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PREVALENCE OF GIARDIA LAMBLIA CYSTS AND CRYPTOSPORIDIUM PARVUM OOCYSTS AMONG SUBJECTS USING PONDS AND STREAMS FOR RECREATION IN THREE SELECTED AREAS OF KADUNA STATE, NIGERIA

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DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

AHMADU BELLO UNIVERSITY,

ZARIA

JUNE, 2018

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

AHMADU BELLO UNIVERSITY,

ZARIA

JUNE, 2018

DECLARATION

I declare that the work in this dissertation entitled "Prevalence of *Giardia Lamblia* Cysts and *Cryptosporidium Parvum* Oocysts among Subjects using Ponds and Streams for recreation in Three selected areas of Kaduna State, Nigeria" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

_____ _____

SAEED, SANI Date

(P16LSMC8122)

CERTIFICATION

This dissertation entitled "Prevalence of *Giardia Lamblia* Cysts and *Cryptosporidium Parvum* Oocysts among Subjects using Ponds and Streams for recreation in Three selected areas of Kaduna State, Nigeria" by SAEED, SANI (P16SCMC8122) meets the requirement for the award of Master of Science Degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

This study; Prevalence *Giardia lamblia* Cysts and *Cryptosporidium parvum O*ocysts among subjects using ponds and streams for recreation in Three selected areas of Kaduna state, Nigeria was carried out to assess the prevalence of *Giardia lamblia* cysts and *Cryptosporidium parvum oo*cysts among subjects using ponds and streams for recreation in Three selected areas of Kaduna state, Nigeria. Stool samples from 210 subjects using ponds and streams for recreational purposes were collected and examined for *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts microscopically. Similarly, 210 water samples were collected from ponds and streams used for recreational purposes and examined for the presence of the parasites. Forty (40) Stool samples that were positive to microscopy were screened for *G. lamblia* specific antigen and *C. parvum* specific antigen by Enzyme immunoassay which confirms Thirteen (13) samples positive. Out of the 210 stool samples examined, 14.76% and 7.14% of the samples were positive for *G. lamblia* cysts and *C. parvum* oocysts respectively. Based on gender, the prevalence of *G. lamblia* was higher among females (22.22%) as compared to males (14.43%). However, the males had 7.46% for *C. parvum*, while females had no *C. parvum* infection. The highest prevalence of *G. lamblia* and *C. parvum* was observed among members of the Age group 6-11years (18.84% and 9.42% respectively). Out of the 210 water samples examined, 5.24% and 2.86% of the samples were positive for *G. lamblia* cysts and *C. parvum* oocysts respectively. Enzyme immunoassay (EIA) detected coproantigen of the parasites in 13 (32.50%) of the positive samples by microscopy. The study concludes that proper hygienic practice and early treatment of infectious giardiasis and cryptosporidiosis are suggestive of protective factors against the disease.

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LIST OF ABBREVIATIONS AND SYMBOLS

df	-	degree of freedom
DNA	-	Deoxyribonucleic acid
ELISA	-	Enzyme Linked Immunosorbent Assay
kDa	-	kilo Dalton
LGA	-	Local Government area
mL	-	milliliter
mm	-	millimeter
OD	-	Optical Density
Rpm	-	revolution per minute
USA	-	United State of America
χ	-	chi

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Cryptosporidium Parvum and *Giardia Lamblia* are among the highest reported causative agent of waterborne parasitic infections and they are the common cause of waterborne outbreaks of parasitic infection around the world (Karani *et al*., 2007).

These two protozoans cause common waterborne outbreaks in the USA (Kumar *et al.,* 2016). Cryptosporidiosis and Giardiasis can be primarily transmitted via direct contact with contaminated water (diving, swimming, bathing, among other water-based activities), via contact with water that has been deficiently treated, and via accidental ingestion of water containing (oo)cysts; infection with either can lead to potentially fatal diseases in humans (Kumar *et al.,* 2016).

Microscopic identification requires trained Microscopists and involves time and labour for preparing, staining, and examining smears. As a result, immunoassays for the detection of *Giardia* and *Cryptosporidium* stool antigens have replaced microscopy as the routine diagnostic procedure of choice in many hospitals and public health laboratories. These immunoassays are reported to be as sensitive and specific as traditional microscopic methods and increase laboratory efficiency by reducing labor, time, and costs (Johnston *et al.,* 2003).

1.2 Statement of the Problem

Morbidity and mortality due to diarrhoeal diseases in developing countries remain the main public health problems that need due attention. Although there could be many other causes of diarrhoea, the enteric protozoa; *Cryptosporidium parvum* and *Giardia lamblia* have been recognized as important causes of both outbreak-related and sporadic diarrhoea among humans. Both immunocompetent and immunocompromised individuals could be the victims of diarrhoeal diseases caused by these parasites (Tigabu *et al.,* 2010).

1991-1992 there were five documented outbreaks: one *Giardia* outbreak occurred in June of 1991 involving 14 people in a swimming pool and in July 1991, a *Giardia* outbreak involved 16 persons who became infected via wading pool in a daycare center. In July of 1991 there was yet another *Giardia* outbreak contracted from a lake at a camp ground. In Oregon, 1992 there was a *Cryptosporidium* outbreak in a wave pool infecting 500 persons.

The Centre for Disease Control and Prevention in Atlanta, USA attributed 71% of waterborne disease outbreaks in 1993 and 1994 to *Cryptosporidium parvum* and *Giardialamblia*, which caused cryptosporidiosis and giardiasis, respectively (Gostin *et al.,* 2000).

Individual infections with protozoa like *Cryptosporidium* and *Giardia* are costly. New Zealand spends approximately NZ$1.5 million per year to treat approximately 2,400 people (Snel *et al.,* 2009).

1.3 Justification for the Study

The diagnosis of cryptosporidiosis is usually achieved by conventional microscopic examination of modified acid-fast stained faecal smears. The need for rapid and cost-effective diagnosis has led to the development of immunoassay techniques like immunofluorescence assay (IFA) and enzyme linked immunosorbent assay (ELISA). These methods are relatively easy to perform and interpret (Cho *et al*., 2012).

Jombo *et al*. (2011) report a prevalence rate of 10.5% for *Giardia lamblia* among undernourished children aged between 6 to 70 months attending paediatric clinics in Jos, Nigeria.

