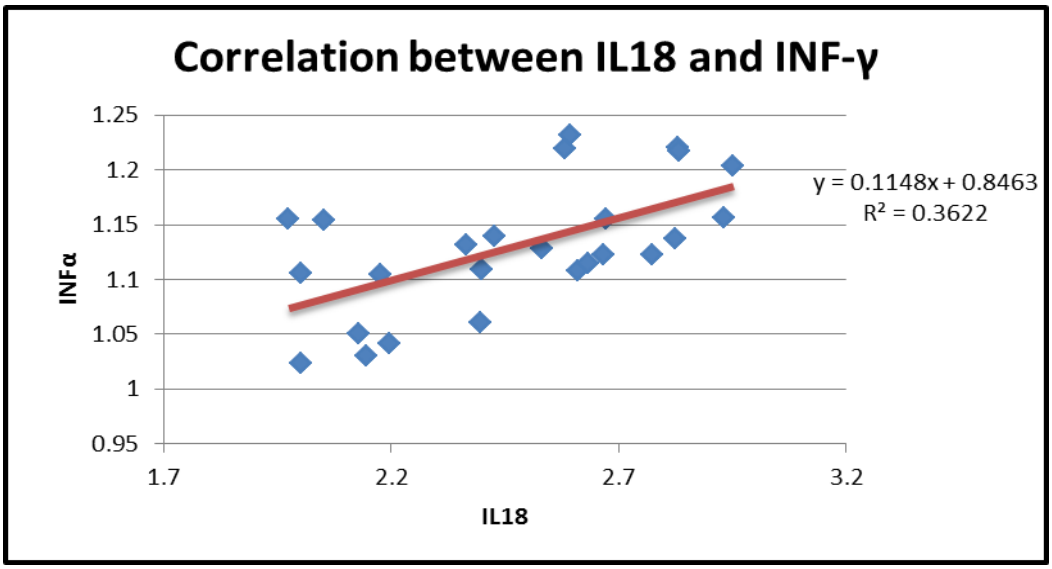


Figure (3-6): the correlation between IL-8with INF-γ

3.4.4 The correlation of MBL with TGF:

There was confident correlation between MBL with TGF ($R^2 = 0.0345$, $p<0.05$) and this weak correlation as shown in Figure (3-7)

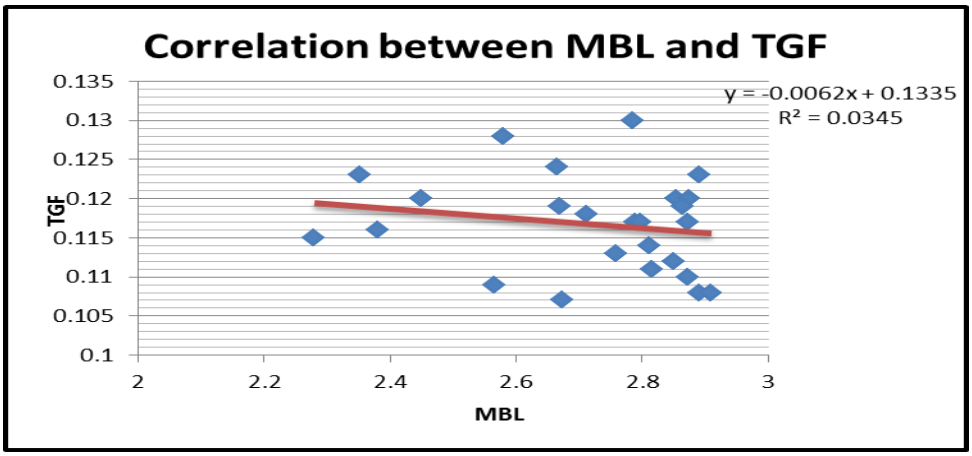


Figure (3-7): the correlation between MBL with TGF

3.4.5 correlation of MBL with INF-γ :

