# Chapter one

## Introduction and literature review

### 1.1 Introduction:

Human cryptosporidiosis is caused by infection with apicomplexan protozoans of the genus *Cryptosporidium*. Human illness was formerly thought to be caused by a single species, but molecular studies have demonstrated that it is caused by at least 15 different species. Among the more common species are *Cryptosporidium hominis*, for which humans are the only natural host, and *C.parvum*, which infects bovines as well as humans (Checkly *et al.*, 2015).

Outbreaks of cryptosporidiosis should be detected by vigilant observation for increased case numbers at primary and public health care levels. The genus *Cryptosporidium* consists of a group of protozoan parasites within the protist subphylum Apicomplexa. There are more than 26 known *Cryptosporidium* species, as recognized by host specificity, morphology, and molecular biology studies (Bouzid *et al.*, 2013). Besides humans, the parasite can infect many other species of animals, such as mammals, birds, and reptiles, and is pathogenic to immunocompetent and immunocompromised hosts. *Cryptosporidium* species are able to infect and reproduce in the epithelial cell lining of the gastrointestinal and respiratory tracts without causing cytopathic effects. *C hominis* and *C parvum* cause most human infections. In immunocompetent individuals, the organisms are primarily localized to the distal small intestines, whereas in immunocompromised hosts, the parasites have been identified throughout the gut, biliary tract, and respiratory tract (Checkly *et al.*, 2015).

## 1.2 Literature review:

### 1.2.1 Historical perspective:

*Cryptosporidium* was first described in the laboratory mouse by Tyzzer in 1907 (Tyzzer, 1907), the medical and veterinary significance of this protozoan was not fully appreciated for another 70 years. The early history of *Cryptosporidium* is extensively documented in several review articles and book chapters published recently (O'Donoghue, 1995). Like other enteric coccidia of vertebrates, *Cryptosporidium* has a monoxenous life cycle that is primarily completed within the gastrointestinal tract of a single host. However, many unique features that distinguish *Cryptosporidium* from other coccidia, of which the relative lack of host and organ specificity, resistance to antimicrobial agents, ability for autoinfection and the curious location it occupies within the host cell membrane are the most obvious (Tzipor, 1983) cryptosporidiosis became recognized (Tzipor, 1998). From 1983 onwards, with the onset of the acquired immune deficiency syndrome (AIDS) epidemic, *Cryptosporidium* emerged as a life-threatening disease in this subpopulation. In 1993, it reached the public domain when it became widely recognized as the most serious, and difficult to control, cause of waterborne-related diarrhoea. The first glimpse of the seriousness of *Cryptosporidium* in mammals, mainly in calves, was provided in the late 1970s (Morin *et al.,* 1976). Until then, *Cryptosporidium* was mostly identified histologically in infected gut sections or in biopsy specimens and was considered to be an opportunistic protozoan that caused a few or no symptoms (Nime *et al.*, 1976).

### 1.2.2 Taxonomy:

*Cryptosporidium parvum* (*C. parvum*) belongs to the phylum apicomplexa (which possess an apical complex), class sporozoasida (which reproduce by asexual and sexual cycles, with oocyst formation), subclass coccidiasina (with a life cycle involving merogony, gametogeny andsporogeny), order eucoccidiida (in which schizogony occurs), suborder eimeriina (in which independent micro-

and macrogamy develop), family cryptosporiidae (contain four naked sporozoites within oocysts but with no sporocyst) (Levine *et al.*, 1985).

### 1.2.3 Morphology:

The oocyst of *C.parvum* is small, measuring approximately in a range of 4 to 6 μm (figure 1.1) and when mature the oocyst contains four sporozoites that may or may not be visible. A thick double-layered wall protects the oocyst from environmental stresses and no sporocysts are visible but darkly stained granules may be present (Ridley, 2012).

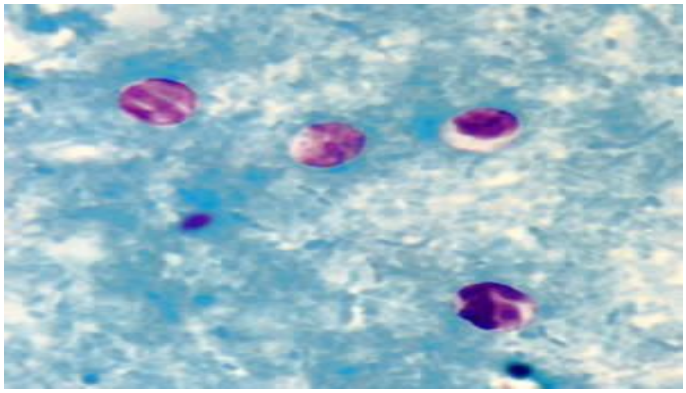

***Figure (1.1):*** *Cryptosporidium Sp.* oocysts stained withmodified acid-fast stain (CDC, 2013-a).

### 1.2.4 Life cycle:

*Cryptosporidium* does not multiply outside of the host (Checkley *et al.*, 2015). *Cryptosporidium* can complete its life cycle (figure1.2) within a single host, including its asexual (merogony) and sexual (sporogony) reproductive cycles. Infection is initiated by ingestion of oocysts, which are activated in the stomach and upper intestines to release 4 infective sporozoites. These motile sporozoites bind to the receptors on the surface of the intestinal epithelial cells and are ingested into a parasite phorous vacuole near the surface of the epithelial cell, separated from the cytoplasm by a dense layer. Once inside the epithelial cell, the parasite goes through a series of sexual and asexual multiplication steps leading to the production of oocysts. Two morphologic forms of the oocysts

have been described: thin-walled oocysts (asexual stage) excyst within the same host (causing self-infection), whereas the thick-walled oocysts (sexual stage) are shed into the environment. Oocyst shedding can continue for weeks after a patient experiences clinical improvement (Bouzid*et al.*, 2013).