Most people who ingest *Giardia* are either not infected or become asymptomatic excretors of cysts. With symptomatic infection, which is the most common scenario in young children, a 1- to 2-weeks incubation period is followed by either acute self-limited diarrhea or chronic diarrhea with malabsorption. Explosive, watery and foul-smelling diarrhea that is free of blood or mucus occurs in almost all children who become symptomatic. Often the stools are greasy and may float. Crampy abdominal pain and bloating are common, and about 50% of patients complain of nausea and anorexia early in the illness. Vomiting is seen in fewer than one third of patients, and fever is even less common. Generally, symptoms clear within a few days, even without treatment (Stevens, 2004).

1.4 Aim

The aim of the study was to detect *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts among Subjects using ponds and streams for recreation in selected areas of Kaduna State, Nigeria

1.5 Specific Objectives

The objectives of this study were to:

1. Identify the prevalence of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in the stool and water samples of Subjects using Ponds and Streams for recreation in the Three selected LGAs of Kaduna State.

2. Identify the risk factors associated with *Giardia lamblia* and *Cryptosporidium parvum* infections through structured questionnaire.

3. Identify Coproantigens of *Giardia lamblia* and *Cryptosporidium parvum* in Stool samples of the enrolled Subjects by Enzyme Linked Immunosorbent Assay (ELISA).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Giardia lamblia*

Giardia lamblia is a flagellated unicellular eukaryotic microorganism that commonly causes diarrhoeal disease throughout the world. It is the most common cause of waterborne outbreaks of diarrhoea. In developing countries, there is a very high prevalence and incidence of infection, and data suggest that long-term growth retardation can result from chronic giardiasis. In certain areas of the world, water contaminated with *G. lamblia* cysts commonly causes travel related giardiasis in tourists (Barwick *et al.,* 2000).

Giardia lamblia(*G. lamblia*)is the most commonly isolated intestinal parasite throughout the world. Prevalence rates of 20-40% have been reported in developing countries, especially in children (Fraser, 1994). Analysis of 722 faecal DNA samples revealed that a prevalence rate of 9.3% of *G. lamblia* by PCR, as compared to 5.7% by microscopy in the Netherland (ten Hove *et al.*, 2007).

In a study conducted in northern Ghana by Klaus *et al*. (2007), *Giardia lamblia* were observed more than twice as frequently in asymptomatic individuals 12(9.7%) than in symptomatic individuals 9(3.7%). The diplomad *Giardia* exist in two morphological forms, the trophozoite and the cyst. The trophozoite is easily recognized under a microscope. It is about 12 to 15 μm long, shaped like a pear cut in half lengthwise, and has two nuclei, structures called median bodies, and four pairs of flagellae. The flagellae help these organisms to migrate to a given area of the small intestine, where they attach by means of an adhesive disk to epithelial cells and thus maintain their position despite peristalsis. *Giardia* cyst-the form usually seen in the faeces, is

ovoid, 6 to 12 μm long, and can often be seen to contain two to four nuclei at one end and prominent diagonal fibrils (Ayalew, 2006).

2.1.1 Taxonomy

Based on the new systematic data for the taxonomical classification, *Giardia lamblia* belongs to the:

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Metamonada

Subphylum: Trichozoa

Class: Trepomonadea;

Subclass: Diplozoa

Order: Giardiida

Family Giardiidae

Genus: *Giardia*

Species: *lamblia* (Cavalier-Smith, 2003).

2.1.2History of the Discovery and Species Designation of *Giardia*

An appropriate classification for *Giardia* spp. is critical to an understanding of the pathogenesis and epidemiology of infection, as well as the biology of the organism. This process has been difficult for a number of reasons (Adam, 2001).

(i) The (presumed) asexual nature of the organism does not allow mating experiments to allow species designation. For clonal organisms in the same clade, there are no well-defined criteria for species designation; these designations remain controversial.

(ii) Many of the earlier descriptions of *Giardia* spp. assumed a different species for each host and consequently overestimated the number of species. Subsequent species descriptions based on morphologic differences detected by light microscopy have probably underestimated the differences among isolates, strains, or species.

(iii) Cross-transmission experiments of *Giardia* from one host to another have yielded inconsistent results.

(iv) The available tools for distinguishing *Giardia* isolates have been inadequate until the recent introduction of molecular and electron micrographic techniques for classifying *Giardia* spp. In view of these concerns, a review of the history of the description of *Giardia* and the designation of *Giardia* species is warranted.

Giardia was initially described by van Leeuwenhoek in 1681 as he was examining his own diarrhoeal stools under the microscope. The organism was described in greater detail by Lambl in 1859, who thought the organism belonged to the genus *Cercomonas* and named it *Cercomonas intestinalis* (Adam, 2001).

Giardia lamblia is pear shaped and has one or two transverse, claw-shaped median bodies; *G. agilis* is long, slender and has a teardrop-shaped median body; and the *Giardia muris* trophozoite is shorter and rounder and has a small, rounded median body. *G. lamblia* is found in humans and a variety of other mammals, *G. muris* is found in rodents, and *G. agilis* is found in amphibians (Ayalew, 2006).

For the *Giardia* isolates grouped with *G. lamblia* on the basis of morphologic criteria discernible by light microscopy, differences that can be detected by electron microscopy have allowed the description of additional species, *G. psittaci* from parakeets and *G. ardeae* from herons. Another

species, *Giardia microti*, has been suggested on the basis of host specificity for voles and muskrats, differences in the cyst as assessed by electron micrography, and by differences of the 18S rRNA sequences compared with *G. lamblia* of human origin (van Keulen *et al.*, 1998).

2.1.3 Multiplication and life cycle of *Giardia*

Giardia species have two major stages in the life cycle. Infection of a host is initiated when the cyst is ingested with contaminated water or, less commonly, food or through direct fecal-oral contact. The cyst is relatively inert, allowing prolonged survival in a variety of environmental conditions. After exposure to the acidic environment of the stomach, cysts excyst into trophozoites in the proximal small intestine. The trophozoite is the vegetative form and replicates in the small intestine, where it causes symptoms of diarrhoea and malabsorption. After exposure to biliary fluid, some of the trophozoites form cysts in the jejunum and are passed in the feces, allowing completion of the transmission cycle by infecting a new host (Adam, 2001).

