Brief Overview about Protozoa Contaminating Drinking Water or Protozoal Diseases Transmitted Through Drinking Contaminated Water

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Abstract

Background: The first published report of *Cyclospora* infection in humans can probably be dated to 1979. Ashford described coccidian organisms causing diarrhea in two children and a woman in Papua New Guinea and concluded that they could be a coccidian of the genus *Isospora*. In 1991 and 1992, Ortega and collaborators characterized this controversial organism as a new coccidian species capable of infecting humans and belonging to the genus *Cyclospora*. In reports published in 1993 the name *Cyclospora cayetanensis* (*C. cayetanensis*) was proposed. Cyclospora infection is characterized by anorexia, nausea, flatulence, abdominal cramping, diarrhea, low-grade fever and weight loss. Cyclospora infection induces several pathological alterations of the intestinal epithelial tissue, which include; focal vacuolization at the tips of the villi of the surface epithelium, loss of the brush border, and alteration of cells from a columnar to cuboid shape. In addition, the intestinal architecture may show variable degrees of villous atrophy and crypt hyperplasia, characterized by shortened blunted villi and increased crypt length. Human cryptosporidiosis is caused by the infection with the Apicomplexa protozoan of the genus Cryptosporidium. Cryptosporidium hominis (C. hominis) and Cryptosporidium parvum (C. parvum) are among the commonest species of this genus. Humans are the only natural hosts for C. hominis, while C. parvum infects bovines as well as humans. Some studies indicate that C. parvum exhibits geneticpolymorphism, with one genotype infecting only humans and another infecting humans, cattle, and mice. At present, the infection of Cryptosporidium has been reported in more than 70 countries. The world-wide prevalence of Cryptosporidium infection in man ranged from 0 % to 86%. The highest prevalence was found in Mexico, Nigeria, Bangladesh and Republic of Korea among general residents, patients, school children and healthy population.

Keywords: Cyclospora infection, Cryptosporidium

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Introduction:

Cyclosporiasis

Historical review:

The first published report of *Cyclospora* infection in humans can probably be dated to 1979. **Ashford** described coccidian organisms causing diarrhea in two children and a woman in Papua New Guinea and concluded that they could be a coccidian of the genus *Isospora* **(Ashford, 1979)**. In 1991 and 1992, **Ortega and collaborators** characterized this controversial organism as a new coccidian species capable of infecting humans and belonging to the genus *Cyclospora*. In reports published in 1993 the name *Cyclospora cayetanensis* (*C. cayetanensis*) was proposed **(Ortega *et al.,* 1993**).

Taxonomy: (Ortega *et al.,* 1994; Ortega and Sanchez, 2010).

Cyclospora belongs to the following taxonomy:

- **Subphylum:** Apicomplexa
- **Subclass:** Coccidiasina
- **Family:** Eimeriidae
- **Genus:** Eimeria
- **Species:** Thirteen *Cyclospora* species have been described for vipers, moles, myriapodes, and rodents, including *Cyclospora viperae*, *C. glomericola*, *C. babaulti*, *C. tropidonoti*, *C. anglomurinensis*, *C. caryolytica*, *C. talpae*, *C. ashtabulensis*, *C. megacephaly*, *C. parascalopi*, *C. niniae*, *C. scinci*, and *C. zamenis.*

Morphology:

Cyclospora unsporulated oocysts are spherical and measure about 8–10 µm in diameter. The *Cycolospora* occysts are thin, colorless and bilayered. They contain a polar body and oocyst residuum. It takes more than 1 week for the oocysts to sporulate. Sporulation occurs outside the host. When sporulated, the oocyst has two sporocysts, stieda and sub-stieda bodies. Each sporocyst contains two sporozoites that are ovoidal and lack crystalloid or refractile bodies **(Ortega *et al.,* 1994)**.

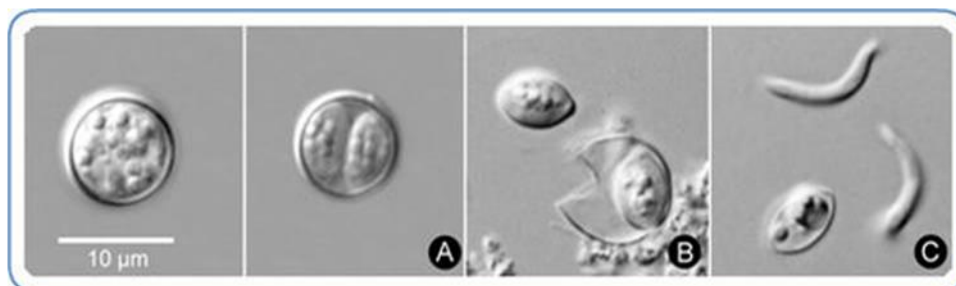

Fig. 1: Morphology of *Cyclospora* and steps of sporulation (A, B and C) (CDC, 2020).

Transmission and life cycle:

- **Transmission:**

The faecal–oral course is the major route of *C. cayetanensis* transmission **(Ortega *et al.,* 1998)**. Direct person-to-person transmission is unlikely. Indirect transmission can occur if an infected person contaminates the environment, the oocysts sporulate under the right conditions, and then contaminated food and water are ingested. Soil is a potentially important source of *C. cayetanensis* infection **(Chacín-Bonilla *et al.,* 2007; Chacín-Bonilla, 2008)**.

Water contaminated with faecal matter may act as another method of transmitting *C. cayetanensis* infection. The source of drinking water has been determined as a risk factor for cyclosporiasis in endemic areas **(Tanduka *et al.,* 2013; Bhandari *et al.,* 2015)**. The oocysts of *C. cayetanensis* have been detected in several types of water including; chlorinated and wastewater in both endemic and non-endemic areas, which suggests the potential spread of the parasite via drinking and recreational water. The water-borne oocysts of *C. cayetanensis* act as a potential source of infection in areas where water and sewage treatment systems are inefficient. Oocysts can pass through physical barriers and are not affected by chlorine and other water disinfectants **(Mansfield and Gajadhar, 2004)**.

Cyclospora cayetanensis can contaminate plant crops via different pathways, including black water used for the irrigation or spraying of crops, contact with contaminated soil, infected food handlers, or hands that have been in contact with contaminated soil **(Dawson, 2005)**. Irrigation of crops by using untreated or poorly treated water is a likely source of contamination for fruits and vegetables **(Mota *et al.,* 2000)**.

- **Life cycle:**

Individuals with *Cyclospora* infection excrete unsporulated oocysts in their faeces. These oocysts require 7 to 15 days to sporulate under ideal conditions (23 to 27°C) and become infectious to a susceptible host. When food or water contaminated with infectious oocysts is ingested by a susceptible host, the oocysts excyst and sporozoites are released to infect epithelial cells of the duodenum and jejunum. Asexual multiplication results in type I and II meronts. The latter differentiate into sexual stages or gametocytes. The macrogametocyte is fertilized by the microgametocyte and produces a zygote. Oocysts are then formed and excreted into the environment as unsporulated oocysts **(Eberhard *et al.,* 2000)**.

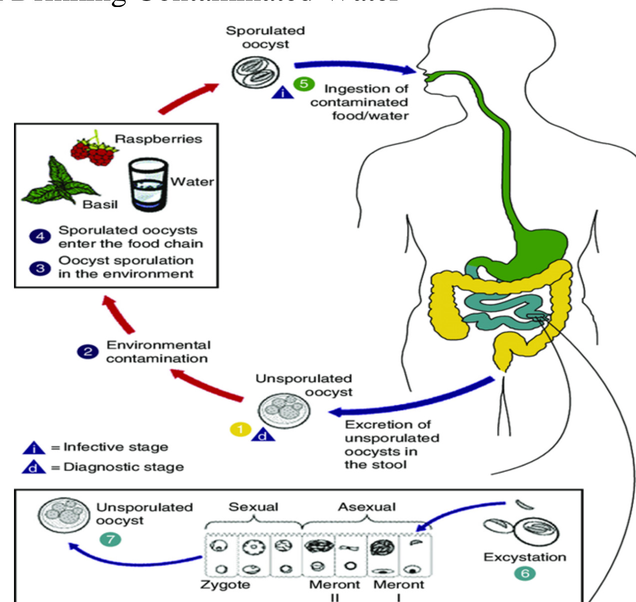

Fig. 2: Life cycle of *Cyclospora* in human host (CDC, 2019).

Clinical picture:

Cyclospora infection is characterized by anorexia, nausea, flatulence, abdominal cramping, diarrhea, low-grade fever and weight loss **(Herwaldt and Ackers, 1997; Fleming *et al.,* 1998)**.

The clinical presentation is somewhat different in areas of endemicity, where asymptomatic infections are more frequent. Nevertheless, younger children have more severe clinical symptoms. Symptoms associated with cyclosporiasis are more severe in HIV/AIDS patients. Moderate weight losses (3.5 kg) were reported for non-AIDS patients **(Shlim *et al.,* 1991; Sifuentes-Osornio *et al.,* 1995)**, whereas losses (7.2 kg) were more severe in AIDS patients **(Shlim *et al.,* 1991)**.