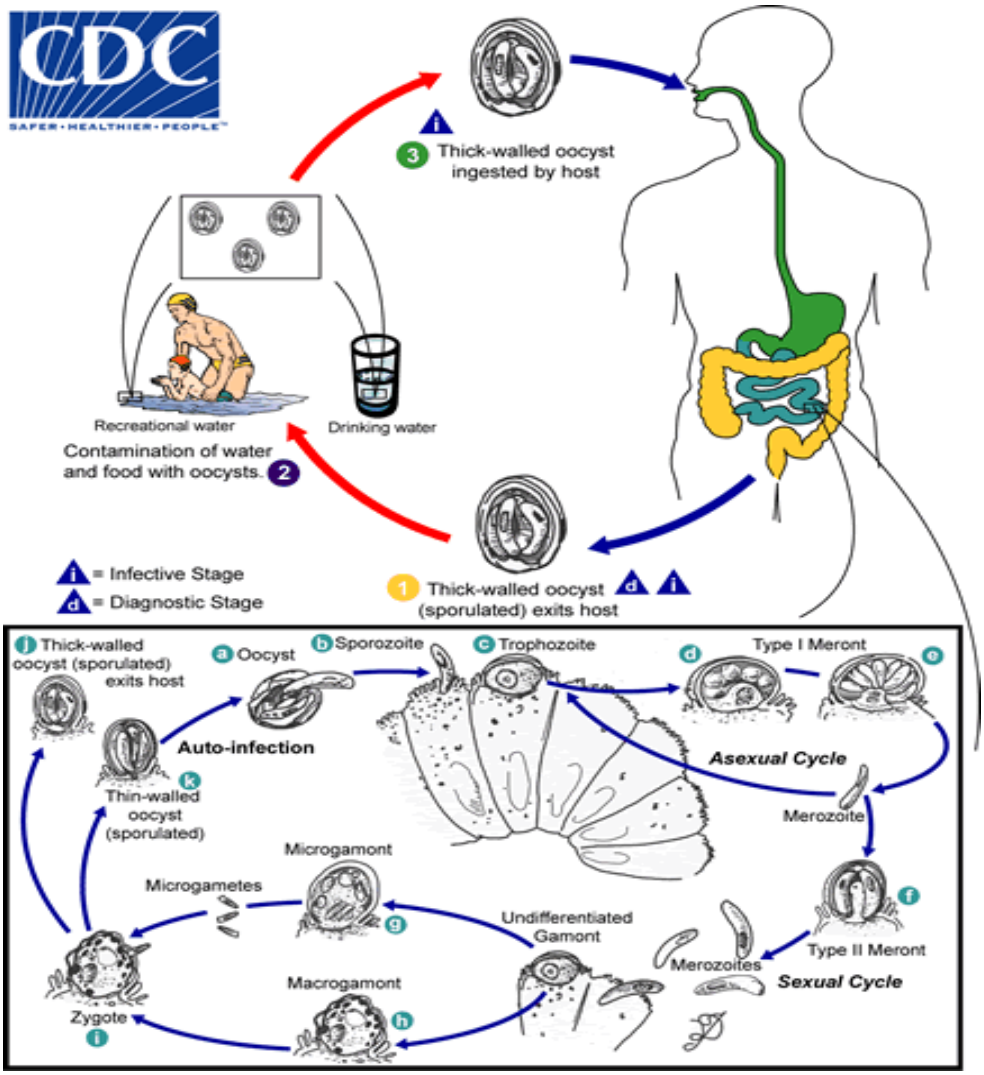


**Figure (1.2): Life cycle of *Cryptosporidium*** (CDC-b, 2013).

### 1.2.5 Characteristics of *Cryptosporidium*:

Many aspects of the biology and the nature of *Cryptosporidium* interaction with the host cell remain unclear. At least six *Cryptosporidium* species are currently

recognized, based largely on genotyping and a limited number of transmission experiments. These six species include two mammalian (C. *parvum* and C. *muris*) and two avian (C. *meleagridis* and C. *baileyi*) species, a species seen in reptiles (C. *serpentis*) and a species seen in fish (C. *nasorum*) (Fayer *et al.*,1998). Other less clearly defined species include those from guinea pigs (C. *wrairii*), cats (C. *felis*), dogs (C. *canis*) and marsupials (unnamed). The current differentiation of isolates into valid species is based on their genetic profile and the species of the host from which they were originally isolated. There are serious limitations associated with speciation of *Cryptosporidium* based on the above criteria (Tzipori and Ward, 2002). For instance, since C. *meleagridis*, C. *canis* and C. *felis* were subsequently also observed in humans with cryptosporidiosis, these species would most likely have been named in relation to their human hosts rather than their current respective animal hosts. While these criteria, after some rigorous testing, are appropriate to other microorganisms, including other members of the apicomplexa, they currently are premature with regard to *Cryptosporidium*. The reasons for this are several, including the ubiquitous nature of *Cryptosporidium* (at least C. *parvum*, the most extensively investigated species) and the technical barriers associated with propagation and maintenance. While there appear to be clear genetic and pathogenic differences among isolates of *Cryptosporidium* obtained from different classes of vertebrates, from different species there in or even from the same species of animals, these differences are inadequately characterized, particularly with respect to infectivity for various animals and the degree of virulence (Tzipori and Ward, 2002). The broader epidemiological and epizootiological implications of studies conducted on a few isolates, often using one animal species, carry major risks to public health. These can lead to a complacent view that humans are safe from exposure to *Cryptosporidium* that originates from non-mammalian vertebrates (Tzipori and Ward, 2002). Recent reports of human infections with C. *meleagridisare* a good example. On the

other hand, the existence of diversity within C. *parvum* casts further doubts on speciation based on transmission experiments as well. C. *parvum* isolates, even when obtained from the same host (e.g. humans), display diversity in the range of mammalian species they infect. They have consequently been divided into genotype 1, found exclusively in humans and a few other primates, and genotype 2, found in most, if not all, mammals, including humans. Given such differences in genetic and infectivity profiles, they qualify to be considered as two distinct species. The application of restriction fragment length polymorphism helped identify these two genotypes within C.*parvum*. This segregation was confirmed by a multilocus analysis based on polymorphisms (in microsatellites) located at five unlinked loci in the genome of C.*parvum*, applied to isolates from a variety of hosts and geographic origin (Widmer, 1998, Peng *et al*., 1997).

Demonstration of significantly greater sequence homologies within species than among species at multiple unlinked loci, in isolates obtained from a large and diverse range of host species and locations, could provide, in the future, a solid genetic basis for elucidating the taxonomy of the genus *Cryptosporidium*. Confirmatory evidence would require an experimental system to test whether putative species are reproductive entities. The few C.*parvum* isolates that are currently being used in laboratory investigations are maintained by passage through animals, mostly calves. There are only a handful of C.*parvum* isolates that are widely used and partially characterized genetically and phenol typically (Tzipori and Ward, 2002).