The trophozoite, the actively metabolizing, motile form, lives in the duodenum and jejunum and multiplies by binary fission. Trophozoites that are swept into the faecal stream lose their motility, round up, and are excreted as dormant, resistant cysts. The cyst is sufficiently hard to survive host-to-host transfer. *Giardia* infection is acquired by ingestion of mature cysts (infective dose varies from 10-100 cysts) via contaminated water or food. The exposure of cysts to host stomach acidity and body temperature triggers excystation, with the emergence of trophozoites which rapidly multiply and attach to the host small intestinal villi by means of a disk on their posterior or ventral surface (Meyer, 1990).

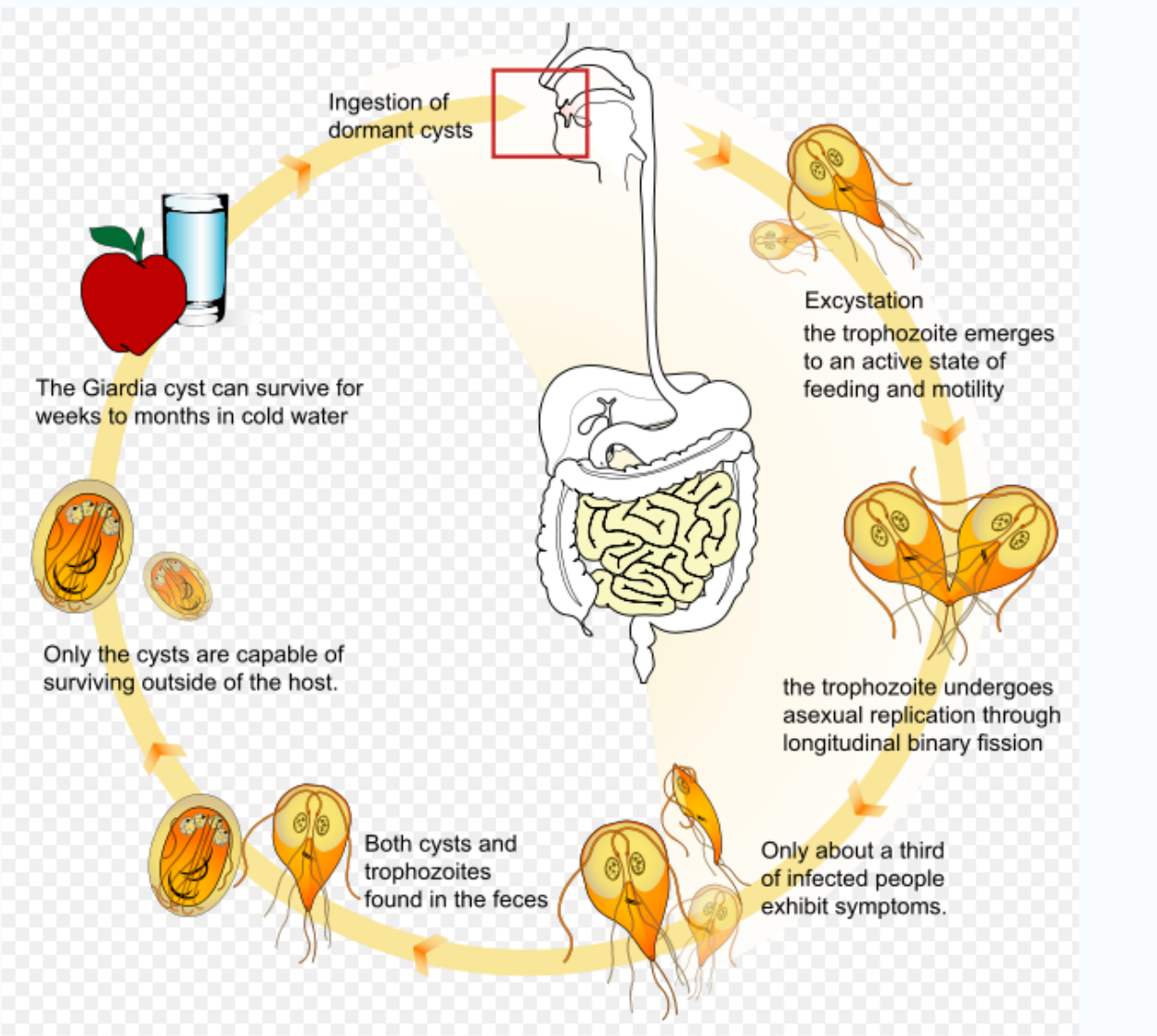
2.1.4 Cell Biology

2.1.4.1Trophozoite structure

The *G. lamblia* trophozoites are pear-shaped and are approximately 12 to 15 mm long and 5 to 9 mm wide. The cytoskeleton includes a median body, four pairs of flagella (anterior, posterior, caudal, and ventral), and a ventral disk. Trophozoites have two nuclei without nucleoli that are located anteriorly and are symmetric with respect to the long axis. Lysosomal vacuoles, as well as ribosomal and glycogen granules, are found in the cytoplasm. Golgi complexes become visible in encysting trophozoites but have not been confirmed to be present in vegetative trophozoites. However, stacked membranes suggestive of Golgi complexes have been demonstrated (Adam, 2001).

2.1.4.2 Nuclear structure and replication

G. lamblia trophozoites have two nuclei that are nearly identical in appearance. They replicate at approximately the same time and are both transcriptionally active as determined by uridine incorporation into nuclear RNA. Both nuclei have approximately equal numbers of rDNA genes as determined by in situ hybridization using the rDNA probe. Both have approximately equal amounts of DNA as determined by the intensity of nuclear staining with 49,6-diamidino-2-phenylindole (DAPI) or propidium iodide, although the possession of equal amounts of DNA by the two nuclei has been questioned (Kabnick and Peattie, 1990).