There was confident correlation between MBL with INF-γ ($R^2 = 0.2383$, $p<0.05$) as shown in Figure (3-8)

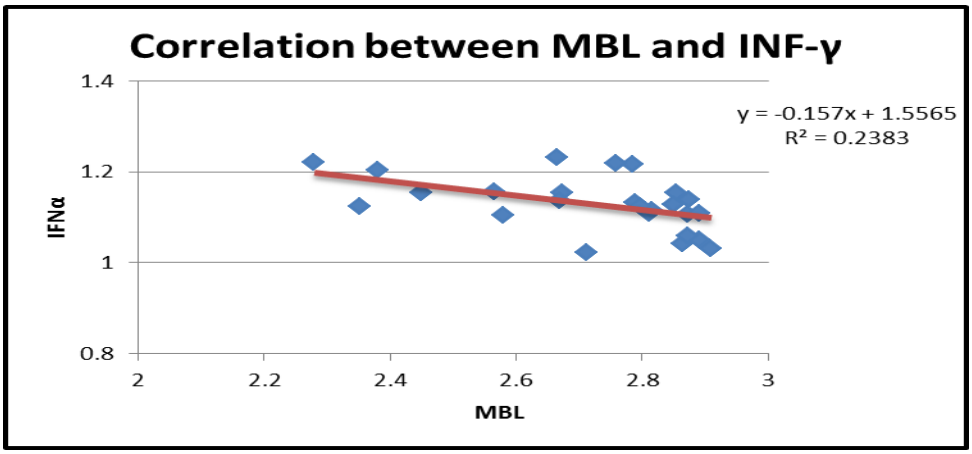


Figure (3-8): the correlation between MBL with INF-γ

3.4.6 correlation of INF-γ with TGF:

There was confident correlation between **of INF-γ with** TGF ($R^2 = 0.061$, $p<0.05$) as shown in Figure (3-9)

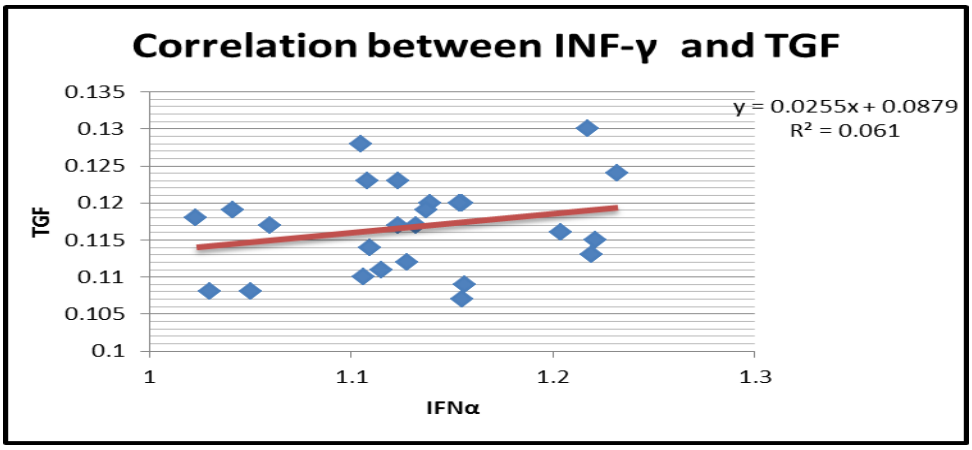


Figure (3-9): the correlation between INF-γ with TGF

4.5 Genetic diagnosis of *Cryptosporidium parvum* in stool specimen

To determine if parasite isolates was *C. parvum* ,the present study characterized the isolates on the basis of the presence of heat shock protein genes(HSP70) by using PCR technique. Figure (3-10) show Agarose gel electrophoresis image that showed the PCR product analysis of HSP70 gene in *C.parvum* isolated from Stool samples of diarrheal patient . out of 75 only 50(66.66%) samples of patient observed carried the heat shock protein genes