**1.2.6 Disease transmission:**

The infective form from animals and humans is the resistant oocyst that is passed in feces. Ingestion of contaminated food and water as well as person-to-person transmission are the main routes of infection. Municipal water systems that become contaminated with fecal material have also been implicated in widespread outbreaks of diarrhea in recent years. Municipal water supplies may

sometimes become contaminated with many organisms, including *E*. *Dispar* and other organismssuch as coccidia, that are not filtered out in the treatment plants. Standard chlorination levels by water treatmentplants do not control this organism and levels of up totimes what is normal are necessary in water suppliesto destroy the organisms. Most of the coccidian infections in man are zoonoses, a term indicating that a distinct possibility of contracting the disease from infectious animals exists. Immunocompetent individuals usually suffer from mild, self-limiting infections that are sometimes not even noticed to an appreciable extent, but for those with an immunocompromised defense system, the symptoms may progress to a much more severe condition (Ridley, 2012).

### 1.2.7 Aetiology and pathology:

Exposure to *C. parvum* oocysts, either directly through contact with infected humans or animals, or indirectly by drinking or eating food washed with contaminated water, may lead to acute diarrhea. *C. parvum* causes an acute, self-limiting infection and diarrheal disease in immunocompetent people, in whom the onset may be rapid (3-7 days)depending on a combination of host (age, presence of maternal antibodies or previous exposure, and infectious dose) and parasite (origin and age of oocysts, and species/genotype) factors (Tzipori and Ward, 2002). Infection presumably begins in the small intestine, where the emerging sporozoites infect enterocytes, and after amplification, endogenous forms spread throughout the epithelial surfaces of both villi and crypts. The infection may spread throughout the gut, which includes the gastric mucosa and the small and the large intestines, or it may remain localized in segments of the small and/or large intestine. The extent of spread and the sites involved determine whether the infection is clinical or subclinical as well as the overall intensity of the disease (Tzipori and Ward, 2002).Generally, the more proximal in the small intestine the location is the more severe and watery is the manifestation of diarrhea. Infections confined to the distal ileum and/or the

large bowel can often result in intermittent diarrhea or even be asymptomatic. Infections may often involve the pyloric region of the gastric mucosa. Parasite forms displace the microvillus border and eventually lead to the loss of the mature surface epithelium. The rapid loss of surface epithelium causes marked shortening and fusion of the villi and lengthening of the crypts due to acceleration of cell division to compensate for the loss of cells. The combined loss of microvillus border and villus height diminishes the absorptive intestinal surface and reduces uptake of fluids, electrolytes and nutrients from the gut lumen. The loss of the microvillus border in the proximal small intestine leads, in addition, to loss of membrane-bound digestive enzymes, whose role in children, in particular is crucial, and contributes to marked maldigestion in addition to the malabsorption (Theodos *et al.,* 1997). Diarrhoea lasting 7-10 days results in serious dehydration and loss of body weight. Specific antibodies are not considered to be a major factor in recovery from infection, although they may play a role in protection against reinfection. Although the immune factors that contribute to recovery from cryptosporidiosis in the immunocompetent host are not well understood, clearly the absence of optimal number of circulating or mucosal CD4 T lymphocytes, or interferon gamma (IFNγ) is critical (Theodos *et al.,* 1997).

**1.2.8 Risk factor:**

Although healthy individuals can become ill from exposure to *Cryptosporidium*, immunodeficiency places an individual at increased risk for cryptosporidiosis, particularly for more severe and disseminated disease. Immunodeficiency may be congenital or may be secondary to HIV infection, malnutrition, cancer chemotherapy, diabetes mellitus, or bone marrow or solid organ transplantation. The following people have greater exposure to contaminated materials and are at more risk for infection (Painter *et al.,* 2015, Chalmers *et al.,* 2011). Child care workers parents of infected children international travellers, including backpackers and hikers who drink unfiltered, untreated water, swimmers who

swallow contaminated recreational water, people who handle infected animals, people exposed to human feces through sexual contact, hospital-associated infection in patients and health-care providers has also been reported. Pregnancy is another predisposing factor for cryptosporidiosis. In developing nations, the prevalence of *Cryptosporidium* infection is significantly higher than in industrialized countries because of a lack of clean water and sanitary facilities, crowding and animal reservoirs in close proximity to residences (Yoder and Beach, 2010).

**1.2.9 Prognosis:**

After an incubation period of 5-10 days (range 2-28 days), an infected individual develops watery diarrhea, which may be associated with abdominal cramps. In sporadic cases, fever may be low grade or nonexistent; however, during outbreaks, fever may occur in 30-60% of patients.In most healthy individuals, *Cryptosporidium* -induced diarrhea is usually self-limited. However, diarrhea is often prolonged (>1 week) or persistent (>2 weeks). In patients who are severely immunocompromised, cryptosporidiosis may be chronic, severe, sometimes fatal, and with extraintestinal manifestations.Individuals with AIDS and cryptosporidiosis tend to develop chronic symptoms more often and about 10% have a fulminant course (Nair *et al.,* 2008). Antiretroviral treatment improves outcome. Immunocompetent children infected with *Cryptosporidium* generally do well. However, persistent abdominal pain, loose stools, and extraintestinal sequelae (eg, joint pain, eye pain, headache, dizzy spells, fatigue) have been reported, especially with *C.hominis* infection (Semenza and Nichol, 2007).

**1.2.10 Morbidity and mortality:**

Complications of cryptosporidiosis include the following:

Cryptosporidiosis is an important cause of persistent diarrhea in developing countries; children with persistent diarrhea develop worsening malnutrition,

which may result in cognitive and fitness problems that persist for years (Mondal *et al.,* 2009, Guerrant *et al.,* 1999). Sclerosing cholangitis, acalculouscholecystis, papillary stenosis, and pancreatitis may develop with biliary involvement in immune-compromised subjects respiratory track involvement has been described both in AIDS patients and in otherwise-healthy children with intestinal cryptosporidiosis. Although the main symptoms of cryptosporidiosis are related to the gastrointestinal tract, in immunocompromised patients respiratory symptoms may also develop. Respiratory tract involvement is often asymptomatic, but it may manifest as bilateral pulmonary infiltrates with dyspnea. Non specific respiratory symptoms, including shortness of breath, wheezing, cough, hoarseness, and croup, may be a manifestation of respiratory infection. Rarely, conjunctival irritation is also present. In water-borne outbreaks, immunocompetent patients present with subclinical or milder illness that lasts for less than 5 days. Chronic cryptosporidiosis may be complicated by malabsorption, malnutrition, and death in individuals with AIDS and in malnourished children (Amadi *et al.,* 2002).