Biliary disease has also been reported after *Cyclospora* infections **(de Gorgolas *et al.,* 2001; Sifuentes-Osornio *et al.,* 1995)**. Calculous cholecystitis was reported for HIV/AIDS. These patients presented with right upper quadrant abdominal pain and elevated alkaline phosphatase levels **(Shlim *et al.,* 1991)**. Yet, the disease resolved after initiation of treatment **(Sifuentes-Osornio *et al.,* 1995; Zar *et al.,* 2001)**. Guillain-Barre syndrome (GBS) and Reiter syndrome have also been reported following *Cyclospora* infection **(Richardson *et al.,* 1998; Connor *et al.,* 2001)**.

Pathological changes:

Cyclospora infection induces several pathological alterations of the intestinal epithelial tissue, which include; focal vacuolization at the tips of the villi of the surface epithelium, loss of the brush border, and alteration of cells from a columnar to cuboid shape. In addition, the intestinal architecture may show variable degrees of villous atrophy and crypt hyperplasia, characterized by shortened blunted villi and increased crypt length **(Connor *et al.,* 1993)**.

In **1996, Deluol** and co-authors described supranuclear intracytoplasmic vacuoles with 6 to 8 comma-shaped merozoite in biopsy samples of *Cyclospora* infected patients. Also, diffuse edema and inflammatory cellular infiltrations of plasma cells, eosinophils and lymphocytes were recorded.

In **1999, Connor *et al.*** described an accumulation of myelin-like material (MLM) between the base and sides of the enterocytes.

Diagnosis:

1. **Direct microscopic examination:**

   Cyclospora protozoan can be identified using light, phase-contrast or bright-field microscopy. The excreted oocysts measure about 8 to 10 µm. They appear as spherical refractile bodies with a central morula **(Ortega *et al.,* 1994)**. Under an epifluorescence microscope, *Cyclospora* oocysts appear fluorescent green when using an excitation filter of 450-490 (Dichroic mirror) DM, while when using a 330-380 DM excitation filter, they give a white-blue appearance **(Dixon *et al.,* 2005)**.

- **Staining methods:**

  Cyclospora oocysts stain variably with the MZN acid-fast stain. Some oocysts stain dark red, whereas others stain pale pink or do not take the stain at all. Safranin stains oocysts uniformly when the faecal smears are heated by microwave. Other stains used in parasite detection, such as Giemsa, Trichrome and Gram-chromotrope stains, do not stain *Cyclospora* oocysts **(Visvesvara *et al.,* 1997)**.

2. **Molecular diagnosis:**

   In 1996, **Relman *et al.*** developed a nested PCR approach, which targeted a segment of the 18S rRNA gene of the parasite. It has been used widely to examine clinical specimens in different settings and during outbreak investigations.

   By using MZN and modified acid-fast trichrome (MAFT) staining methods, the severity of *Cyclospora* infection in symptomatic patients was identified as mild (16%), moderate (24%) and severe (60%). After targeting the 18S rRNA gene sequence using RT-PCR technique, the DNA of *C. cayetanensis* was in 100% of symptomatic and asymptomatic patients and in 20% of controls **(Hussein *et al.,* 2007)**.

3. **Serological diagnosis:**

   Specific IgG and IgM antibodies were tested via ELISA **(Wang *et al.,* 2002)**.

4. **Flowcytometry:**

   FC detection of *C. cayetanensis* was developed on the basis of morphological and autofluorescence properties of oocysts **(Dixon *et al.,* 2005)**. Furthermore, the FC is characterized by being easy, rapid and simple **(Li *et al.,* 2014)**.

   In 2007, **Hussein *et al.*** observed that there is no significant differences between FC and qPCR assays for the detection and quantification of *Cyclospora* oocysts.

Treatment:

1. **Trimethoprim–sulfamethoxazole (TMP–SMX):**

   This drug combination was first used to treat cyclosporiasis in 1993 **(Madico *et al.,* 1993)**. Since 1995, it has been the drug combination of choice **(Hoge *et al.,* 1995a)**. For immune-competent patients, a 7-day course of trimethoprim–sulfamethoxazole (160 and 800 mg, respectively) is given. For immunocompromised patients, the same dosage is recommended, but is given for 10 days **(Guerrant *et al.,* 2001)**.

2. **Ciprofloxacin:**

It is less effective than the (TMP–SMX) combination, but is suitable for patients who are intolerant to sulfonamide drugs **(Verdier *et al.,* 2000)**.

3. **Nitazoxanide:**

Successful treatment of *C. cayetanensis* infections with NTZ has only been reported in a small number of patients **(Diaz *et al.,* 2003)**. However, NTZ is an important treatment option for patients with a sulfa allergy or for patients who did not show any responsiveness to treatment with ciprofloxacin or sulfa **(Zimmer *et al,* 2007)**.

4. **Other agents:**

Norfloxacin, metronidazole, tinidazole and quinacrine have been shown to be ineffective in several studies of human cyclosporiasis **(Escobedo *et al.,* 2009)**.

Prevention and control:

a) **Health education:**

Cyclosporiasis could be prevented by improved personal hygiene and sanitary conditions to avoid faecal-oral transmission from contaminated food, water and soil in endemic areas. Avoiding consumption of raw fresh fruits and raw vegetables is another preventive measure **(Almeria *et al.,* 2019)**.

b) **Disinfection of food:**

Using gamma-irradiation, at 1.0 KGy and higher, was effective in the decontamination of raspberries **(Lee and Lee, 2001)**; hydrostatic pressure (550 MPa at 40 ◦C for 2 min) in raspberries and basil appeared effective **(Kniel *et al.,* 2007)**. Sodium dichloroisocyanurate solution (1g/liter) has been used in disinfection of raw vegetables and fruits which resulted in reduction of parasite numbers **(El Zawawy *et al.,* 2010)**.

Also, magnesium oxide nanoparticles could be used for treatment of *C. cayetanensis* oocysts as they resulted in reductions in sporulation rates compared to untreated oocysts. So, they could be used safely as disinfectant for food and water **(Hussein *et al.,* 2018)**.

c) **Treatment of water:**

Water used for drinking, food preparation and washing of fresh fruits and vegetables, should be boiled or filtered **(Almeria *et al.,* 2019)**.

Cryptosporidiosis

Historical review of *Cryptosporidium* parasite:

Human cryptosporidiosis is caused by the infection with the Apicomplexa protozoan of the genus *Cryptosporidium*. *Cryptosporidium hominis (C. hominis*) and *Cryptosporidium parvum (C. parvum)* are among the commonest species of this genus. Humans are the only natural hosts for *C. hominis,* while *C. parvum* infects bovines as well as humans **(Ramirez *et al.,* 2004)**.

The genus *Cryptosporidium* was first named and reported in 1907, after its discovery in the stomachs of mice by **Tyzzer (Tyzzer, 1907)**. More than 20 species of *Cryptosporidium* were described. The current valid species are *C. muris* (rodents), *C. parvum* (bovines and humans), *C. canis* (dogs), *C. felis* (cats), *C. galli* (birds), *C. hominis* (humans), *C. andersoni* (cattle) and *C.*

baileyi (chicken and some other birds). The only species that affects both humans and other mammals is *C. parvum* **(Barriga, 1997)**. Other morphologically distinct *Cryptosporidium* spp. have been found in reptiles **(Upton *et al.,* 1989)**, birds **(Lindsay and Blagburn, 1990)**, mammals **(Fayer *et al.,* 2000)** and fish **(Alvarez-Pellitero and Sitja-Bobadilla, 2002)**, but have not been named.

Some studies indicate that *C. parvum* exhibits geneticpolymorphism, with one genotype infecting only humans and another infecting humans, cattle, and mice **(Peng *et al.*, 1997)**. After its original identification in animals, the first human *Cryptosporidium* infections were not reported until 1976 **(Meisel *et al.,* 1976; Nime *et al.,* 1976)**. By 1979, only seven cases of human cryptosporidiosis were confirmed **(Lasser *et al.,* 1979; Weisburger *et al.,* 1979)**.

Taxonomy (Manson, 2009):

- **Phylum:** Apicomplexa.
- **Class:** Sporozoasida.
- **Subclass:** Coccidiasina.
- **Order:** Eucoccidiida.
- **Suborder:** Eimeriina.
- **Family:** Cryptosporidiidae.
- **Genus:** *Cryptosporidium*.

Epidemiology:

At present, the infection of *Cryptosporidium* has been reported in more than 70 countries. The world-wide prevalence of *Cryptosporidium* infection in man ranged from 0 % to 86%. The highest prevalence was found in Mexico, Nigeria, Bangladesh and Republic of Korea among general residents, patients, school children and healthy population **(Dong *et al.,* 2020)**.

In developed countries, like in USA, a study done by **Le Chevallier *et al.* (1991)** confirmed the presence of *Cryptosporidium* in 27% of drinking water samples.