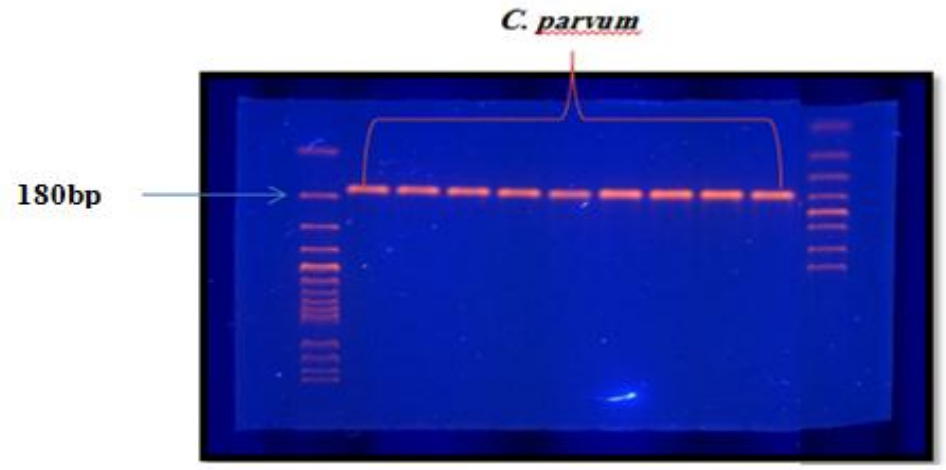


Figure (3-10): Agarose gel electrophoresis image that showed the PCR product analysis of heat shock protein genes from stool samples of patient

The *Cryptosporidium* HSP70 gene appeared as a good target for genotyping, due to its high level of heterogeneity spread over the entire sequence, The *Cryptosporidium* HSP70 gene refers to a specific gene encoding the heat shock protein 70 (HSP70) in the *Cryptosporidium* parasite. Heat shock proteins are a family of proteins that play a crucial role in protecting cells from various environmental stresses, such as heat, cold, toxins, and other forms of cellular damage(Al-Musawi *et al.*,2022)

Cryptosporidium is a genus of microscopic parasites that can cause a gastrointestinal illness called cryptosporidiosis in humans and animals. *Cryptosporidium* species have complex life cycles involving different developmental stages within their hosts. During certain stages, such as when the parasite is exposed to stressful conditions, it may induce the expression of HSP70 genes(Mason *et al.,2022*)

Cryptosporidium and other organisms, the HSP70 gene is part of the cellular response to stress, including high temperatures (heat shock) and other environmental challenges. The gene is unregulated when the cell experiences stress to help prevent protein misfolding and aggregation, stabilize existing proteins, and assist in proper folding of newly synthesized proteins.

HSP70 gene in *Cryptosporidium* is important because it provides insights into the molecular mechanisms that these parasites use to adapt to their environment and cause disease.

3.6 *G*ene Polymorphism and Susceptibility to the *C. parvum*

Using the amplification refractory mutation system- polymerase chain reaction (ARMS-PCR) method, the genotyping of *MBL* polymorphism was determined in patient in both diarrheic and control groups.

3.6.1 *MBL* gene Polymorphism and Susceptibility to *Cryptosporidium parvum* infection :

In present study, investigated a possible association between *MBL*(rs 1800450) polymorphisms and *C. parvum* in population of Babylon provinces/Iraq. The results of the present study demonstrate that the polymorphisms in *MBL* gene may affect susceptibility to *C. parvum* and increase risk of developing the disease. Figure (3-11) show the ARMSPCR product analysis of *MBL* (rs 1800450 p) polymorphism from blood samples.