Waterborne outbreaks of cryptosporidiosis have been documented in countries around the world. Between the years 1986 and 1996, 16 total cryptosporidiosis outbreaks in drinking water were reported in Europe, the majority in England and Wales, and 14 in North America, the majority in the United States of America (USA). Two outbreaks were reported in Japan. Most of the *Cryptosporidiuim* recreational outbreaks have been documented in the USA and the United Kingdom (UK). Rainfall was a strong variable in drinking water outbreaks and fecal accidents in recreational outbreaks. In the USA there have been 10 drinking water outbreaks of cryptosporidiosis documented from 1984 to 1996. Only in England and Wales have been there more reported outbreaks (Smith and Rose, 1998, Craun *et al.*, 1998, Kramer *et al*., 2001). In most countries where outbreaks have been documented only a few have been identified as caused by *Cryptosporidium*. In Canada, between 1993 and 1996,

four drinking water outbreaks occurred with 361 laboratory cases reported and an estimated 31 900 people affected. A significant number of cryptosporidiosis outbreaks were associated with contaminated ground-water (wells and springs not properly protected from sewage and run off or wells located adjacent to rivers and streams) and many of the documented outbreaks associated with surface water contamination were linked to human sewage discharge and run off, which occurred during heavy rainfall events in the USA, the UK, and Canada. Craun *et al*. have reviewed some of the outbreaks in North America and the UK (Craun *et al*., 1998). Where there was information, they reported attack rates ranging from 1 to 60% (average 22%), and hospitalization rates from 1 to 44% (average 13%), and there was no correlation between these two indices, suggesting that the dose (the level of contamination and the distribution) was not associated with the organism's virulence (Rose *et al*., 2002).

### 1.2.11 Immunology of pregnancy:

The maternal immune system during pregnancy is altered to actively tolerate the semi-allo geneicfetus. These alterations include changes in local immune responses, that is, in the uterine mucosa (decidua) (Trundley and Moffett, 2004) and changes in peripheral immune response (Van Nieuwenhoven *et al.*, 2003). After implantation, the uterine endometrium is rapidly infiltrated by fetal trophoblast cells; the endometrium will then develop into the decidua and ensure anchorage of the placenta and therefore proper fetal nutrition. However, this invasion needs to be properly regulated to protect the corporal integrity of the uterine wall of the mother. Both shallow and over-invasion will lead to problematic pregnancies (Lala and Chakraborty, 2003, Wells, 2007). Local decidual immune cells, such as uterine natural killer (uNK) cells and macrophages, are important regulators of this balance between tolerance of fetal trophoblasts and limitation of their invasion (Nagamatsuu and Schust, 2010,

Lash *et al.,* 2010). When placental circulation is established, the peripheral blood also comes into close contact with fetal cells, specifically, villous trophoblasts. This may affect the peripheral maternal immune response. During weeks 8-12, when placental circulation is established, the maternal peripheral blood is in close contact with semi-allogeneic villous trophoblasts. These trophoblasts are able to produce and shed factors, such as IL-4 and syncytiotrophoblast microfragments (Sacks *et al.,* 2001) or fetal cells (Bianchi *et al.,* 1996) into the maternal circulation. Such factors affect the immune system. The presence of proinflammatory factors in the plasma of pregnant women has been shown by Faas *et al.*,( 2008) who demonstrated that incubation of monocytes with plasma from pregnant women activated this cell type. Moreover, the passage of maternal blood through the placenta activates inflammatory cells such as granulocytes and monocytes (Mellembakken *et al.,* 2002).

### 1.2.12 *Cryptosporidium* diagnosis:

To determine the minimum number of *Cryptosporidium* oocysts that can be detected in stool specimens by diagnostic procedures, stool samples seeded with known numbers of *C.parvum* oocysts were processed by the modified formalin-ethyl acetate (FEA) stool concentration method. FEA concentrates were subsequently examined by both the modified cold Kinyoun acid-fast (AF) staining and fluorescein-tagged monoclonal antibody (immunofluorescence [IF]) techniques. Oocysts were more easily detected in watery diarrheal stool specimens than they were informed stool specimens. For watery stool specimens, a 100% detection rate was accomplished at a concentration of 10,000 oocysts per gram of stool by both the AF staining and IF techniques (Vohra *et al.,* 2012). In formed stool specimens, 100% of specimens seeded with 50,000 oocysts per gram of stool were detected by the IF technique, whereas 500,000 oocysts per gram of stool were needed for a 100% detection rate by AF staining. Counting of all oocysts on IF slides indicated a mean

oocyst loss ranging from 51.2 to 99.6%, depending on the stool consistency, as determined by the FEA concentration procedure. These findings suggest that the most commonly used copro diagnostic techniques may fail to detect cryptosporidiosis in many immunocompromised and immunocompetent individuals (Vohra *et al.,* 2012).

**1.2.12.1 Laboratory diagnosis:**

**a. Microscopic techniques:**

i) Concentration methods

Stool concentration techniques include:

- Floatation methods - flotation of oocysts in Sheather's sugar solution, in zinc sulfate (1.18 or 1.20 specific gravity), or in saturated sodium chloride (1.27 specific gravity).
- Sedimentation methods- stool concentration techniques using sedimentation include formalin-ether and formalin-ethyl acetate.

ii) Staining techniques: several widely used techniques for demonstrating *Cryptosporidium* oocysts in fecal specimens from humans and other animals are: hematoxylin and eosin staining, romanowsky stains, modified acid-fast staining, safranin- methylene blue staining, negative staining, dimethylsulphoxide (DMSO) modified acid fast staining and fluorescent stains (Vohra *et al.*, 2012).