Giardia_life_cycle_en.svg (SVG file, nominally 900 × 859 pixels, file size: 555 KB)

Figure 2. 1: Life cycle of *Giardia lamblia*

Source: http://en.wikipedia.org/wiki/File:*Giardia*_life_cycle_en.svg

2.1.4.3 Cytoskeleton and Motility

Trophozoites colonize the small intestine of their host, predominating in the mid-jejunum. They attach by their concave ventral surfaces (ventral adhesive disk) to the intestinal wall, where they obtain the necessary nutrients and avoid transport beyond the jejunum. The ventral disk mediates a mechanical attachment not only to the intestinal wall but also to the surface of the container used for axenic growth. Neither cellular invasion nor receptor-mediated attachment has been documented for *Giardia* spp. Therefore, the cytoskeleton and especially the ventral disk play a key role in the survival of the organism in the intestine of the host (Adam, 2001).

The trophozoite has four pairs of flagella that begin at two sets of basal bodies that are near the midline and anteroventral to the nucleus. They emerge from the anterior, posterior, caudal, and ventral regions of the trophozoite. Paraflagellar rods extend along one side of the two ventral flagella. Nine pairs of microtubules encircle two microtubules to form the flagella. The flagella appear to be important for motility but not for attachment. In addition, their early emergence through the cyst wall during the process of excystation suggests their importance in excystation (Lujan *et al.*, 1996a).

2.1.4.4 Cyst structure

Encystation occurs after organisms have undergone nuclear replication but before cytokinesis; therefore, cysts contain four nuclei. They are approximately 5 by 7 to 10 mm in diameter and are covered by a wall that is 0.3 to 0.5 mm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes. The outer portion of the cyst wall is covered by a web of 7- to 20-nm filaments. Four major proteins have been identified in the outer cyst wall, 29, 75, 88, and 102 kDa in size. The sugar component of the outer portion is predominantly

galactosamine in the form of *N*acetylgalactosamine (GalNAc). Earlier claims that the cyst wall is composed of chitin (*N*-acetylglucosamine) have been refuted (Adam, 2001).

As the environmentally stable form of the life cycle, cysts have a metabolic rate only 10 to 20% of that found in trophozoites. The respiration of both cysts and trophozoites is stimulated by ethanol, while trophozoites are stimulated only by glucose (Lujan *et al.*, 1995).

2.1.4.5 Encystation

i. Promoting and inhibiting factors. As trophozoites replicate and colonize the intestinal surface, some encyst in the jejunum after exposure to biliary secretions. Encystation has been performed*in vitro* by exposing trophozoites to an environment that mimics that of the jejunum. Specific conditions that promoted encystation included a mildly alkalotic pH of 7.8 and conjugated bile salts plus fatty acids. Other investigators reported that expression of cyst wall protein, a marker of the encystation process, was detected 90 min after exposure to medium with lipoprotein-deficient serum. Encystation was abolished when cholesterol was added, leading to the proposal that encystation results from cholesterol starvation. The relative importance of cholesterol starvation and addition of bile salts and fatty acids for the induction of encystation remains controversial (Lujan *et al.,* 1996a).

ii. Events of encystation. Encystation can be divided into two phases, early and late. The timetables determined by various studies have differed somewhat, probably because of the different approaches used. After the initiation of encystation conditions, the organisms do not all enter the encystation process at the same time. Therefore, morphologic studies that examine the time course of the first encysting organisms tend to give earlier times for the encystation events than studies that depend on pooled organisms (Que *et al.*, 1996).

The early phase consists of the intracellular synthesis and transport of cyst wall components. A Golgi-like stack of membranes become readily visible by EM and are probably the means of transporting cyst-specific molecules to the encystation- specific vesicles (ESV). The Golgi-like membrane labels with NBD-ceramide and protein secretion is inhibited by brefeldin A, as expected for the Golgi complex. Expression of BiP, the molecular chaperone found in the ER, is increased during encystation. The ESV become visible by light microscopy and are probably involved in the transport of building blocks to the nascent cyst wall. A set of proteins of 21 to 39 kDa are expressed in the early phase. A monoclonal antibody to several bands of 26 to 44 kDa initially labels the ESV and subsequently labels the cyst wall, indicating transport of these molecules to the cyst wall. The genes encoding two of the proteins incorporated into the cyst wall have been cloned and characterized, a 26-kDa CWP1 and a 39-kDa CWP2. They are related leucine-rich proteins and form a stable complex that is transported to the cyst wall by the ESV (Lujan *et al.,* 1996b).

iii. Enzymatic pathways. The major cyst wall sugar, *N*-acetylgalactosamine, is produced by an enzymatic pathway that is induced during encystation. The activities of each of the enzymes in the proposed pathway have been documented and two of the enzymes have been cloned and characterized. In the first step, fructose-6-phosphate from the glucose metabolism pathway is converted to glucosamine- 6-phosphate by glucosamine-6-phosphate-isomerase (Adam, 2001).

One form of this enzyme (gpi2, or Gln6PI-A) is expressed constitutively at low levels during the entire life cycle. Transcription of the other form the enzyme (gpi1, or Gln6PI-B) is greatly increased during encystation. This up-regulated gene has two transcripts with different initiation sites. The shorter transcript is expressed constitutively at low levels, while expression of the longer transcript with the 147 nucleotide 59 untranslated region (UTR) is markedly increased

during encystation. Another enzyme in this pathway, UDP-*N*-acetylglucosamine pyrophosphorylase, is allosterically regulated, with a sixfold increase in activity from activation by glucosamine-6-phosphate. During encystation, the uptake of oxygen and glucose decreases substantially so that glucose uptake is undetectable within 16 h of exposure to encystation conditions. However, aspartate uptake is unchanged, resulting in the suggestion that gluconeogenesis may result from the importation of amino acids (Maroulis *et al.,* 2000).

2.1.4.6 Excystation

i. Promoting and inhibiting factors. In the mammalian host, excystation occurs with exposure to the contents of the proximal small intestine after passage through the acidic environment of the stomach. Excystation was first induced *in vitro* by exposure of animal- and human-derived cysts to an acidic pH. Optimum excystation occurred following exposure to a pH of 1.3 to 2.7. Subsequently, excystation of *G. muris* cysts has been performed at pH 7.5 in phosphate buffer with bicarbonate, indicating that an acidic pH was not required for excystation (Meng *et al.,* 1996).

The inhibition of excystation by 4-49-diisothiocyanatostilbene- 2-29-disulfonic acid (DIDS) suggested that vacuolar acidification was required. Subsequent studies of cysts produced *in vitro* found an optimal pH of 4.0. Excystation was facilitated by pancreatic proteases and inhibited by a trypsin inhibitor, suggesting the importance of proteases in excystation (Ward *et al.,* 1997).