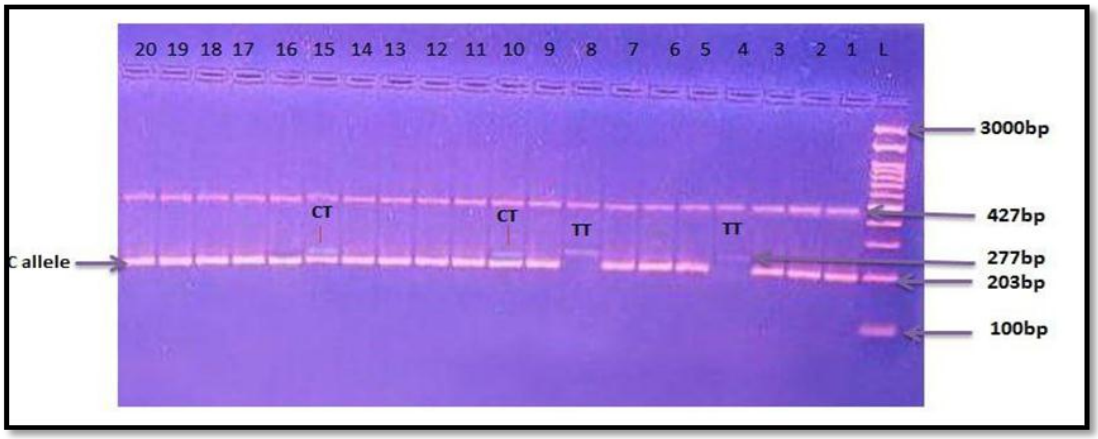


Figure (3-11): Agarose gel electrophoresis image that showed the ARMS PCR product analysis of *MBL* gene(rs 1800450 p) from blood samples of patient. Where Marker ladder (3000- 100bp).

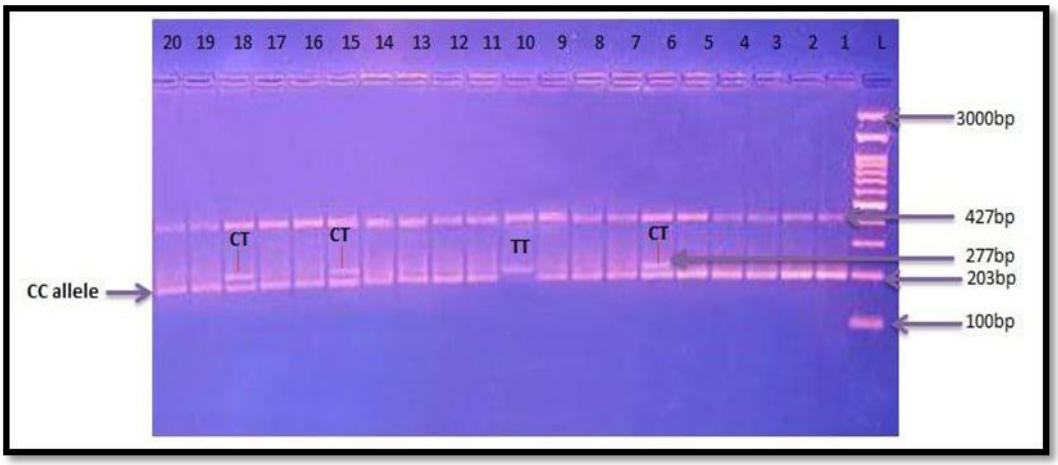


Figure (3-12): Agarose gel electrophoresis image that showed the ARMS PCR product analysis of *MBL* gene (rs 1800450 c) from blood samples of control. Where Marker ladder (3000- 100bp).

The results of genotype and alleles frequencies of *MBL* (rs1800450p) gene polymorphism in cases and(rs1800450c) in control groups are presented in table (3-13).Over all, there was no significant difference in the distribution of *MBL* (rs1800450) genotypes between case and control group(p=0.0001).

Table (3-13): rs1800450 SNP distribution frequencies in the screened population (Control and Patients).