**b. Serological techniques:**

1. Antibody detection methods

The use of sero diagnostic techniques to monitor exposure to *Cryptosporidium* species has been limited to a few laboratories. Antibodies specific to *Cryptosporidium* species have been detected by an IFA procedure in sera obtained from persons who recovered from confirmed infections (Campbell and Current, 1983, Casemore, 1987). Specific anti-*Cryptosporidium* IgG, IgM, or both were also detected, by an enzyme-linked immunosorbent assay (ELISA), in the sera of 95 % of patients with cryptosporidiosis at the time of medical

presentation and in 100% within 2 weeks of presentation (Ungar *et al.,* 1986). According to one serologic survey, >50% of persons with no known infection may have anti *Cryptosporidium* IgG, suggesting recent exposure to the parasite (Ungar *et al.,* 1988).

2. Antigen detection tests:

a) Immunochromatographic dipstick tests (ICT):

- Rida Quick *Cryptosporidium*
- *Cryptosporidium*-strip (Coris BConcepts, Gemb). Advantages: sensitive, cost-effective methods. Limitations: not reliable as routine methods (Vohra *et al.*, 2012).

b) Enzyme immunoassays (EIAs)

I) Rida screen *Cryptosporidium* (R-Biopharm). Compared with the EIA tests, the dipstick kits had the advantage of being less time-consuming and simpler to carry out, and did not require an ELISA microplate reader or other specialized equipment. Microscopy can be false-negative for cases with a low parasite density, or when intact microorganisms are absent. Another important factor influencing the performance of copro-antigen assays is the study population. False-negative copro-antigen test results have been associated with low parasite densities (Wetizzel *et al*., 2006).

II) Prospect *Cryptosporidium* microtiter assay.

III) Color Vue *Cryptosporidium* assay (Kehl *et al.*, 1995)

The Color Vue assay was judged to be slightly better suited for batch testing because it did not require a predilution step. A predilution step is required in the Prospect assay. These pretesting processes add to the complexity of these methods and the time required to perform the tests. Advantages less detection time, economical, lesser skill reacquired. This ELISA will be particularly useful in laboratories not accustomed to diagnosing cryptosporidiosis often, in epidemiologic studies in need of diagnostic standardization, and in situations when batch specimen processing may be crucial (Unger, 1990).

**c. Flow cytometery:**

*Cryptosporidium* oocysts, as discrete particles, can be detected by flow cytometry (FC) if labeled with an appropriate fluorescent tag (Arrowood *et al*., 1995) described the use of this method, which employed a monoclonal antibody for evaluation of experimental cryptosporidial parasite loads in infected mice with severe combined immunodeficiency (SCID). Oocysts can be fluorescently labeled and counted by FC and (Valdez *et al.,* 1997) modified and extended the method of (Arrowood *et al*., 1995) to human stool samples.

**d. Molecular methods:**

Polymerase chain reaction (PCR):

Due to its extreme sensitivity and specificity, the PCR-based detection of microbes in clinical samples is an attractive option (Fricker and Crabb, 1998, Widmer, 1998, Morgan and Thompson, 1998) have reviewed various methods for PCR-based detection of *Cryptosporidium* in clinical samples and drinking water. Some investigators have found high sensitivity for PCR -based assays (one oocyst) and suggest that these assays are more sensitive than microscopic analysis of acid-fast smears; unfortunately, no large comparative study has been performed to determine the ideal primers, PCR conditions, or stool extraction methods to use with clinical samples. Several factors complicate the PCR-based detection of *C.parvum* in stool. Standard fixation in 10% buffered formalin may reduce the sensitivity of the PCR, particularly if fixation occurs over an extended period. Also, extended formalin fixation may alter the buoyancy of *C.parvum* oocysts, interfering with standard methods for purification of *C.parvum* oocysts from stool (Vohra *et al*., 2012). PCR detection of oocysts from frozen stool is also possible, but the sensitivity may be reduced, probably due to rupture of oocysts during thawing. One method for oocyst purification from stool commonly used in the research laboratory involves density gradient centrifugation of stool while this method provides purified oocysts, ideal for PCR analysis, it is more suitable for a

research laboratory than a clinical laboratory and may not be useful for specimens containing few oocysts. The PCR can be inhibited by numerous substances, including some stool components. Several investigators have developed nucleic acid extraction methods for stool to remove these inhibitors. Unfortunately, many of these methods are quite complex, and detailed comparative studies have not been performed to identify the most useful technique (Clark, 1999).

**1.2.12.2 Diagnosis of extra intestinal cryptosporidiosis:**

a. Biliary cryptosporidiosis:

- Ultrasonography-best initial diagnostic method. It is suggestive in most cases by identifying biliary ductal wall thickening and/or gallbladder dilation or both, computerized tomography, endoscopic retrograde cholangio pancreatography (ERCP), percutaneous liver biopsy, serum alkaline phosphatase- most commonly elevated liver biochemical test with mean values in most series of 700 IU/L-800 IU/L and serum amino-transferases mild increase in values (Wilcox and Monkemuller, 1998).

b. Pulmonary cryptosporidiosis:

Oocysts have been identified in sputum samples, tracheal aspirates, bronchoalveolar lavage fluid, brush biopsy specimens, and alveolar exudate obtained from lung biopsy (Current and Garcia, 1991). Due to presence of acid-fast oocysts in sputum sample and absence of any other pathogenic organism by microscopy or culture examination, *Cryptosporidium* was considered as an etiological agent for the pulmonary pathology in a study by Shrikhande *et al.*, (2009). Thus, pulmonary cryptosporidiosis should be considered as one of the differential diagnosis when an immune compromised patient with respiratory symptoms is being investigated (Vohra *et al.*, 2012).

### 1.2.13 Treatment:

Optimal therapy for cryptosporidiosis includes attention to fluids and electrolytes, anti motility agents, anti parasitic drugs, nutritional support, and/or reversal of immune suppression (Checkley *et al.,* 2015).

Nitazoxanide significantly shortens the duration of diarrhea and can decrease the risk of mortality in malnourished children (Amadi *et al.*, 2002).