In UK, a study done to detect *Cryptosporidium* spp. in drinking water using PCR, found the parasite in 100% of the samples **(Nichols *et al.,* 2003)**.

Morphology of *Cryptosporidium*:

Cryptosporidium oocysts are rounded, measuring 4 to 6μm. Each oocyst contains four sporozoites surrounded by a membrane. Oocysts occur in two forms, one with thin wall which accounts for internal autoinfection to the host and is not believed to survive outside the host, and the other form has a thick wall which pass in stool and is capable of surviving for several weeks in the environment **(Beaver *et al.,* 1984)**.

Using transmission electron microscopy (TEM), analysis of oocysts showed three electron- dense layers and an intermediate electron-translucent layer. An irregular outer electron-dense layer (8.5 ± 0.6 nm) was separated from two underlying dense layers (13.0 ± 0.5 nm and 28.6 ± 1.6 nm, respectively) by a thin electron-translucent middle layer (4.0 ± 0.2 nm). The average thickness of the oocyst wall measured in perpendicular cross sections was 54.1 ± 4.1 nm. The irregular outer

dense layer and translucent middle layer appeared to overlay the complex suture structure. This explains the resistance features to environmental factors by this parasite **(Michael *et al.,* 2010)**.

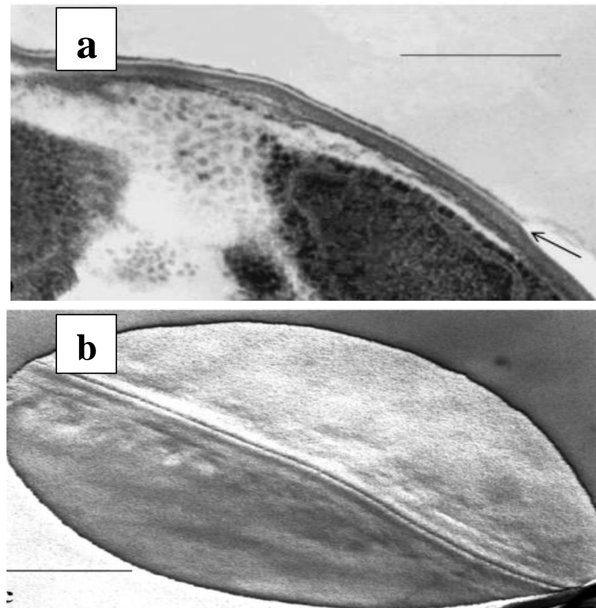

Fig. 3: The morphology of *Cryptosporidium* using transmission electron microscopy (TEM) (Michael *et al.,* 2010).

Fig. 3 (a): TEM showing thin sections of different wall layers of *C.parvum* oocyst, Fig. 3 (b): TEM showing oocyst smooth convex fracture face containing zipper like suture complex in cross section panel.

Transmission and life cycle:

The infection is mainly transmitted through the ingestion of contaminated food and water. Direct contact with faecal matter is an important route of the infection, which could also be transferred from person-to-person or from animal to person. Children, childcare workers and bed-ridden patients are at high risk of the infection **(Pawlowski *et al.,* 2009)**. Infected cattle represent an important source for human infection. Isolates from humans and calves have also been transmitted to kids, puppies, cats and mice **(Current *et al.,* 1983)**.

As few as 30 oocysts can cause infection in an immunocompetent person. Meanwhile, a single oocyst can induce disease in immune compromised individuals **(DuPont *et al.,* 1995)**. Humans, cattle and other domestic animals represent the principal reservoirs for cryptosporidiosis **(Pawlowski *et al.,* 2009)**.

- **Developmental cycle:**

The *Cryptosporidium* parasite normally inhabits the small intestine, where it forms oocysts that are excreted in the host's faeces. Each oocyst contains four small banana-shaped sporozoites. When a susceptible host ingests the oocysts, the sporozoites shed their protective cover and penetrate the epithelial cells of the new host's intestine. Each sporozoite differentiates into the trophozoite stage, which multiplies asexually to form two types of meronts. Type I meronts produce six to eight new banana-shaped parasites (merozoites). Type II meronts form four oval-shaped merozoites, the gametocytes. The gametocytes also invade new intestinal cells, where they differentiate into male

cells (microgametocytes) and female cells (macrogametocytes). The microgametocytes produce numerous filamentous microgametes which fertilize the macrogametocytes, forming a zygote. The zygote matures in the host cell and produces four sporozoites. Most of the mature zygotes (around 80%) develop a tough outer cover and become infective thick walled oocysts. These oocysts are shed by the host in faeces and contaminate the environment. The rest of the zygotes have only a thin outer membrane. Because these thin-walled oocysts are easily ruptured, their sporozoites remain in the intestine re-infecting the same host **(Fayer and Ungar, 1986)**.

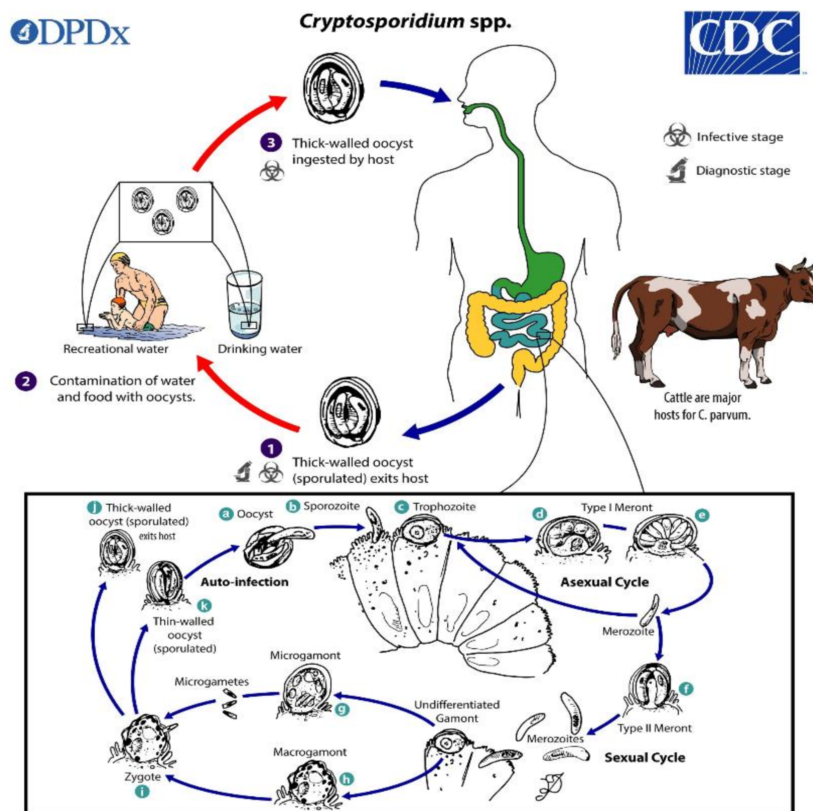

Fig. 4: Life cycle of *Cryptosporidium* in human (CDC, 2019).

Clinical presentation and complications:

Symptomatic cryptosporidiosis typically produces moderate to severe watery diarrhea that begins one or two weeks after infection and generally lasts for 8–20 days. Other symptoms include; abdominal pain, vomiting, nausea, low grade fever, anorexia, dehydration and weight loss **(Tzipori, 1988; Current and Garcia, 1991)**.

Host immune status dramatically alters the course of the disease. Healthy individuals typically experience no symptoms or transient gastroenteritis, which recovers without treatment **(Tzipori, 1988)**. In contrast, immunocompromised patients might suffer fatal cryptosporidiosis. They experience persistent diarrhea of 71 evacuations per day, fluid loss of up to 25 liters and wasting **(Pitlik *et al.,* 1983; Current and Garcia, 1991)**.

The symptoms of watery diarrhea and malabsorption are attributed to sodium malabsorption, chloride secretion and increased intestinal permeability **(Goodgame *et al.,* 1995; Zhang *et al.,* 2000)**.

Disseminated cryptosporidiosis is one of the chief complications of the infection. In addition to the gastrointestinal and respiratory tracts, *Cryptosporidium* infection has been identified in the pancreas **(Kocoshis *et al.,* 1984)**, urinary bladder **(Ditrich *et al.,* 1991)** and hepatobiliary system **(Kutukculer *et al.,* 2003)**.

Cryptosporidium infection has been documented as a major contributors to childhood morbidity and mortality worldwide **(Chalmers and Katzer, 2013; Ryan *et al.,* 2014)**.

<u>Pathology and pathogenesis:</u>

The *Cryptosporidium* parasite predominantly infects the small intestine and colonize the lumen and epithelial surface, but does not invade deeper mucosal layers. The parasite develops within the microvillus layer of intestinal epithelial cells, in the immunocompetent hosts. However, the parasite might spread throughout the gastrointestinal tract and even the respiratory tract in the imunocompromised hosts. Persistent infection is associated with villus atrophy, crypt hyperplasia and variable increases in leucocytes in the lamina propria **(Miyamoto and Eckmann, 2015)**.