SNP	Allele	Frequency	Controls	Patients	P Value	OR (95% CI)	
rs1800450	**C**	68 (0.83)	35 (0.83)	33 (0.82)	0.920	1.061	(0.336-
	T	14 (0.17)	7 (0.17)	7 (0.18)			3.351)
	P value	<0.0001*	<0.0001*	<0.0001*			
	Genotypes						
	C/C	31 (0.76)	15 (0.71)	16 (0.8)	0.14		1.00
	C/T	6 (0.15)	5 (0.24)	1 (0.05)		0.19	(0.02-
	T/T	4 (0.1)	1 (0.05)	3 (0.15)			1.80)
						2.81	(0.26-
							30.10)
	P value	<0.0001*	0.001*	<0.0001*			

* represent a significant difference at p<0.05.

In table (3-13), the results for *MBL2* (rs 1800450) gene polymorphism have shown that the C allele frequency was 68 (0.83%) in the infected patient and 35(0.83) in the control individuals, whereas **T** allele frequency was 14 (0.17 %) in the study groups and 7 (0.18) in the control groups .

From the finding of this study, found higher frequencies of C allele at *MBL* (rs 1800450) in diarrheic Patient compared to control groups . This allele was associated with increased susceptibility to *C. parvum*.

The results of genotypes frequencies of *MBL* (rs 1800450) gene polymorphism in diarrheic , and control groups are presented in table (3-13), and the three genotypes (CC, CT and TT) frequencies of the cases and control groups

The results have shown that CC homozygous genotype frequency was overrepresented among the cases, 31 (0.76) which is more than the other two genotypes CT 6 (0.15) and TT 4 (0.1) respectively. Therefore, the CC homozygous genotype was the most frequent in Patients. The *MBL* (rs1800450) CC homozygous genotype was no significantly associated with increased susceptibility to *C. parvum*.

Patient with CC homozygous genotype were no significantly overrepresented among diarrheic cases, 16 (0.8), as compared with control groups, 15 (0.71%) and had a 1.00-folds increased risk of developing disease than the other two genotypes (CT and TT),(P value= 0.14, OR =1.00, 95% CI: (0.26-30.10) for TT genotype.

In contrast, the Patient with CT heterozygous genotype were obviously more presented among cases, 1 (0.05%), when compared with control, 5 (0.24) . (P value = 0.14, OR: 2.81, 95% CI: (0.02-1.80) for CT genotype.

While, Patient with TT homozygous genotype were obviously more presented among case, 3 (0.15) when compared with control ones, 1 (0.05). (P value= 0.14, OR= 2.81, 95% CI:(0.26-30.10) for TT

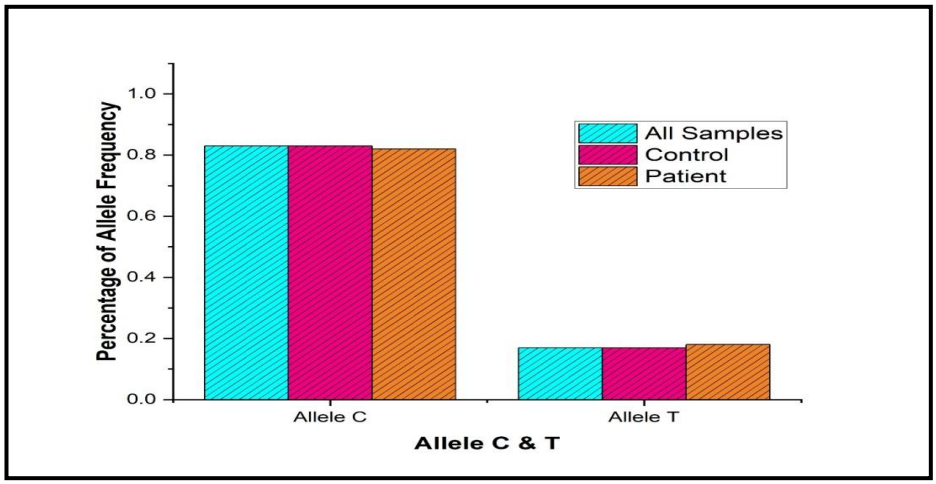


Figure (3-13): Genotype Frequencies of *MBL* Gene Polymorphisms in diarrheic Cases and Control groups