In addition to external proteases, a *G. lamblia* cysteine protease (CP2) of the cathepsin B family is required for excystation. Cysteine protease activity was found in the endosome- lysosome-like vacuoles. Inhibitors of cysteine proteases prevented excystation but did not affect trophozoite growth or replication. Certain calmodulin antagonists (TFP and W7, but not W5) inhibited

excystation, suggesting that calmodulin may be involved in excystation (24). Excystation is inhibited by antibody to cyst wall and by wheat germ agglutinin (WGA), presumably by its reaction with one or more of the glycoproteins found in the cyst wall (Ward *et al.*, 1997).

ii. Morphology of excystation. After exposure to conditions that promote excystation, the process of excystation is rapid, being completed within 10 min for *G. lamblia* or for *G. muris*, and appears to be similar for both species. After the initiation of excystation, one or two pairs of cytoplasmic protrusions (preventral flanges) appear to develop into the ventral flange. The peritrophic space and the preventral flange enlarge as the emerging trophozoite separates from the cyst wall. Externally, the emergence of flagella through the opening cyst wall is followed by the entire trophozoite (Hetsko *et al.,* 1998).

Karyokinesis occurs during encystation, so that the emerging trophozoite has four nuclei but only the eight flagella of a single trophozoite. The oval trophozoite becomes more rounded and then undergoes cytokinesis within 15 to 30 min after the onset of excystation, so that two trophozoites are formed from one cyst (Hetsko *et al.*, 1998).

2.1.5 Pathogenesis of Giardiasis

The pathogenesis of diarrhoea in giardiasis is thought to be related to the following factors: (1) the number of organisms ingested, (2) the specific strain ingested, (3) non antibody protective factors in the gastrointestinal tract, and (4) the immune response of the host (Meyer, 1990).

Lectin, a protein on the trophozoite lining, recognizes specific receptors on the intestinal cell and may be partly responsible for the tight attachment between the parasite and the villi, which is followed by mucosal damage, mechanical obstruction (only caused in the presence of numerous organisms), and deconjugation of bile salts (Washington Winn *et al.*, 2006).

Studies have indicated that inflammatory mast cells may interfere with duodenal growth of *G. lamblia* trophozoites. Other inflammatory cells, as well as CD8+ T cells, contribute to villus-shortening and crypt hyperplasia. Inflammation results in an increased turnover rate of intestinal mucosal epithelium. The immature replacement cells have less functional surface area and less digestive and absorptive ability (Meyer, 1990).

2.1.6 Genotypes of *G. lamblia*

Molecular classification tools have been of great value in understanding the pathogenesis and host range of *Giardia* isolates obtained from humans and a variety of other mammals. The first study of the molecular differences of *G. lamblia* isolates was a zymodeme analysis of five axenized isolates, three from humans, one from a guinea pig, and one from a cat, using six metabolic enzymes. Zymodeme analysis consists of the typing of organisms based on the migration of a set of enzymes on a starch gel in the presence of an electric field. The migration depends on the size, structure, and isoelectric point of these enzymes. Since these properties are a function of the primary amino acid sequence, differences in the zymodemes should reflect differences in the sequences of the genes encoding these enzymes (Adam, 2001).

Likewise, classification by surface antigens is limited by antigenic variation of the variant-specific proteins (VSPs). These studies have been very useful, but the conclusions that can be drawn from these types of data are limited by the semiquantitative nature of the data. To allow a more quantitative comparison of *Giardia* isolates, sequence comparisons of the small-subunit rRNA, triosephosphate isomerase (*tim*), and glutamate dehydrogenase (GDH) genes have been utilized in a number of subsequent studies (Monis *et al.*, 1999).

The small-subunit or 18S rRNA (SS rRNA) sequence shows a 1% divergence between groups 1 and 3, reflecting its more highly conserved sequence. In addition to their marked genetic differences, the two genotypes may have a number of important biologic differences. For example, the GS isolate (group 3) was significantly more pathogenic in infections of human volunteers than was the WB isolate (group 1). Group 3 organisms also appear to grow much more slowly in axenic culture than do genotype 1 organisms (Karanis *et al.,* 2007).

More recently, a number of additional assemblages (genotypes) have been proposed for *Giardia* isolates from a variety of mammals. These isolates are morphologically identical to human *G. lamblia*, but sequences of their protein-coding regions differ. These studies have allowed the identification of a dog isolate that is genetically distinct from human *G. lamblia.Giardia* isolates from dogs have been notoriously difficult to axenize, in comparison to isolates from humans, leading to the proposal that *Giardia* isolates from dogs were different from cat or human isolates. However, a few dog isolates have been axenized and characterized (Hou *et al.,* 1995).

To further evaluate the zoonotic potential of dog *Giardia*, suckling-mouse infections were established from 11 consecutive infected dogs. On the basis of sequence analysis, these were assigned to two assemblages (C and D) that were quite distinct from assemblages A and B. A PCR-based study of nine fecal isolates from dogs found that one of the nine was similar to human isolates while the other eight were different. These results suggest that most dog isolates are genetically distinct from those found in humans and have little or no potential for zoonotic transmission (Monis *et al.,* 1998).

2.1.7 Clinical manifestations of *Giardia lamblia* infection

The length of the incubation period, usually 1 to 3 weeks, depends at least partly on the number of cysts ingested. Normal human hosts with giardiasis may have any or all of the following signs and symptoms: loose, foul-smelling stools, steatorrhea (fatty diarrhoea), malaise, abdominal cramps, excessive flatulence, fatigue and weight loss or a coeliac-disease-like syndrome (Washington Winn *et al.*, 2006).

2.1.8 Host defense to *Giardia lamblia* infection

The fact that many *Giardia* infections in humans and experimental animals resolve spontaneously implies that an effective host immune response develops (Heresi and Cleary, 1997).

There is evidence that both humoral and cellular immune mechanisms are involved. Hosts with gamma globulin deficiencies are prone to severe disease, suggesting that humoral immunity plays a role. *In vitro* studies have found that normal human milk (but not cow milk or goat milk) kills trophozoites *G. lamblia,* and that this killing does not depend on secreted IgA.This finding raises the possibility that even mother's milk that does not have antiprotozoal antibody may protect infants exposed to these parasites (Meyer, 1990).