After the infection, the parasite alters the function of the intestinal barrier, increasing its permeability, absorption and secretion of fluid and electrolytes. The persistence and outcome of the infection depend on the degree of the immunological status of the patients **(Petry *et al.,* 2010; Kumar *et al.,* 2018)**.

A critical initial step in establishing *Cryptosporidium* infection is the attachment of the parasite to host cells. Most of the proteins involved in parasite attachment are present on the surface of the apical complex (micronemes, rhoptries and dense granules) **(O'Hara and Chen, 2011)**. Two classes of proteins mediate the adhesion to target cells; mucin-like glycoproteins and thrombospondin-related adhesive proteins **(Bouzid *et al.,* 2013)**.

Upon *Cryptosporidium* infection, epithelial cells release proinflammatory cytokines and chemokines to the infection site, which in turn, lead to increased epithelial permeability, impaired intestinal absorption and increased secretion **(Di Genova and Tonelli, 2016)**. Marked mucosal inflammation, decreased absorptive surface and malabsorption contribute to the pathogenesis of *Cryptosporidium*-induced diarrhea **(Di Genova and Tonelli, 2016)**.

It has been reported that pathogenesis of cryptosporidiosis, including invasion of intestinal tissue, is also associated with trogocytosis **(Yoshida *et al.,* 2011)**. *Cryptosporidium* induces cytoskeletal changes that modulate actin reorganization **(O'Hara and Chen, 2011)**. Infection of intestinal and biliary epithelial cells requires host cell actin polymerization and cytoskeletal remodeling **(Chen and LaRusso, 2000)**.

- **Modulation of host cell apoptosis by *Cryptosporidium*:**

Cryptosporidium has been shown to modulate apoptosis of the host cell, inhibiting the process at the trophozoite stage and promoting it at the sporozoite and merozoite stages. This suggests that the parasite is able to regulate gene expression of the host cells **(Mele *et al.,* 2004)**.

The completion of the parasite life cycle requires viable host cells while the induction of apoptosis appears to play a host protective role by limiting parasite numbers and clearing the infection. Gene transcript profiles suggested that host pro-apoptotic gene expression is actively down-regulated early in infection, but is favoured at late stages. It has also been demonstrated that *Cryptosporidium*-infected cells exhibit activated nuclear factor kB (NF-kB), which induces anti-apoptotic mechanisms. The inhibition or induction of epithelial cell apoptosis demonstrates the complex interaction between host and parasite **(Mele *et al.,* 2004; O'Hara and Chen, 2011)**.

- **Causes of cell damage and tissue invasion by *Cryptosporidium*:**

Cellular damage, loss of barrier function, release of lactate dehydrogenase and increased rates of cell death have been documented in colonic epithelial cells infected by *C. parvum* in culture. Several molecules, such as phospholipases, proteases and hemolysins have been proposed as potential causes of cellular damage. Proteases have been implicated in protein degradation, invasion of host tissues and evasion of host immunity. Hemolysin H4 of *C. parvum* has sequence similarity to the hemolysin of enterohemorrhagic *Escherichia coli.* The function of H4 is unknown, but its ability to disrupt cell membranes suggests a role in invasion and disruption of vacuolar membranes, which would allow merozoites to spread to other cells **(Bouzid *et al.,* 2013)**.

Immune response:

Although the outcome and severity of infection is critically dependent on the immune status of the host, the nature of the immune response in cryptosporidiosis, particularly in humans, is poorly understood. Much of what is known about immune responses to *Cryptosporidium* is based on studies conducted in animals, particularly in mice **(Theodos, 1998; Riggs, 2002)**. However, there have been very few studies on immune responses in humans. Most studies in humans have focused on systemic antibody responses with a few addressing cell-mediated responses. Other than faecal antibody responses, there have been no studies on mucosal immune responses in humans. Such studies are challenging to perform in humans since they would involve invasive tissue sampling. Thus, most investigations have been done in tissue culture models utilizing human cell lines or on peripheral blood mononuclear cells (PBMCs). *In vitro* studies in cancer-derived human cell lines have limitations since they may not accurately reflect the complicated dynamics of the mucosal immune system *in vivo* **(Pantenburg *et al.,* 2008)**.

I. **Innate immune responses:**

Recent studies in humans as well as *in vitro* in human cell lines suggest that specific innate immune responses may play a role in resistance to cryptosporidiosis.

- **The intestinal innate immune system:**

The gut epithelium and specialized innate immune cells, are the first line of defence against *C. parvum* infection. Innate immunity restricts the expansion and growth of the parasite and initiates the adaptive response **(Laurent and Lacroix-Lamande, 2017; Ivanova *et al.,* 2019)**. IL-18, a product of the inflammasome complex, is elevated in human epithelial cell lines following *C. parvum* infection **(McDonald *et al.,* 2006)**.

a) Antimicrobial peptides:

With improved understanding of mucosal defense it is evident that the intestinal mucosa not only serves as a physical barrier, but plays an active and important role in innate defenses. Antimicrobial peptides (AMPs) are one of the components of the intestinal mucosal barrier. They are small polypeptides (<100 amino acids) that have antimicrobial and immunomodulatory properties. They are conserved effectors of the innate immune system **(Wehkamp *et al.,* 2007)**. AMPs that are expressed and secreted by human intestinal epithelial cells include α- and β-defensins and cathelicidins. There are six known human β-defensins (HBDs), of which HBD-1 is constitutively expressed and HBD-2, -3 and -4 are expressed during infection or inflammation. Another defense against intracellular pathogens is apoptosis of the host cell. Infection by *C. parvum* initiates apoptosis of infected and surrounding epithelial cells through Fas and Fas-L interactions **(Chen *et al.,* 1999)**. Yet. , after the formation of trophozoite within hours post-infection, apoptosis is inhibited to facilitate growth within the host cell, by the production of anti-apoptotic factors B-cell lymphoma 2 **(Mele *et al.,* 2004)**, osteoprotegerin **(Castellanos-Gonzalez *et al.,* 2008)** and survivin **(Liu *et al.,* 2009)**. Inhibition of apoaptosis of host cell ends by the formation of the sporozoite and merozoite life stages and apoptosis of the host cell is promoted **(Mele *et al.,* 2004; Liu *et al.,* 2009)**.

b) Prostaglandins and substance P:

- **Prostaglandins**:

  They have a major role in pathogenesis and protection against cryptosporidiosis. They produce secretory diarrhea by altering chloride uptake and fluid secretion. They also upregulate mucin production from epithelial cells that interfere with parasite adhesion and protect the host intestinal mucosa from being infected with *C. parvum*. Infection of human intestinal epithelial cells with *C. parvum* leads to activation of prostaglandin H synthase 2 expression and increased production of prostaglandin E2 and F2-α **(Laurent *et al.,* 1997)**.

- **Substance P:**

  It is a neuropeptide that is located in the gastrointestinal tract tract **(Mazumdar and Das, 1992)**. It also plays a role in secretory diarrhea through chloride ion secretion. Jejunal biopsies from AIDS patients with cryptosporidiosis showed increased expression of substance P mRNA **(Robinson *et al.,* 2003)**.

II. <u>Adaptive immune response:</u>

a) Humoral immunity:

Specific anticryptosporidial antibodies act against sporozoites and merozoites (invasive stages), so prevent their attachment to host cells. Cryptosporidiosis in human produces specific anticryptosporidial serum IgG, IgM, IgA and faecal IgA **(Riggs, 2002)**. Their role is evidenced by patients with primary immunodeficiencies, such as selective IgA deficiency, have increased susceptibility to *Cryptosporidium* infection **(Winkelstein *et al.,* 2003; Wolska-Kusnierz *et al.,* 2007)**. Hyperimmune bovine colostrum preparations (derived from cows immunized with *C. parvum*) produce partial efficacy against cryptosporidial infections in healthy human and AIDS

patients, which is another evidence about the role of humoral immune response against cryptosporidiosis.

b) **Cell-mediated immunity:**

The crucial role of cell-mediated immune responses in the protection from, cryptosporidiosis has been well established in both murine and human models **(McDonald, 2000; Gomez Morales and Pozio, 2002)**. Patients with CD4+ counts less than 50 cells/mm^3 are more likely to have a fulminant form of the disease, while those with CD4+ counts of 180 cells/mm^3 or more tend to have less severe self-limited disease **(Huang, 2006)**. The increased susceptibility of AIDS patients to *Cryptosporidium* infection and resolution of cryptosporidiosis following immune reconstitution emphasizes the importance of CD4+ T cells in *Cryptosporidium* infection **(Pozio *et al.,* 1997; Schmidt *et al.,* 2001)**.