Also , investigated a possible association between *IL -18* (rs 5744247) polymorphisms and *Cryptosporidium parvum* . The results of the present study demonstrate that the polymorphisms in *MBL* gene may affect susceptibility to *Cryptosporidium parvum* .

3.6.2 *IL18* gene Polymorphism and Susceptibility to *Cryptosporidium parvum*

The results of the present study demonstrate that the polymorphisms in *IL -18* gene may affect susceptibility to *C. parvum* and increase risk of developing the disease. Figure (4-14) show the ARMSPCR product analysis of *IL18* (rs5744247p) polymorphism from blood samples.

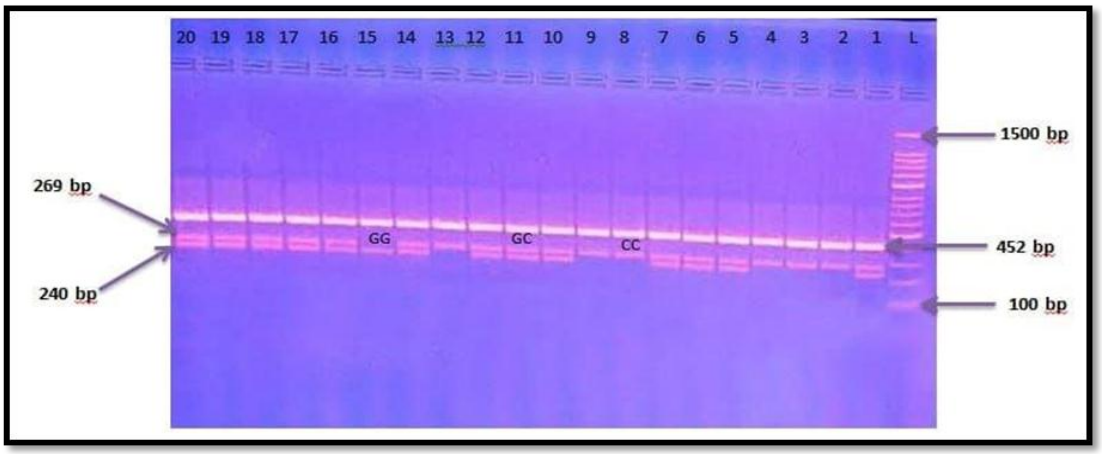


Figure (3-14): Agarose gel electrophoresis image that showed the ARMS PCR product analysis of *IL18 (rs5744247p)* from blood samples of patient . Where Marker ladder (3000- 100bp).