2.1.9 Host-Parasite Coevolution

Whenever members of a group of parasitic organisms parasitize different hosts, it is appropriate to address the question of coevolution of the host and parasite. For *Giardia* spp., the ability to do so has been limited because of the uncertainty about the host specificity of a specific *Giardia* species or genotype as well as the problem of appropriate classification of isolates. The

development of good molecular classification tools and a better understanding of host specificity makes it reasonable to address the possibility of coevolution of different species or genotypes of *Giardia* with their hosts.

The greater difference between *G. lamblia* and *G. ardeae* than among the *G. lamblia* genotypes supports the idea that the divergence between *G. lamblia* and *G. ardeae* accompanied the divergence between birds and mammals. However, *G. lamblia* and *G. ardeae* are closer to each other than to *G. muris*, the opposite of the expected finding, since mice diverged from other mammals more recently than from birds. Sequence information is not yet available from *G. agilis* to allow a similar comparison of the amphibian and mammalian *Giardia* species. The closer than-expected relationship between *G. ardeae* and *G. lamblia* could perhaps be explained if transmission of *Giardia* between birds and mammals was followed by divergence of the two lineages with their hosts (Adam, 2001).

2.1.10 Genome Structure

The genome of *G. lamblia* has the features expected of eukaryotic cells, including linear chromosomes flanked by telomeres that are similar in sequence to those of other eukaryotes (TAGGG). Chromosomal DNA of eukaryotes forms chromatin by associating with four core histones (H2a, H2b, H3, and H4) and a linker histone (H1) to form nucleosomes (Adam, 2001).

G. lamblia has all four of the core histones, and these histones are very similar to those of other eukaryotes and have no particular similarity to the histone-like proteins of the Archaea. The GC-rich rDNA genes, as well as certain protein-coding genes that were sequenced in some of the initial molecular work on *G. lamblia*, suggested a GC-rich genome. However, the G1C content

of the contigs that have been assembled as part of the genome project have yielded a GC content of 49%, excluding the rDNA genes (Mc Arthur *et al.,* 2000).

PFGE separations of chromosomal DNA have been used to demonstrate that *G. lamblia* trophozoites have five chromosome linkage groups ranging in size from approximately 1.6 to 3.8 Mb. The initial report of PFGE separations of *G. lamblia* chromosomes identified four intensely staining ethidium bromide-stained chromosomal bands for most isolates (including ISR and WB) and five bands for JH. Probes specific for each of the five chromosomal bands of the JH isolate were used to demonstrate that the smallest of the four bands of the ISR and WB isolates represented two comigrating chromosomes (Adam, 2001).

2.1.11 Surface antigens and antigenic variation

2.1.11.1 Occurrence and biological significance of antigenic variation

Giardia lamblia trophozoites undergo antigenic variation of a family of immunodominant cysteine-rich surface antigens *in vitro* and *in vivo*. The initial studies of the surface antigens of *G. lamblia* showed differences among strains by crossed immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA) and marked differences in the molecular masses of "excretory-secretory products" from different surface- iodinated *G. lamblia* isolates. These surface antigens varied in number and in size from approximately 50 to 200 kDa in a study of 19 isolates. A monoclonal antibody (MAb 6E7) for a 170-kDa surface antigen (initially called CRP170 but now called VSPA6) from the WB isolate was cytotoxic for WB trophozoites but not for isolates expressing other surface antigens. WB trophozoites were doubly cloned by limiting dilution and incubated with MAb 6E7; some organisms survived and were totally resistant to the cytotoxic effect of MAb 6E7. One of these cloned lines of organisms (WB1269) had a 68-kDa surface-

labeled antigen (initially called CRP68 but now called VSP1269), while another (WB1267) had a 64-kDa surface antigen (VSP1267) (Nash and Mowatt, 1992).

A monoclonal antibody (MAb 5C1) reactive with the surface antigen of WB1267 was cytotoxic for WB1267 trophozoites. These trophozoites were incubated with MAb 5C1, and organisms surviving the cytotoxic antibody reacted with neither of the initial MAbs and expressed surface antigens in the 60- to 100-kDa size range. Subsequent data have confirmed that individual organisms express only one VSP at a time; the detection of multiple surface-labeled bands in some of the studies resulted from subpopulations of trophozoites expressing several different VSP types. In axenic culture, variation occurs approximately once every 6 to 12 generations for a frequency of 1023 to 1024 (Nash and Mowatt, 1992).

2.1.11.2 Other Surface Antigens

G. lamblia trophozoites also have a 49-kDa antigen (GP49) that is attached to the membrane surface by a glycosylphosphatidylinositol (GPI) anchor. During synthesis of the GPI anchor, exogenous phosphatidylinositol is incorporated, but *myo*-inositol is converted to phosphatidylinositol prior to incorporation. The GPI anchor is not subject to cleavage by phospholipase C, in contrast to a variety of other GPI anchored surface molecules, including the trypanosome variant surface glycoprotein. GP49 is constant among different isolates and does not demonstrate variation within single isolates (Adam, 2001).

2.2 *Cryptosporidium parvum*

Cryptosporidium parvum is a member of Apicomplexa that has been found in human and animal populations worldwide. People from both developed and developing countries are vulnerable to these important opportunistic protozoa. It has a predilection for epithelial cells in the digestive

tracts of a wide variety of hosts which include humans, livestock, companion animals, wildlife, birds, reptiles and fish (O'Donoghue, 1995). The protozoan is responsible for moderate to severe opportunistic infection in both immunocompetent and immunocompromised individuals, the latter group being more susceptible with fatal consequences. The immunocompetent individuals usually experience self-limiting disease often manifested by acute profuse, watery diarrhoea accompanied by abdominal pain and other enteric symptoms like vomiting, low grade fever, general malaise, weakness, fatigue, loss of appetite, nausea, chills and sweats. In contrast, the disease may be chronic and even life-threatening for undernourished infants and AIDS patients. Other groups at risk include people who have chronic disease, malnutrition or other debilitating conditions, which lead to compromised immune system. Further, the problem becomes more critical and complex by the lack of curative therapy (Siddiki and Masuduzzaman, 2009).