Individuals with immunodeficiency disorders that affect T cells, including HIV/AIDS, severe combined immunodeficiency syndrome and CD4 lymphopenia are particularly at risk. In AIDS patients, severe disease occurs when the CD4 cell count is less than 50. The role of T cells has been further confirmed with the introduction of the highly active anti-retroviral therapy (HAART) that had a major impact on the crypto-sporidiosis in AIDS patients by restoring cellular immunity (increase in CD4 counts) and also by a direct effect on the parasite. In contrast, immune deficiencies affecting B cell function have not been associated with severity of cryptosporidiosis **(Bouzid *et al.,* 2013)**.

<u>**Some immunological aspects in cryptosporidiosis:**</u>

- **Interferon gamma (IFN-γ):**

IFN-γ is a major component not only in cell-mediated immunity, but in the early innate immune responses as well **(Lean *et al.,* 2002; Riggs, 2002)**. *In vitro* studies using the human intestinal cell lines have demonstrated that IFN-γ directly prevents *Cryptosporidium* from invading host cells. *C. parvum* infection may downregulate the expression of IFN-γ, thus facilitating cellular invasion **(Choudhry *et al.,* 2002)**. Cellular immune response, by the CD4+ cells, is mediated in large part by the cytokine IFN-γ **(Riggs, 2002)**. In a human volunteer study of adults experimentally infected with *Cryptosporidium*, mucosal IFN-γ production correlated significantly with the presence of pre-existing anti-*Cryptosporidium* antibodies, and reduction in oocyst shedding, suggesting that prior exposure to *Cryptosporidium* may be important in developing protective IFN-γ-mediated memory responses in subsequent infections **(White *et al.,* 2000)**.

- **Toll-like receptor-mediated pathways:**

Toll-like receptors (TLRs) are a family of conserved molecules that play an important role in mediating resistance to a wide array of pathogens by recognizing specific pathogen-associated molecular patterns **(Akira, 2001)**. *Cryptosporidium parvum* induces recruitment of TLR2 and TLR4 to the site of infection leading to activation of downstream effectors in a human cholangiocyte model *in vitro* **(Chen *et al.,* 2005)**. Knockdown of TLR2 and TLR4 expression results in inhibition of downstream signaling pathways and increased *C. parvum* infection **(Chen *et al.,* 2007)**.

- **Mannose-binding lectin (MBL):**

Mannose-binding lectin (MBL) is another highly conserved component of the innate immune system **(Takahashi *et al.,* 2006)**. It is a collagenous lectin found in serum that binds to specific carbohydrate residues on the surface of infectious organisms including *Cryptosporidium*, resulting in activation of the lectin complement pathway in an antibody-dependent manner via mannose-binding lectin-associated serine proteases (MASPs), thereby promoting opsonization and phagocytosis **(Kelly *et al.,* 2000)**.

- **Chemokines:**

They are a family of small 8–10 kDa protein molecules that are produced by epithelial cells. They exert their effects by interacting with G-protein-linked transmembrane chemokine receptors and function as chemo-attractants for inflammatory cells. They are divided into two major groups, CC chemokines with adjacent cysteine residues and CXC chemokines with cysteine groups separated by an amino acid. The CC chemokine ligand (CCL)-5 is a potent chemo-attractant that is upregulated in human intestinal epithelial cells infected with *C. parvum* **(Maillot *et al.,* 2000)**.The role of Chemokine-induced immune cell recruitment is proved by the increased susceptibility to infection of mice deficient in chemokine receptors, in spite of intact immune cell recruitment processes **(Lacroix-Lamande *et al.,* 2008; Lantier *et al.,* 2013)**.

<u>Diagnosis:</u>

- **Detection of *Cryptosporidium* in water and environmental samples:**

1- **Samples processing and concentration:**

Procedures for recovering and identifying *Cryptosporidium* in water samples are highly variable, inefficient and time-consuming. The currently recommended practice involves passing large volumes of water through special filters, followed by centrifugation and purification of the concentrate in a Percoll-sucrose gradient. The samples are next stained with fluorescent antibodies, and, finally, examined microscopically **(Malorny and Hoorfar 2005)**.

2- **Microscopic examination:**

Direct microscopy remains the most widely used method for the detection of *Cryptosporidium* oocysts in water, food and faecal samples **(O'Donoghue, 1995)**. 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 **(Fall *et al.,* 2003)**. Moreover, microscopic examination does not allow the determination of oocyst viability **(Fall *et al.,* 2003)**.

Due to the very low concentration of *Cryptosporidium* spp. oocysts present in water samples, and the lack of available concentration methods, their recovery and detection rely on filtration of the water sample, followed by selective concentration of oocysts. The Safe Drinking Water Act (1996) of the US Environmental Protection Agency (USEPA) evaluates the risk to public health posed by drinking water contaminants, including *Cryptosporidium* spp. To implement the Act, the USEPA developed Method 1623 **(USEPA 2005)** to detect *Cryptosporidium* spp. and *Giardia duodenalis* in raw surface waters.

Water samples are routinely tested using the USEPA method 1623 **(USEPA, 2005)**, which involves filtration of large volumes of water (minimum 10 L) to concentrate suspended particles, followed by selective concentration of *Cryptosporidium* oocysts and *Giardia* cysts using immunomagnetic separation (IMS), and staining with fluorescently labeled monoclonal antibodies. The samples are then examined under fluorescence microscopy **(USEPA, 2005)**.

3- **Direct fluorescent antibody (DFA) assay:**

This method uses a fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* monoclonal antibody (FITC-C-mAb) to recognize surface epitopes on the oocysts. Fluorescing oocysts are then identified using immunofluorescence microscopy **(Jex *et al.,* 2008; Smith and Nichols 2010)**. Even though most commercially available (DFA) kits have exhibited high specificity and sensitivity, none of them can specifically identify *Cryptosporidium* species pathogenic to humans or livestock **(Jex *et al.,* 2008)**.

The majority of the commercially available FITC-C-mAbs are of the immunoglobulin M (IgM) class, which is used regularly for detecting and enumerating *Cryptosporidium* oocysts in environmental or faecal samples **(Smith and Rose 1998)**. Nonetheless, IgG1 antibodies have shown a better diagnostic specificity compared with IgM for the detection of *Cryptosporidium* oocysts in environmental and water samples. This was attributed to the fact that IgG antibodies have a higher affinity to oocyst surface antigens compared to IgM antibodies **(Ferrari *et al.,* 1999; Weir *et al.,* 2000)**.

4- **Indirect immunofluorescence assay:**

This test requires additional steps compared to the DFA assay. The primary antibody in the indirect assay is not conjugated; instead, a second fluorophore- conjugated antibody is used to act against the primary antibody for the detection of *Cryptosporidium* oocysts. The indirect assay involves additional incubation time, making the process longer. Additionally, the use of the indirect assay produces more non-specific binding as a result of using a second antibody **(Iacovski *et al.,* 2004)**.

One of the main problems encountered during using the immuno immunofluorescence assay (IFA) is the presence of inert particles in water samples. Such particles have strong auto-fluorescence that can compete with the signal of labeled cells. Although fluorescent dyes have been suggested to effectively label oocysts in water samples, they have high susceptibility to photo degradation and have broad excitation and emission spectra **(Zhu *et al.,* 2004; Lee *et al.,* 2008)**.

5- **Immunomagnetic separation (IMS):**

This procedure uses magnetic beads coated with an antibody raised against a particular species of *Cryptosporidium*, such as *C. parvum*. This method is often used to isolate oocysts from environmental samples **(Smith and Grimason, 2003)**. The sample is added to antibody conjugated magnetic beads. After applying a magnetic field, oocysts are isolated and studied by other immuno-assays **(Smith and Nichols, 2010)**.

IMS is a valuable technique to increase the sensitivity of oocyst detection in samples where the abundance of oocysts is low, such as surface water and waste water samples **(Robinson *et al.,* 2008;**

Coklin *et al.,* 2011). However, as an antibody-based method, IMS does not provide species or genotype identification of the captured microorganisms. Moreover, IMS is incapable of assessing the infectivity of the captured microorganisms **(Allen *et al.,* 2000; Simmons *et al.,* 2001)**.

6- **Immunochromatographic lateral-flow assay (ICLFA):**

This diagnostic method has several advantages which include; simplicity, cost-effectiveness, no requirement for bulky expensive equipment (e.g., microscopes) or highly trained personnel to analyze the data. Furthermore, it has sensitivities of higher than 97% and specificities of 100% **(Johnston *et al.,* 2003; Shams *et al.,* 2016)**. Despite the high sensitivity of ICLFA kits, many false-positive cases have been reported **(Garcia *et al.,* 2000; El-Moamly and El-Sweify 2012)**. Also, false-negative results were recorded, particularly in cases of light infections **(Garcia *et al.,* 2003)**.

7- **Enzyme-linked immunosorbent assay (ELISA):**

Detection of *C. parvum* in water samples is not reliable using the ELISA measure, as these samples may be complex in their constituents and the number of oocysts may be very low. Therefore, ELISA is mainly used for testing faecal samples **(Esther *et al.,* 2018)**.