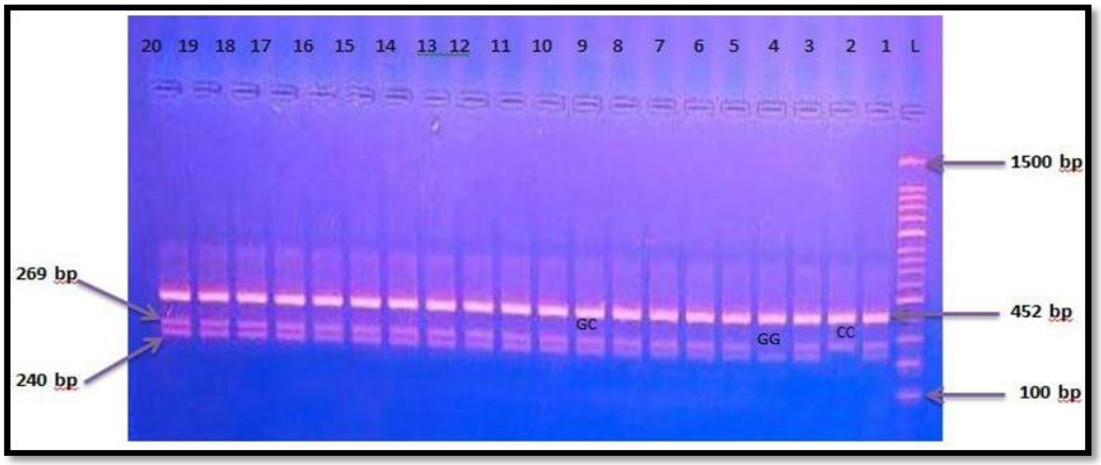


Figure (3-15): Agarose gel electrophoresis image that showed the ARMS PCR product analysis of *IL18 (rs5744247c)* from blood samples of control. Where Marker ladder (3000- 100bp).

Table (3-14): rs5744247 SNP distribution frequencies in the screened population (Control and Patients).

SNP	Allele	Frequency	Controls	Patients	P Value	OR (95% CI)
rs5744247	**G**	45 (0.56)	20 (0.50)	25 (0.62)	0.260	0.600 (0.246-1.463)
	C	35 (0.44)	20 (0.50)	15 (0.38)		
	P value	0.264	1	0.114		
	Genotypes					
	C/C	2 (0.05)	1 (0.05)	1 (0.05)	0.092	1.00
	G/C	31 (0.78)	18 (0.9)	13 (0.65)		0.12 (0.01-1.12)
	G/G	7 (0.18)	1 (0.05)	6 (0.3)		0.17 (0.01-5.45)
	P value	<0.0001*	<0.0001*	0.004*		

* represent a significant difference at p<0.05.

In table (3-14), the results for *IL18* (rs5744247) gene polymorphism have shown that the G allele frequency was 25 (0.62) in the infected Sample and 20 (0.50) in the control individuals, whereas **C** allele frequency was 15 (0.38) in the study groups and 20 (0.50) in the control groups with no significant difference (P value= 0.0001).

In present study, we found higher frequencies of C allele at *IL18* (rs5744247) in diarrheic Patient compared to control groups . This allele was associated with increased susceptibility to *C. parvum.*

The results of genotypes frequencies of *IL18* (rs5744247) gene polymorphism in diarrheic , and control groups are presented in Table

(4-5), and the three genotypes (CC, GC and GG) frequencies of the cases and control groups were shown in Figures (3-13).

The results have shown that CC homozygous genotype frequency was overrepresented among the cases, 25 (0.62) which is more than the other two genotypes GC 31 (0.78) and GG 7(0.18) respectively. Therefore, the CC homozygous genotype was the most frequent in Patients.

Patient with CC homozygous genotype were no significantly overrepresented among diarrheic cases, 2 (0.05), as compared with control groups, 1 (0.05) and had a 1.00-folds increased risk of developing disease than the other two genotypes (GC and GG),(P value= 0.092, OR =1.00, 95% CI: (0.01-5.45) for GG genotype.

In contrast, the Patient with GC heterozygous genotype were obviously more presented among cases, 13 (0.65),when compared with control, 18 (0.9) . (P value = 0.092, OR: 0.12, 95% CI: (0.01-1.12) for GC genotype.

While, Patient with GG homozygous genotype were obviously more presented among case, 6 (0.3) when compared with control ones, 1 (0.05). (P value= 0.092, OR= 0.17, 95% CI:((0.01-5.45) for G