Cryptosporidium parvum, an important zoonotic protozoan has been found to infect both human and animal populations throughout the world. The zoonotic form of infection caused by this protozoan has also been reported by many investigators and has led to many cross transmission studies to understand the complex epidemiology of cryptosporidiosis. The waterborne transmission through drinking water or swimming pool is quite common resulting in a number of outbreaks in many countries worldwide. While many physical and chemical methods are employed to remove the oocysts from drinking water, it is still difficult to completely remove it through any single technique. The parasite has a unique intracellular but extracytoplasmic location and several other unusual features like presence of relict mitochondria, unique life cycle features like production of two types of oocysts, ability to autoinfection and lack of host specificity. More importantly, the opportunistic pathogen can produce an infection that may be chronic and even life-threatening for undernourished infants and AIDS patients.Numerous *in*

vivo and *in vitro* drug trials have been conducted against this important opportunistic pathogen but none was found to be completely effective against cryptosporidiosis (Siddiki and Masuduzzaman, 2009).

Cryptosporidiaare intracellular parasites, occurring in large numbers in small intestinal mucosal epithelial cells. The oval oocyst (the form passed in the feces) is 4µm to 5µm long and may be seen to contain four sporozoites (Navin and Juranek, 1984; Current and Owens, 1989).

Cryptosporidium infection is common worldwide, and its frequency may be related to the inadequacy of sanitation. In general, the infections are more common in developing countries (Meyer, 1990).

2.2.1 Taxonomy of *Cryptosporidium parvum*

Cryptosporidium parvum belongs to the:

Kingdom: Protozoa

Phylum: Apicomplexa

Class: Coccidea

Order: Eucoccidiorida

Family: Cryptosporidida

Genus: *Cryptosporidium*

Species: *parvum*(Fayer, 2008)

2.2.2 Historical Perspective

Taxonomically *C. parvum* belongs to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Eimeriorina, family Cryptosporidiidae (Siddiki and Masuduzzaman, 2009). However, recent molecu-lar studies have shown that members of the genus are actually more closely re-lated to the gregarines than to eimerias or plasmodium (Carreno *et al.,* 1999)**.** After the first report in mice, in 1955 the organ-ism was recognised as a potential cause of diarrhoea in turkeys (Siddiki and Masuduzzaman, 2009). Subsequently it was identified in other animal species in which the infection was thought to be either opportunistic and harmless or associated with individual cases of diarrhoea (Tzipori and Griffiths, 1998). Cryptosporidiosis has long been a veterinary problem, mainly in young farm animals, such as calves. The first two cases of human cryptosporidiosis were reported in 1976 (Siddiki and Masuduzzaman, 2009).

Until 1982 however, *Cryptosporidium* was rarely reported in humans. Later, it has been reported to infect humans of 95 different countries throughout the world (Morgan-Ryan *et al.,* 2002). The number of detected cases began to rise rapidly along with the AIDS epidemic and the development of methods to identify the parasite in stool samples. Until now at least 22 species of *Cryptosporidium* have been named based on host occurrence, parasite morphology, host predilection and site of infection. However, only 13 species are considered valid by most investigators (Ramirez *et al.,* 2004). Again, an increasing number of genetically distinct intraspecific variants or genotypes of *C. parvum* have been described, many of which appear to be host-specific and could repre-sent distinct species. *C. parvum* has some unique features like unusual location within the host cell, sequestered between the cell cytoplasm and cell membrane, lack of host specificity, and innate antimicrobial resistance which distinguish it from other

enteric protozoa. Unlike other Apicomplexa, *C. parvum* produce two types of oocysts (thin and thick walled) and the thin walled oocyst is responsible for autoinfection within the host. Thus, the repeated first generation schizogony contributes considerably to its pathogenesis (Siddiki and Masuduzzaman, 2009).

2.2.3 Multiplication and life cycle of *Cryptosporidium parvum*

The life cycle of *C. parvum* has been out-lined in a number of studies. In experimental animals, the prepatent period (PPP), i.e. the interval between infection and the first appearance of oocysts in the faeces, is generally 4 days (3 days in heavy infections). However, human infection with low oocyst ingestion can extend the PPP up to 6 days depending on the dose size and frequency. Patency, which is the length of time oocysts are shed in the faeces, generally lasts 6−18 days (4−10 days of diarrhoea) in immunocompetent individuals but may be prolonged in immuno-suppressed patients. Some individuals shed oocysts but appear asymptomatic which is the subclinical form of cryptosporidiosis (Siddiki and Masuduzzaman, 2009).

Cryptosporidium is monoxenous, that is, its life cycle is completed within one host. The parasite moves from host to host via the faecal-oral route (Siddiki and Masuduzzaman, 2009). The life cycle begins with the ingestion of oocysts which excyst in the intestine, releasing sporozoites. Thereafter, two cycles of schizogony are followed by gametogony with the production of male and female gametocytes (Tzipori and Griffiths, 1998). The asexual cycle of *C. parvum* life cycle starts with the ingestion of thick walled oocysts from the external source or autoinfection with the thin walled oocysts of earlier infection. The sporozoite is the infective stage and after its invasion into the epithelial cells, schizogony takes place with production of two different types of merozoites. After ingestion, the oocysts excyst in the gastrointestinal tract releasing the infective sporozoites. Various factors including reducing conditions, carbon dioxide,

temperature, pancreatic enzymes and bile salts are thought to be associated with triggering the excystation. The release of sporozoites takes place through a slit-like opening created at one end of the oocyst by dissolution of a special suture in the oocyst wall. The sporozoites then are ready to attach with epithelial cells where they become enclosed within parasitophorous vacuoles and develop trophozoites (Siddiki and Masuduzzaman, 2009).

Cryptosporidium species live inside the epithelial cells of the intestinal tract of a variety of vertebrates, but some species can infect the respiratory tract (Meyer, 1990). Infection starts with ingestion of an oocyst containing four sporozoites. The excystation procedure usually requires reducing conditions. Digestive enzymes such as pancreatic enzymes and bile salts trigger the release of sporozoites from the oocyst; however, it is possible for *Cryptosporidium* oocysts to exist in warm aqueous solutions without any special stimuli (Sterling and Arrowood, 1986).