8- **Flow cytometry (FC):**

It is an automated technique that has become popular due to its many advantages which include; the analysis of large number of samples in a short time, and its high sensitivity and specificity. In FC, the sample containing the pathogens is suspended in a fluid and directed past a laser beam. The light scattered is picked up by detectors and is characteristic of the cells and their components **(Esther *et al.,* 2018)**. *Cryptosporidium* oocysts were first detected by using this approach **(Vesey *et al.,* 1991)**.

Shams *et al.* (2016) showed that flow cytometric analysis of stool samples from persistently infected mice is 10 times more sensitive than that of the DFA test. Yet, the use of the FC method is not common in the diagnostic parasitology laboratories because of the cost of the instrumentation and the need for technical expertise **(Jex *et al.,* 2008)**.

9- **Nanotechnology-Based Platforms:**

The potential of nanotechnology-based materials has been utilized to improve specificity and sensitivity of other detection methods such as ELISA, PCR, lateral flow assays and immunofluorescent-antibody microscopy **(Il *et al.,* 2013; Checkley *et al.,* 2015)**.

The use of semiconductor quantum dots (QD) nanoparticles for immunofluorescent labeling of *C. parvum* oocysts in water samples has shown to provide excellent and more consistent results than IFA microscopy which results are affected by the presence of inert particles or algal cells with strong auto-fluorescence that can compete with the signal of labeled cells and photo degradation of fluorescent dyes used in this technique **(Zhu *et al.,* 2004; Lee *et al.,* 2008)**. QD labeled oocysts exhibit better photostability after being exposed to continuous ultraviolet (UV) excitation for 5 min. **(Zhu *et al.,* 2004; Lee *et al.,* 2008)**.

Oligonucleotide gold nanoparticles were used in diagnosis of *Cryptosporidium* through molecular detection without the need for amplification of nucleic acids and proteins **(Weigum *et al.,* 2013)**,

such as; electrochemical-based sandwich enzyme-linked immunosensor **(Chinnasamy *et al.,* 2011)** and rapid immunodot blot assay **(Thiruppathiraja *et al.,* 2011)** by using a dual labeled gold nanoparticles (alkaline phosphatase and anti-oocyst monoclonal antibody) functionalized indium tinoxide electrode **(Weigum *et al.,* 2013)**.

10- **Assessment of the viability of *Cryptosporidium* oocyst:**

To date, the viability of *Cryptosporidium* sporozoites has been assessed based on morphology or motility using phase-contrast microscopy **(Woodmansee *et al.,* 1987)** and, more recently, fluorogenic stains **(Brown *et al.,* 1996; Schmidt and Kuhlenschmidt, 2008)**. It was suggested that excysted sporozoites became smaller and swollen in cell-free medium **(King *et al.,* 2009)**. For evaluating the viability of *Cryptosporidium* oocysts in water sample, excystation assay as well as vital dye stains, like PI (propidium iodide) have been used **(Slifko *et al.,* 1997; Fricker and Crabb, 1998)**.

11- **Molecular diagnosis:**

Polymerase chain reaction (PCR) is now gaining acceptability as the method of choice in the detection of *Cryptosporidium* infection due to its higher sensitivity compared to other methods. PCR has the capacity to detect co-enteropathogens (multiplex), to quantify the amount of parasites present in the sample when utilizing quantitative PCR (qPCR), and discriminate between infecting strains. The simultaneous detection in a single assay provides substantial savings in cost and time in identifying the specific infectious agent. Also, it does not require visual determination or antibody binding, thus permitting early and appropriate therapy initiation in a timely and effective manner **(Van Lieshout, 2015)**.

In environmental samples with low amounts of target pathogens, there is a need for more sensitive probe technologies in order to detect very few target DNAs in the presence of large background flora in often PCR inhibitory sample matrices **(Malorny and Hoorfar 2005)**.

Detection of *C. parvum* by PCR was first reported in 1991. Since then, several techniques have been developed to detect and differentiate *Cryptosporidium* at the species and subtype level. Nucleic acid-based methods to screen or detect the presence of *Cryptosporidium* involves isolating its DNA, combining the isolate with PCR mixture, amplifying the target sequence and then detecting the product via gel or fluorescent dyes. Prior to DNA extraction, it is necessary to break and open the oocysts. Various methods have been reported such as freeze-thawing or boiling **(Lindergard *et al.,* 2003)**.

- **Types of PCR techniques:**

The introduction of molecular tools has not only produced new insights into the epidemiology of cryptosporidiosis, but has also led to the identification of common modes of transmission such as zoonotic or anthroponotic transmission via the faecal-oral route or via contaminated food and water **(Xiao and Feng, 2017; Ryan *et al.,* 2018)**.

Several types of PCR techniques were introduced like; Conventional PCR, Nested-PCR, Single-Stranded Conformation Polymorphism PCR (PCRSSCP), DNA probes hybridization, Restriction Fragment Length Polymorphism PCR (PCR-RFLP), multiplex-PCR and real-time PCR **(Xiao,**

2010). These techniques are generally associated with sequencing of amplified fragments for results confirmation and to analyze the genetic diversity between species **(Xiao, 2010; Khurana and Chaudhary, 2018)**. These methods use several genetic markers, among them, the most reported are the small subunit rRNA (SSU) gene that encodes the smaller ribosomal subunit of 18S rRNA; the *cowp* gene, which encodes an oocyst wall protein; the hsp70 gene, which encodes the heat shock proteins; ITS-1 and ITS-2, corresponding to internal transcribed spacer regions of ribosomal DNA; the trap gene (Thrombospondin-related adhesive protein of *Cryptosporidium*), and the gene encoding the GP60 or GP15/40 glycoproteins **(Ryan, 2014; Xiao and Feng, 2017; Khan, 2018)**. Direct DNA sequencing is considered the gold standard for the evaluation of polymorphisms and the genetic variability of a given gene. In addition, it enables analysis of phylogeny in comparative genetic investigations. The first analysis on the comparison between the genetic sequences of *C. hominis* and *C. parvum* was carried out in 1997 by **Morgan** and colleagues.The authors recognized discriminatory regions, including; the TTTTTTTTTTT sequence in *C. hominis* and the TATATTT sequence in *C. parvum*. They observed that the sequences are rich in bases A and T. Years later; these results were confirmed in another phylogenetic study of *Cryptosporidium* **(Xiao and Feng, 2017)**.

1) **Conventional PCR:**

In the conventional PCR technique, a portion of a target gene is amplified into several million detectable copies in a short time. The product is detected after the amplification ends. Conventional PCR-based assays have been used extensively to detect *Cryptosporidium* oocysts and to determine the species and genotypes in various types of environmental and clinical samples **(Smith and Nichols, 2010; Ahmed and Karanis, 2018; Esther *et al.,* 2018)**.

2) **Nested PCR:**

Nested PCR uses two sets of primers, where the first primer set binds to sequences outside of the target DNA, and the product serves as a template for the second pair of primers. Nested PCR increases sensitivity and specificity by decreasing the non-specific binding resulting from the first set of primers. Nested PCR has been used to detect *Cryptosporidium* oocysts in surface water, wastewater and in both human and animal faecal samples **(Mirashemi *et al.,* 2015; Koehler *et al.,* 2017)**.

3) **PCR-restriction fragment length polymorphism (PCR-RFLP):**

This technique determines the species or genotype utilizing specific primer pairs for the selective amplification of the variable region (genetic loci), followed by digestion of the amplified segment with restriction enzymes and analysis by gel electrophoresis. It consists of restriction enzymes or endonucleases to digest PCR products. These enzymes cleave the DNA at a specific site promoting its fragmentation in certain sizes, resulting in different patterns according to the species of the analyzed parasite. Nested PCR is usually followed by RFLP to increase the sensitivity of the assay **(Quintero-Betancourt *et al.,* 2008)**.

This method is used to determine the genotype of *Cryptosporidium* by the detection of many different genetic loci, including SSU rRNA genes, COWP, the 70 kDa heat shock protein

(HSP70), 18S rRNA and the 60 kDa glycoprotein (gp60) gene. Using a multi-locus approach in characterizing *Cryptosporidium* isolates increases accuracy and confidence in the diagnosis. **(Nichols *et al.,* 2003; Xiao *et al.,* 2004).**

4) **Digital PCR (dPCR):**

This method is based on the principle of amplifying a single DNA template from maximally diluted samples, therefore, generating amplicons that are exclusively derived from one template **(Pomari *et al.,* 2019)**. The development of the droplet digital PCR now provides absolute quantification without the need for calibration curves or normalization to reference genes. Unlike qPCR, which produces an exponential signal, dPCR generates linear, digital signals. This techqnique allows quantitative analysis of the PCR product and detection of very rare mutations with unprecedented precision and sensitivity **(Pohl and Shih, 2004)**. Despite the reported precision of dPCR which is superior to that of qPCR, it is a more expensive measure **(Yang *et al.,* 2014)**.

Application of dPCR for the detection and quantification of *Cryptosporidium* in human samples is limited to a single study. In 2014, **Yang *et al.*** have initially applied (dPCR) for the detection and quantification of *Cryptosporidium* oocysts in faeces.