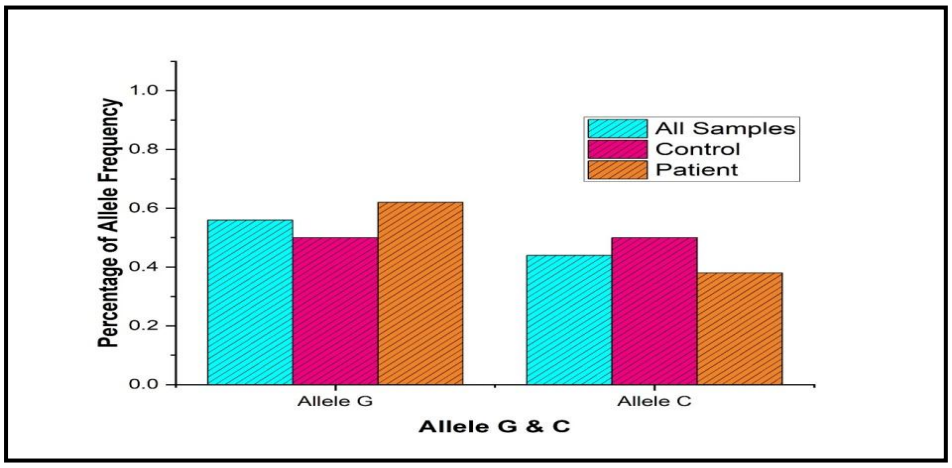


Figure (3-16): Genotype Frequencies of *IL-18* Gene Polymorphisms in diarrheic Cases and Control group

In present findings revealed that there were statistically no significant differences between study groups and control groups for CC genotypes frequencies regarding MBL(polymorphism ($P \leq 0.05$). that there were no statistically no significant differences between diarrheic and control groups for CT and TT genotypes frequencies regarding MBL polymorphism ($P > 0.05$). But, that there were statistically significant differences between cases and control groups for C and T alleles frequencies regarding MBL polymorphism ($P < 0.05$).

The results have an agreement with Jahan *et al*., (2022) they found that frequencies of C alleles at rs1800450 significantly higher in Cryptosporidium than healthy groups, and also found that the CC homozygous genotype was predominant for rs8193046 position, and also they found that the frequency of CC genotype at MBL (rs1800450) polymorphic site was higher in cases than in the controls, suggesting an association with disease susceptibility; whereas the CT genotype was more common in controls than in cases, suggesting a protective association with Cryptosporidium.

Zhou *et al*., (2015); Kothavade,(2011) reported that the genotype TT is associated with resistance to *Cryptosporidium*, while genotype CC is associated with the risk of developing disease.

Conclusions and Recommendations

Conclusions and Recommendations

1) Conclusions:

1) The finding of the current study showed high infection rate of Cryptosporidiosis (25%) in children of Babylon province.
2) The result showed importance of using rapid chromatography test for diagnosis of Cryptosporidiosis
3) There were increasing in immunological marker (IL-18,MBL,TGF-β and INF in patient with C.*parvum.* in compare with control group
4) *IL18* (rs5744247) homologous genotypes was no significantly associated with increasing susceptibility to C.*parvum.* infection
5) *MBL* ((rs1800450) homologous genotypes was no significantly associated with increasing susceptibility to C.*parvum.* infection

2) Recommendations:

1) Using ZNS and rapid test in diagnosis of C.*parvum* in rotiene work in hospital.
2) PCR-RFLP method recommended used to characterize parasite at species and genotype level to achive a better understanding of epidemiology and transmission of infection.
3) Molecular detaction of some virulence factor associated with C.*parvum* pathogenesis
4) Study Other immunological markers in immune pathogenesis of C.*parvum* in children.
5) Direct detection of C.*parvum* by real time PCR.

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جـــــــــامعة بابل
كـــــــــــلية الطب

تعدد الأشكال الجينية لبعض المعايير المناعية في الأشخاص المصابين بطفيلي الأبواغ الخبيئة

رسالة مقدمة إلـــى

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إلى مجلس كلية الطب / جامعة بابل كجزء من متطلبات
نيل شهادة الدكتوراه فلسفة في العلوم / الأحياء المجهرية
الطبية

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أشراف
أ. د هيام خالص المسعودي

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