### 1.2.14 Prevention and control:

Quantitative microbial risk assessment (QMRA) provides a tool for estimating the disease burden from pathogenic microorganisms in water, using information about the distribution and occurrence of the pathogen or an appropriate surrogate (Howard *et al.*, 2006). This information may then be used to formulate appropriate management practices for the water supply system (Howard *et al.*, 2006). Malnutrition, immunosuppression, young ageand an increase in the preceding diarrhea burdens are all risk factors for the development of persistent diarrhoea. Patient management strategies include rehydration, adequate diet, micronutrient supplementation, and antimicrobials (Ochoa *et al*., 2004). Persistent diarrhoea seriously affects nutritional status, growth, and intellectual function. Meeting these challenges is profoundly important, particularly in developing countries. Aggressive treatment of infectious diarrhoea is required in severely immune compromised children (Huang and White, 2006).

### 1.2.15 Cryptosporidiosis in Sudan:

Protozoa are eukaryotic cells distributed worldwide in nature and are receiving increasing attention as reservoirs and potential vectors for the transmission of pathogenic bacteria. In the environment, on the other hand, many genera of the protozoa are human and animal pathogens. It is necessary to establish a molecular identification of species of the protozoa from drinking and environmental water. 600 water samples were collected from five states (Gadarif, Khartoum, Kordofan , Juba and Wad Madani) in Sudan and

analysed by polymerase chain reaction (PCR) and sequencing 57 out of 600 water samples were PCR positive for protozoa. 38 out of the 57 positive samples were identified by sequencing to contain 66 protozoa species including 19 (28.8%) amoebae, 17 (25.7%) apicomplexa, 25 (37.9%) ciliates, and 5 (7.6%) flagellates. This study utilized molecular methods identified species belonging to all phyla of protozoa and presented a fast and accurate molecular detection and identification of pathogenic as well as free-living protozoa in water uncovering hazards facing public health (Shanan *et al*., 2015). *Cryptosporidium*, a protozoan parasite that causes watery diarrhea, is found worldwide and is common in areas with low water hygiene. In February 2014, 866 stool samples were collected from the inhabitants of 2 rural areas in White Nile State, Sudan. These stool samples were assessed by performing modified acid-fast staining, followed by examination under a light microscope. The overall positive rate of *Cryptosporidium* oocysts was 13.3%. *Cryptosporidium* oocysts were detected in 8.6% stool samples obtained from inhabitants living in the area having water purification systems and in 14.6% stool samples obtained from inhabitants living in the area not having water purification systems. No significant difference was observed in the prevalence of *Cryptosporidium* infection between men and women (14.7% and 14.1%, respectively). The positive rate of oocysts by age was the highest among inhabitants in their 60-69 years (40.0%). These findings suggest that the use of water purification systems is important for preventing *Cryptosporidium* infection among inhabitants of these rural areas in Sudan (Sim *et al*., 2015). In 2007, the prevalence of cryptosporidiosis among patients presenting with non-bloody diarrhoea to Nyala medical laboratory Eleven (15.3%) out of 72 patients were positive for *Cryptosporidium* oocysts. Seven of them were below five years of age (Adam *et al*., 2007). The stool specimens were examined for *Cryptosporidium* oocysts by using Safranine/ Methylene blue stain technique.

## Rationale:

Pregnancy is characterized by a state of immune suppression. All individuals affected by immune suppression are at risk of infection by opportunistic parasites that may gain the capacity to invade even the immune competent as well as immune compromised individuals. Thus, the study of this phenomenon is strongly considered. This study aimed to provide knowledge on the opportunistic parasitic protozoa such as *Cryptosporidium* as a cause of disease in pregnant women and also provide guidance on recent advances in diagnosis of this important parasite.

**Objectives:**

**General objective:**

To determine prevalence of *Cryptosporidium* among pregnant women in Medical Military Hospital-Khartoum state.

**Specific objectives:**

1. To demonstrate the occurrence of cryptosporidiosis among pregnant women according to age groups and gestation age.
2. To investigate the prevalence of cryptosporidiosis according to source of drinking water, diarrheal sign and to the contact with animals.
3. To compare between stained direct smear and stained formal ether concentration technique for detecting oocyst of *Cryptosporidium*.

# Chapter two

# Materials and methods

## 2.1 Study design:

It is a cross-sectional study.

## 2.2 Study area and study duration:

The study was conducted in Medical Military Hospital-Khartoum state, during the period between May-December 2016. Khartoum State lies between longitude 31.5-34 east and latitude 15-16 north in an area about 28.165 square kilometres. It is bordered on the north and the east sides by the River Nile State, on the north-western side by the Northern State and on the eastern and southern sides by Kassala, Gedaref and Gezira States (Ministry of Human Development and Labour, 2015).

## 2.3 Study population:

The study was carried out on pregnant women who attended in pregnancy clinic for follow up.

## 2.4 Sample size:

The sample size was obtained according to the following equation:

$N = (t^2 * P(1-p) / M^2)$

N = Sample size

t = the normal standard deviate (t =1.96)

P = the frequency of occurrence of *cryptosporidium* (1.1%)

M = degree of precision (0.05)

N = 1.96*1.96*0.11*(1-0.11)/0.05*0.05 = 150

According to the above finding, the study was conducted on 150 pregnant women.

**2.5 Sample collection:**

One hundred and fifty stool samples were collected from all participants. After the direct saline preparation and direct smear were prepared, specimens were fixed with 10% formal saline for further concentration and staining techniques.

**2.6 Data collection:**

150 designed questionnaires were filled by participants (appendix).

**2.7 Methods:**

**2.7.1 Direct wet preparation:**

The technique was used to detect the parasites. All specimens were initially subjected to direct saline preparation and then examined.

**2.7.1.1 Procedure:**

One drop of 0.8 5% sodium chloride (physiological saline), were placed on a clean slide. A small amount of stool specimen (2 mg) was taken to make smooth thin preparation. A cover glass was placed on the suspension. Each suspension was systematically scanned with the low-power objective (x10) then high-power objective (x40) was used for detailed study.

**2.7.2 Direct stool smear:**

A small amount of stool specimen was taken to make a thin smear. The smears were allowed to air dry then examined after staining steps were done.

**2.7.3 Formal-ether concentration technique (F.E.C.T):**

This is recommended for use in district laboratories because it is rapid and can be used to concentrate a wide range of faecal parasites from fresh or preserved faeces (Cheesbrouph, 1987).