5) **Quantitative PCR:**

Quantitative PCR (qPCR) is widely used in the detection of different types of pathogens in environmental and clinical samples **(Hanabara and Ueda 2016; Singh *et al.* 2016)**. Unlike conventional PCR, qPCR allows real-time monitoring of DNA amplification. Also, qPCR is sensitive enough to detect exceptionally low copy number of the organism **(Jothikumar *et al.,* 2008)**.

qPCR offers rapid, cost-effective, and sensitive identification and quantification of *Cryptosporidium* species. qPCR has been used to detect *C. parvum* and *C. hominis* isolates separately or in a mixture, with no cross-reaction with other genera **(Yang *et al.,* 2013)**. qPCR was also effectively used to detect and distinguish between *C. hominis* and *C. parvum* in human faecal samples by targeting the small subunit (SSU) ribosomal RNA (rRNA) gene **(Hadfield *et al.,* 2011)**. Moreover, qPCR was used to detect *C. hominis* and *C. parvum* in environmental and sewage samples through targeting the *Cryptosporidium* oocyst wall protein (COWP) gene **(Guy *et al.,* 2005; Anceno *et al.,* 2007)**.

6) **Multiplex qPCR assays:**

The multiplex PCR procedure was developed to detect multiple parasites simultaneously **(Nurminen *et al.,* 2015)**. These assays have the ability to detect *Cryptosporidium* along with other parasites **(Ryan *et al.,* 2017)**. The sensitivity of this technique ranged from 95 to 100% and specificity from 99.6 to 100% **(Ryan *et al.,* 2017)**.

12- **Fluorescence in situ hybridization (FISH):**

This method utilizes fluorescently labeled complementary DNA oligonucleotide probes that target-specific sequences of cellular rRNA for direct identification of microorganisms. The rRNA is used because it is a naturally amplified target for hybridization probes due to its high copy

number. rRNA targeted oligonucleotide probes provide an advantage because they can be designed to various degrees of specificity, reaching from genus to species and even subspecies level. Fluorescently labeled rRNA targeted probes applied in FISH have emerged as a powerful tool for the detection of microorganisms in a wide range of faecal or environmental samples **(Dorsch and Veal, 2003; Alagappan *et al.,* 2008)**.

- **Detection of *Cryptosporidium* infection in stool samples:**

I) Microscopic examination:

Diagnosis of cryptosporidiosis is usually conducted microscopically by identifying the oocysts (4 to 6 μm) in the stool samples of the infected subjects **(Ahmed and Karanis, 2018; Khurana and Chaudhary, 2018)**.

Since the detection of *Cryptosporidium* oocysts can be difficult, three faecal samples collected on separate days should be microscopically examined for the detection of oocysts before excluding a *Cryptosporidium* infection in subjects with severe diarrhea **(Omoruyi *et al.,* 2014; Khurana and Chaudhary, 2018)**. The use of microscopy as a diagnostic tool for *Cryptosporidium* remains to be the most common technique to confirm active infection. It is the only technique that can detect the oocysts. Despite being a low-cost practical measure, direct microscopic examination has a low sensitivity (≤ 30%). Moreover, accurate diagnosis of cryptosporidiosis using this technique is largely dependent on the availability of experienced personnel.

1. **Direct smear (Wet mount) measure:**

Diagnosis of *Cryptosporidium* oocysts in wet preparations is difficult to achieve because they are indistinguishable from yeast cell and fungal spores. Special optical systems such as phase contrast can help in their identification by showing the parasites as round, refractile structures with internal morphological features **(Kar *et al.,* 2011)**.

2. **Concentration techniques:**

Several concentration methods were developed to improve the detection of *Cryptosporidium* parasite in stool samples. The commonly used techniques include; Sheather's flotation (SF), normal saline sedimentation staining (NSSS), and Sheather's flotation sedimentation staining (SFSS) which has sensitivity and specificity values of 82.6 % and 98.8 %, respectively **(Paul *et al.,* 2009)**.

3. **Staining methods:**

a) **Acid-fast stains**

The oocysts of *Cryptosporidium* can be also observed by acid-fast (modified Ziehl–Neelsen method) or phenol–auramine staining, where the oocysts stain red and bright yellow, respectively **(Omoruyi *et al.,* 2014; Khurana and Chaudhary, 2018)**.

The most widely used acid-fast stains are Kinyoun **(Ma and Soave, 1983)** and modified Ziehl-Neelsen **(Henriksen and Pohlenz, 1981)**, which stain *Cryptosporidium* oocysts red on a counterstained background. The Ziehl–Neelsen stain is the most popular direct stain used in microbiology laboratories to stain *Cryptosporidium* oocysts **(Henriksen and Pohlenz, 1981)**. Yet, when using such staining procedures, the oocysts may appear as "ghost" cells **(Vanathy *et al.,***

2017). Additionally, these methods cannot distinguish between different *Cryptosporidium* species **(Elsafi *et al.,* 2013; McHardy *et al.,* 2014)**.

Although the oocysts of *Cryptosporidium* are half the size of those of *Cyclospora cayetanensis* (about 4–5 µm in diameter vs. 9–10 µm in diameter), much attention should be given when evaluating stool samples since the oocysts of both parasites are acid-fast **(Pacheco *et al.,* 2013; Ahmed and Karanis, 2018)**.

In stool samples, dimethyl sulfoxide can be incorporated in the acid-fast procedure to produce pink oocysts to distinguish them from the stool debris **(Vohra *et al.,* 2012)**.

Most laboratories use a modified acid-fast or Safranin-Methylene blue stain (SMB) to detect oocysts. *Cryptosporidium* oocysts appear as spherical, orange to pink structures with 5 µm diameter and sporozoites within the oocyst stain slightly darker **(Shams *et al.,* 2016)**. SMB is characterized by its rapidness and simplicity. It has the ability to differentiate between the yeasts and oocysts **(Rekha *et al.,* 2016; Shams *et al.,* 2016)**. On the other hand, the main disadvantages of this stain are the requirement for acid-methanol treatment before the addition of safranin and strong heating during the safranin addition **(AL-Ezzy and Khadim, 2021; AL-Ezzy *et al.,* 2021)**.

b) Phenol–auramine stain:

Phenol-auramine staining is a very reliable measure with a relatively high sensitivity of 92.1 %. Yet, it requires a fluorescence microscope with appropriate filters. Using this stain, *Cryptosporidium* oocysts appear bright yellow on a red background. Strict morphologic criteria must be used in the diagnosis, so as to avoid confusion with other coccidian oocysts, such as those of *Cyclospora* spp. which are significantly larger (8-10 µm) **(Casemore, 1991; Chalmers *et al.,* 2011)**.

c) Negative stains:

Nigrosin **(Pojola, 1984)**, merbromine **(Chichino *et al.,* 1991)**, light green, and malachite green **(Elliot *et al.,* 1999)** are rapid negative stains for background yeast and bacteria, but not for oocysts. The malachite green method is a practical and safe method for detecting *Cryptosporidium* oocysts in stool specimens than others. Negative staining techniques are faster but less sensitive than permanent staining methods **(Garcia, 2001)**. Uncertain or confusingly positive samples can be confirmed, using the modified Ziehl-Neelsen staining or other more traditional techniques **(Potters *et al.,* 2010)**.

II) Immunodiagnosis:

Immunological methods can be based on either antigen detection or antibody detection. These methods have reported to yield good sensitivity and specificity in the range of 93%–100% **(Graczyk *et al.,* 1996; Chan *et al.,* 2000)**. While antigen detection tests are useful for diagnosing acute infection, antibody detection tests are useful in sero epidemiological surveys.

a- Antibody detection methods:

Different techniques have been performed to demonstrate anti-cryptosporidial antibodies. The most widely used are enzyme immunoassay (EIA) and indirect immunofluorescence assay (IIF). Serological screening using IIF or EIA has shown that the specific antibodies of all major immunoglobulin classes persist for various periods of time in the sera of convalescent patients

(Petry, 2000). EIA tests were mainly applied in epidemiological studies to denote the seroprevalence in selected populations **(Zu *et al.,* 1994; Petry, 1998)**.

b- Antigen detection tests in faecal samples "Coproantigen" detection:

The laboratory diagnosis of cryptosporidiosis in watery or mushy stools using different techniques such as the enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT), have good sensitivity and specificity for the detection of *Cryptosporidium* antigens **(Christy *et al.,* 2012; Hawash, 2014)**.

Advantages that some coproantigen detection assays have over microscopy is that they can detect prepatent infections in animals not excreting oocysts in faeces **(Smith and Nichols, 2007)**. Moreover, these tests can be employed for the rapid and cost-effective screening of large numbers of faecal samples **(Garcia *et al.,* 2003; Johnston *et al.,* 2003)**. However, like other immunological methods, they do not allow the species or genotype differentiation of *Cryptosporidium* species.

➢ **Immuno-chromatographic Tests (ICTs):**

The development of rapid antigen point of care (POC) tests for the diagnosis of *Cryptosporidium* addresses the challenges posed by resource-limited settings and the patient's need for immediate intervention. The majority of these test kits that have been commercially available over the past decade utilizing the principle of (ICT) in the form of test strips **(Zaglool *et al.,* 2013; Yansouni *et al.,* 2014)**.

Generally, these rapid tests are based on the principle that *Cryptosporidium* antigen-specific antibodies are bound to a membrane and uses capillary flow to move the labeled antibody-antigen complex **(El-Moamly *et al.,* 2012)**. Despite its increasing commercial availability and simplicity, a few clinical laboratories in resource-limited regions apply ICT rapid testing. These laboratories still consider microscopy as the standard diagnostic tool. **(Chalmers *et al.,* 2010; Manser *et al.,* 2014)**.

➢ **Enzyme-linked immunosorbent assay (ELISA):**

ELISA test is widely used in the detection of *C. parvum* in stool samples obtained from humans and animals **(Elgun and Koltas, 2011)**.

In ELISA an antibody against *C. parvum* (or any targeted parasite) is immobilized on the surface of a plate, and then the antigen (i.e., the sample) is introduced. Secondary antibodies and enzymes are added to allow for detection by absorbance spectroscopy. The procedure does not require the concentration of oocysts from faecal samples before processing. Therefore, ELISA allows for rapid examination of large numbers of stool samples **(Ghoshal *et al.,* 2018)**. It has been reported that the sensitivity of ELISA is 10 times higher than acid-fast staining **(Shams *et al.,* 2016; Ghoshal *et al.,* 2018)**.

➢ **Immunofluorescence assays (IFA):**

This method uses polyclonal or monoclonal antibodies against specific oocysts epitopes. This test has some advantages over the traditional acid-fast stain. It is easier to read for inexperienced laboratory personnel. Besides, the screening is more rapid to read at low magnification than conventionally stained smears **(Petry, 2000)**.

In addition, it allows the identification of *Cryptosporidium* oocysts and *Giardia intestinalis* cysts simultaneously on the basis of their size and morphology. IFA has a high sensitivity which ranges between 95% and 100%. Its specificity is 100%. Yet the main disadvantages of the (IFA) test are the high cost of the kit compared to acid-fast stain, and the need of a fluorescence microscope **(Petry, 2000)**.

III) Molecular diagnosis:

PCR techniques represent an excellent alternative to the conventional diagnosis of *Cryptosporidium* spp. in faecal and environmental specimens. Biomolecular methods are more sensitive and specific than direct microscopic diagnostic techniques. But, they require more time and expertise as well as being more expensive **(Morgan *et al.,* 1998)**. In addition, faecal samples contain various PCR inhibitors, such as bile components, which may interfere with DNA extraction. PCR on formalin-fixed stools is less sensitive than on fresh samples **(Morgan *et al.,* 1998)**.

<u>Treatment:</u>

1- **Paromomycin:**

Paromomycin was the first drug tested in humans for treating cryptosporidial diarrhea. It is an aminoglycoside antibiotic, which is poorly absorbed from the gut epithelium. Yet, it can be absorbed in small quantities across the apical membrane bounding the extracytoplasmic parasite. Its mechanism of action is targeting the ribosome, where it disrupts protein synthesis **(Theodos *et al.,* 1998)**.

2- **Nitazoxanide (NTZ):**

NTZ [2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide] is a broad-spectrum anti-parasitic compound. It acts against a wide range of cestodes, nematodes and protozoa with notable effects on apicomplexan parasites, particularly, *C. parvum* **(Esposito *et al.,* 2005)**.

NTZ and its two metabolites, tizoxanide (TZ) and tizoxanide-glucuronide (TZglu) inhibited the growth of *C. parvum* sporozoites and oocysts at concentrations lower than 10 lg/ml **(Theodos *et al.,* 1998)**. NTZ inhibits pyruvate-ferredoxin oxidoreductase (PFOR) enzyme which is necessary for anaerobic metabolism, without the formation of free radicals or deterioration of the host cells **(Dupouy-Camet, 2004)**.

The drug was tested against the three different stages of the developmental cycle of the parasite (asexual, sexual and the completely developed parasite). It elicited notable inhibitory effects on the complete development of the parasite **(Giacometti *et al.,* 1999; Giacometti *et al.,* 2000)**.

NTZ and TZ account for activity in the intestine, while in extra-intestinal locations, like (the biliary tract), TZglu is the most important.High concentrations of TZglu are excreted in bile and it is believed to be responsible for the activity of the compound in the cholangitis produced by *C. parvum* in disseminated cryptosporidiosis in immunocompromised subjects **(Abubakar, 2007)**.

Two double-blind placebo controlled studies of NTZ were carried out in the treatment of cryptosporidial diarrhea in adult AIDS patients. The result showed a statistical significance ($P < 0.05$) in both clinical resolution of diarrhea and suppression of the oocyst shedding **(Rossignol, 1998; 2006)**.

NTZ is effective in treating cryptosporidiosis in immunocompetent patients as shown in three double blind placebo-controlled clinical studies carried out in more than 140 immunocompetent adults and 150 immunocompetent children from Egypt and Zambia. Clinical and parasitological cures were recorded following a three-day course of treatment in adults and children **(Rossignol, 2001; Amadi, 2002)**.

3- **Highly active antiretroviral therapy (HAART):**

The use of highly active antiretroviral therapy (HAART) in patients with AIDS has dramatically reduced the prevalence and severity of *Cryptosporidium* infection. This effect has been attributed to the recovery of the host immune response. Some studies using protease inhibitors such as ritonavir, saquinavir and indinavir claim a drastic reduction of *Cryptosporidium* infection both *in vivo* and *in vitro* **(Hommer *et al.,* 2003; Mele *et al.,* 2003)**.

4- **Azithromycin and roxithromycin:**

These two antibiotic agents were the subject of some clinical investigations in the treatment of cryptosporidial diarrhea in AIDS patients. Treatment with azithromycin has shown a weak efficacy against *C. parvum* infection **(Giacometti *et al.,* 1999)**. Clinical trials have shown that these antibiotic agents did not produce a reduction in the oocyst shedding, but some transient reduction of diarrhea, which was most likely due to the cyclical nature of the disease **(Dionisio *et al.,* 1998; Kadappu *et al.,* 2002)**.

5- **Antibody therapy:**

The close relationship between *Cryptosporidium* infection and host immune response has led to the emergence of antibody therapy. Some investigators hypothesized that immunoglobulins in bovine colostrum immunized against particular pathogens might provide protection against some specific pathogens such as *Cryptosporidium* spp. Unfortunately, bovine colostrum supplements vary widely in terms of their specific constituents. Results obtained from bovine colostrum antibody therapy are contradictory. **Riggs *et al.* (2002)** hypothesized that targeting the apicalcomplex and surface antigens could passively immunize against cryptosporidiosis. They tried to evaluate the effect of neutralizing monoclonal antibodies against persistent infection in experimentally infected mice. Their results showed that the antibody therapy has exhibited a highly significant efficacy in reducing, but not eliminating, persistent *Cryptosporidium* infection.

Prevention and control:

1) **Environmental control:**

- **Water treatment:**

a- **Physical and chemical treatment:**

Water treatment includes coagulation/flocculation, sedimentation, filtration and disinfection methods which may be insufficient to clear contamination. Because of the small size of *Cryptosporidium* (4–6µ), makes oocysts more difficult to filter than larger protozoa such as *Giardia* and *Cyclospora*. Therefore, filters with pore sizes less than 1 micron are necessary for effective filtration **(Betancourt and Rose, 2004)**.

Solar and UV-light disinfection are effective tools in reducing oocyst viability and infectivity **(King and Monis, 2007; Mendez-Hermida *et al.,* 2007)**. Solar photocatalytic disinfection using titanium oxide coated plastic inserts improves oocyst inactivation in water **(Mendez-Hermida *et al.,* 2007)**. Chlorination also, has been found to be insufficient to clear *Cryptosporidium* spp. **(Betancourt and Rose, 2004)**.

b- **Mechanical treatment:**

Constructed wetlands are a cost-effective way to treat wastewater. These wetlands remove and biodegrade pathogens like *Cryptosporidium* through sedimentation, filtration, predation and UV inactivation **(Graczyk *et al.,* 2009)**.

2) **Health education:**

For an individual, prevention of cryptosporidiosis consists of avoiding the ingestion of raw foods or water that may be contaminated with human or animal faeces and avoiding contact with faeces. Cooking high-risk foods and washing hands carefully before eating should also reduce the danger of infection. People should avoid immersion in water containing effluents from sewage systems or cattle farms **(Juraneck, 1995)**.

3) **Veterinary control:**

Passive immunization (by receiving colostrum from hyperimmunized cows) **(Fayer *et al.,* 1989)** and active immunization (by vaccinating calves after birth with a killed or attenuated preparation of *C. parvum*), protect against oocyst shedding and diarrhea caused by *C. parvum* **(Harp and Goff, 1995)**.

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