**2.7.3.1. Procedure:**

The specimen was preserved in 10% formalin then the preservative mixture was stirred. Depending on the amount and viscosity of the specimen, a sufficient quantity of stool were sieved through wet gauze (no more than two layers) into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) for later steps. Approximately 3 to 4 ml of the preserved-stool mixture was

used. 10% formalin almost to the top of the tube was added, and for 10 min at 500 rpm was centrifuged. The amount of sediment obtained approximately 0.5 to 1 ml. The supernatant fluid was discarded, and the sediment in formalin was resuspended, formalin almost to the top of the tube was added and again for 10 min at 500 rpm was centrifuged. The supernatant fluid discarded, and the sediment on the bottom of the tube in 10% formalin was resuspended. The tube half full only was filled. 4 to 5 ml of diethyl ether were added. The tube with stopper was closed and the suspension vigorously for at least 30 s was mixed. After a 15s to 30s waited, carefully the stopper removed. For 10 min at 500 rpm was centrifuged. For four layers was achieved, a small amount of sediment (containing the parasites) in the bottom of the tube, a layer of formalin; a plug of faecal debris on top of the formalin layer and a layer of diethyl ether the top. The plug debris freed by the plug with an applicator stick was ringed, all of the supernatant fluid was discarded. After proper decanted a drop or two of fluid remained on the side of the tube down into the sediment. The fluid was mixed with the sediment. A small amount of mixure were added to a slide, to make thin smear was spread it, then left it to air dry and after staining steps done was examined.

### 2.7.4. Modified Ziehl-Neelsen (M-Zn) stain:

#### 2.7.4.1. Reagents:

- Carbolfuchsin stain
- Malachite green stain
- Acid alcohol 1% v/v
- Methanol

#### 2.7.4.2. Procedure:

After the direct smear from stool specimens was prepared and a smear from the sediment obtained by the formol ether concentration technique. The smear was fixed with methanol for 2–3 minutes. With carbol fuchsine for 15 minutes (cold method) was stained. The stain was washed with water. 1% acid alcohol was

used decolorized for 10–15 seconds, then was washed by water. Then a malachite green stain was used as counter stain for 30 seconds, the slide was washed water and the slide were stood in a draining rack to dry. The smear was examined microscopically for oocyst, a low power magnification was used to detected the oocyst and the oil immersion objective was used identified them (Cheesbrough, 1987).

**2.8 Data analysis:**

Results were analysed using the computerized program of statistical package of social science (SPSS) version 16. Then data were presented in tables.

**2.9 Sensitivity and specificity of the techniques used:**

Sensitivity and specificity was calculated according to the formula (table 2.1) (Indrayan, 2008):

Sensitivity = TP/(TP+FN)x 100%

Specificity = TN/(TN+FP) x 100%

TP= True positive

TN= True negative

FP= False positive

FN= False negative

**Table (2.1): Formula of sensitivity and specificity**

| Technique | Positive | Negative | Total |
|---|---|---|---|
| Positive | TP | FP | TP+FP |
| Negative | FN | TN | FN+TN |
| Total | TP+FN | FN+TN | TP+FP+FN+FP |

**2.10. Ethical consideration:**

Approval was taken from the College of Medical Laboratory Science-Sudan University of Science and Technology and hospital management. The pregnant women were informed for the purpose of the study before collection of the specimen and verbal concept was taken from them.

# Chapter three

# Results

## 3.1 General characteristics of studied population:

The study was conducted on 150 subjects. Study subjects were divided into 4 age groups as follows:<20, 20-29, 30-39 and 40-49 years old. The frequency of each age group was 26 (17.3%), 86 (57.4%), 36 (24.1%) and 2 (1.3%) respectively (table 3.1). The age ranged between 16-43 years old. The mean age was 26± 10 years old. Studied subjects were classified to gestation age as follows: 23 (15%) for first trimester, 66 (44%) for second trimester and 61(41%) for third trimester (table 3.2).

**Table (3.1): Frequency of study subjects according to age groups**

| Age groups years | Frequency | Percentages % |
|---|---|---|
| < 20 | 26 | 17.3 |
| 20-29 | 86 | 57.4 |
| 30-39 | 36 | 24 |
| 40-49 | 2 | 1.3 |
| Total | 150 | 100% |

**Table (3.2): Frequency of study subjects according to gestation age**

| Gestational age | Frequency | Percentage (%) |
|---|---|---|
| First trimester | 23 | 15.3 |
| Second trimester | 66 | 44.0 |
| Third trimester | 61 | 40.7 |
| Total | 150 | 100% |

### 3.2 Parasitological results:

#### 3.2.1 Prevalence of cryptosporidiosis by using M-Zn stain for direct smear and FECT smear prepared technique:

Out of 150 stool samples, 1 (0.66%) was positive for *Cryptosporidium* by using stained direct smear and 23 (15.3%) were positive for *Cryptosporidium* by using stained FECT smear. The difference in rate was found to be statistically significant at p value=0.018 (table 3.3).

**Table (3.3): Prevalence of cryptosporidiosis by using M-Zn stain for direct smear and FECT smear prepared technique**

| Technique | Percentage% |
|---|---|
| Direct smear | 0.66% |
| FECT smear | 15.3% |

p=0.018

#### 3.2.2 Sensitivity and specificity of techniques and assuming the FECE as the gold standard:

According to the formula described in materials and methods (table2.1) sensitivity and specificity of stained direct smear method was 4% and100% respectively (table 3.4).

**Table (3.4): Sensitivity and specificity of stained direct smear**

| Technique | | FECT | |
|---|---|---|---|
| | | Positive | Negative |
| Direct smear | Positive | 1 | 0 |
| | Negative | 22 | 127 |

#### 3.2.3 Prevalence of cryptosporidiosis according to age groups using M-Zn stain FECT smear:

Out of 150 stool samples, the prevalence rates of cryptosporidiosis were 5 (3.3%), 15 (10.0%), 3(2.0%) and 0(0%) in age groups <20, 20-29, 30-39 and

40-49 years old respectively. The difference in rates was found to be statistically insignificant at p value=0.509 (table 3.5).

**Table (3.5): Prevalence of cryptosporidiosis according to age groups using M-Zn stain FECT smear**

| Age groups (years) | FECT | | Total |
|---|---|---|---|
| | Positive | Negative | |
| >20 | 5 | 21 | 26 |
| 21-29 | 15 | 71 | 86 |
| 30-39 | 3 | 33 | 36 |
| 40-49 | 0 | 2 | 2 |
| Total | 23 | 127 | 150 |

p=0.509

### 3.2.4 Prevalence of cryptosporidiosis according to gestation age:

Out of the 23 positive samples, 4 (17.3%) were in the first trimester, 7 (10.6%) were in the second trimester and 12 (19.6%) were third trimester. The difference in rates was found to be statistically insignificant at p value=0.351 (table 3.6).

**Table (3.6): Prevalence of cryptosporidiosis according to gestation age**

| Gestational age | M-ZN stain | | Total |
|---|---|---|---|
| | Positive | Negative | |
| First trimester | 4 | 19 | 23 |
| Second trimester | 7 | 59 | 66 |
| Third trimester | 12 | 49 | 61 |
| Total | 23 | 127 | 150 |

p=0.351.

### 3.2.5 Prevalence of cryptosporidiosis according to the source of drinking water:

Out of 150 subjects, 141were used tap water as source of drinking water. Out of them 20 (14.1%) were positive, while 9 who used other sources, 3 (33.3%) were

positive. The difference in rates was found to be statistically insignificant at p value=0.122 (table 3.7).

**Table (3.7): Prevalence of cryptosporidiosis according to source of drinking water**

| Source of drinking water | Cryptosporidiosis | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Tap water | 20 | 121 | 141 |
| Others | 3 | 6 | 9 |
| Total | 23 | 127 | 150 |

p=0.122

### 3.2.6 Prevalence of cryptosporidiosis according to diarrheal sign:

Out of 150 subjects, 62 had a diarrheal sign. Out of them 14 (22.6%) were positive for cryptosporidiosis while 9 (10.2%) were positive but had no diarrheal sign. The difference in rates was found to be statistically significant at p=0.039 (table 3.8).

**Table (3.8): Prevalence of cryptosporidiosis according to diarrheal sign**

| Diarrheal sign | Cryptosporidiosis | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Yes | 14 | 48 | 62 |
| No | 9 | 79 | 88 |
| Total | 23 | 127 | 150 |

p=0.039

**Table (3.9): Prevalence of cryptosporidiosis according to contact with animals**

| Contact with animals | Cryptosporidiosis | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Yes | 3 | 19 | 22 |
| No | 20 | 108 | 128 |
| Total | 23 | 127 | 150 |

p=0.811

**3.3 Detection of other parasites using wet preparation and stained FECT smear:**

Out of 150 stool samples 17 (11.3%) were positive for *Giardia lamblia* and 5 (3.3%) were positive for *Entamoeba histolytica* by using wet preparation, while 4 (2.7%) were positive for *Cyclospora.spp* using stained FECT smear (table 3.11).

**Table (3.10): Prevalence of other parasites among pregnant women using wet preparation and stained FECT smear**

| Parasites / Total | *Giardia lambila* | *Entamoeba histolytica* | *Cyclospora spp* |
|---|---|---|---|
| Frequency | 17 | 5 | 4 |
| Percentage % | 11.3% | 3.3% | 2.7% |

### 3.3.1 Prevalence of Co-infection of *Cryptosporidium* and other parasites:

From 23 positive cases for *Cryptosporidium* 3(13%), 2(8.7%) and 4(17.4%) were positive for co-infection *Cryptosporidium/ Giardia lambila*, *Cryptosporidium/ Entamoeba histolytica* and *Cryptosporidium/ Cyclospora spp* respectively (Table 3.11).

**Table (3.11): Prevalence of Co-infection of *Cryptosporidium* and other parasites**

| Parasites / *Cryptosporidium* | | *Giardia lambila* | *Entamoeba histolytica* | *Cyclospora spp* |
|---|---|---|---|---|
| Co-infection | Frequency | 3 | 2 | 4 |
| | Percentage % | 13% | 8.7% | 17.4% |

# Chapter four

## Discussion

The present study was carried out on 150 stool samples collected from pregnant women who attended the Medical Military Hospital-Khartoum State. The results showed that the overall prevalence rate of cryptosporidiosis was 15.3% using stained FECT smear. This finding agreed with the finding obtained by Sim *et al.* (2014) in the White Nile State. They found that the prevalence rate was 13.3% and was also similar with the finding obtained by Adam *et al.* (2007) in Nyala medical laboratory, who found that the prevalence was 15.3%. The results showed the prevalence of cryptosporidiosis in the study subjects according to gestation age was higher (19.6%) in the third trimester, compared to other gestation ages due to their lack of efficient immunological response against the infection. The present study showed a strong relationship between the source of drinking water and prevalence of *Cryptosporidium* infection. This finding was inagreement with Sim *et al.* (2015), Adamska (2015) and Shanan *et al.* (2015). The results showed that from the 62 subjects who had diarrhoea, 14 (22.6%) were positive for cryptosporidiosis. This difference in rate was found to be statistically significant at p value=0.039, while there is insignificant difference between prevalence of cryptosporidiosis and contact with animals at p value=0.811. This finding was in agreement with Sim *et al.*(2014). Out of 150 stool samples, 23(15.3%) were positive cases for cryptosporidiosis using stained FECT smear while only one sample (0.66%) was positive using stained direct smear. From this result, stained FECT smear is more accurate than stained direct smear.

The study concluded that the prevalence of co-infection of *Cryptosporidium* with other parasites was detected among pregnant women as *Cryptosporidium/ Giardia lambila* (13%), *Cryptosporidium/ Entamoeba histolytica* (8.7%) and *Cryptosporidium/ Cyclospora spp* (17.4%)

# Chapter five

# Conclusion and Recommendations

## 5.1 Conclusion:

The study concluded that the prevalence rate of cryptosporidiosis among pregnant women was 15.3%. The prevalence was high among age group 20-29 years. Also, the prevalence was high in third trimester according to gestation age.

M-Zn stain FECT smear was more accurate than direct smear in the diagnosis of *Cryptosporidium* parasite.

## 5.2 Recommendations:

- Detection and examination of intestinal coccidian parasites should be considered as a routine working for patients with abdominal disturbances.
- Concentration and staining of faecal smears should be included for routine diagnosis in the laboratories.
- More studies should be done to investigate intestinal coccidian parasites.