Sporozoites enter epithelial cells, taking up a position that is inside the cell (intracellular) but outside the cytoplasm (extra-cytoplasmic). They develop as trophozoites and undergo an asexual multiple budding process (schizogony) to produce a schizont, which releases merozoites. Merozoites invade other epithelial cells, develop into trophozoites and also undergo schizogony to release more merozoites. Merozoites of this second cycle of asexual reproduction infect further epithelial cells but mature into either ‘male (microgamonts) or female (macrogamonts)’ gametes to begin the sexual part of the life cycle. Microgamonts produces microgametes which, on release, fertilize the macrogamont to produce a zygote. The zygote may follow two different oocysts generating developmental routes. It can either transform into an oocyst by secretion of a thick wall and development of sporozoites, or into a thin walled oocyst containing sporozoites. Thin walled oocysts are able to liberate sporozoites, thus allowing further rounds of asexual reproduction within the host (Mahon and Manuselis, 2000).

Approximately 20% of the zygotes develop into thin-walled oocysts, which represent auto infective life cycle forms that can maintain the parasite in the host. *Cryptosporidium* is an obligate parasite and cannot grow outside the body except in tissue culture (Current and Garcia, 1991; Slifko *et al.*, 2002).

Figure 2.2 shows the life cycle of *Cryptosporidium parvum* where1-4shows the Asexual cycle of the endogenous stage: (1) sporozoite or merozoite invading a microvillus of a small intestinal epithelial cell; (2) a fully grown trophozoite; (3) a developing schizont with eight nuclei; (4) a mature schizont with eight merozoites. So as 5 and 6 shows the sexual cycle; (5) microgametocyte with many nuclei; (6) macrogametocyte. Number 7 shows a mature oocyst containing four sporozoites without sporocyst while 8 shows the Oocyst discharged in the feces. (a) Merozoite released from mature schizont; (b) sporozites released from mature oocyst (Tzipori, 1983).

2.2.4 Outbreaks of *Cryptosporidium* infection

Although the first report of *Cryptosporidium* infection in mice was published by Tyzzer in 1907, it was not until the 1980s when it was reported as a cause of death in AIDS patients. The earliest cases of human cryptosporidiosis were diagnosed in animal handlers. An outbreak at a day care centre was first documented in 1983. In 1987, 13,000 people in Carrollton, Georgia became ill with cryptosporidiosis. This was the first report of its spread through a municipal water system that met all state and federal drinking water standards. It also sparked great public health concern after the large human waterborne outbreak in Milwaukee in 1993 when over 40,000 people were affected with nearly 100 deaths.

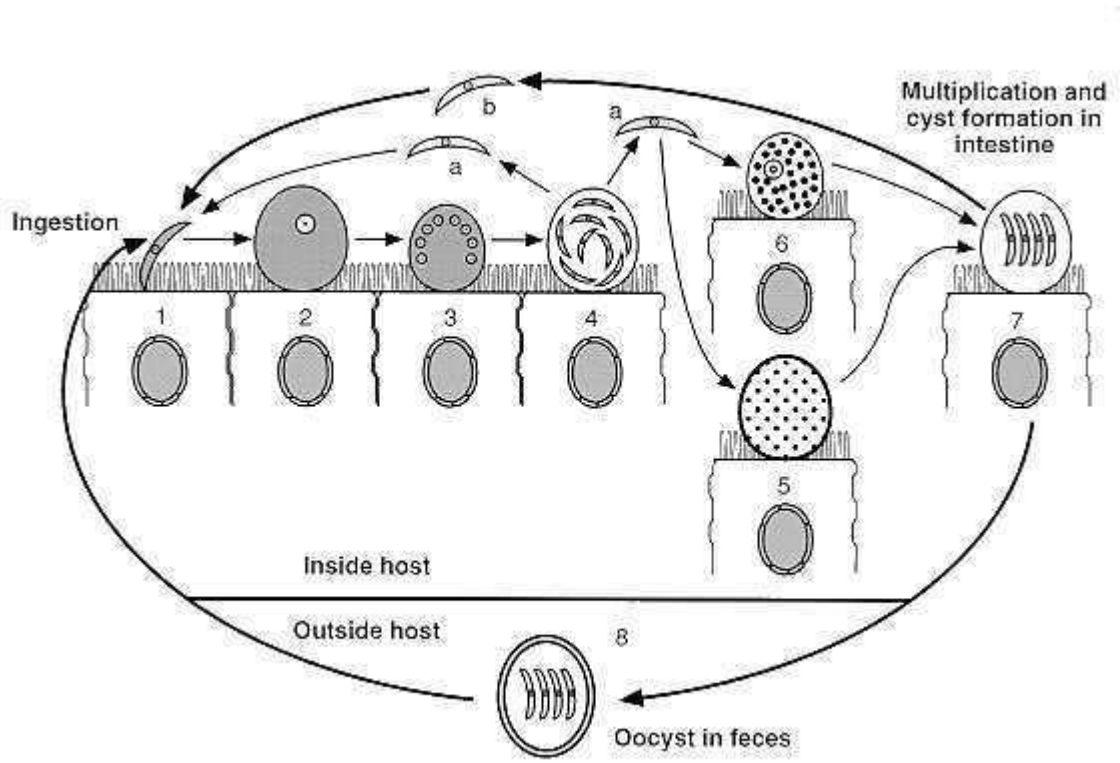


Figure 2. 2: Life Cycle of *Cryptosporidium parvum*

Source: Tzipori, 1983.

The total cost of outbreak-associated illness was $96.3 million: $31.7 million in medical costs and $64.6 million in productivity losses. The average total costs for persons with mild, moderate, and severe illness were $116, $475, and $7,808, respectively (Corso *et al.,* 2003).

These outbreaks focused attention on the risk of waterborne cryptosporidiosis and the possible need for stricter drinking water standards. Again, the impact of cryptosporidiosis on animals can be devastating, resulting in morbidity, poor growth, and even mortality, with associated economic losses. The important thing is that infection in domes-tic and wild ruminants provides the biggest sources of environmental contamination. Apparently healthy calves can also become subclinically infected and contribute to oocyst excretion in faeces (Siddiki and Masuduzzaman, 2009). Since the Milwaukee outbreak, until 1998 there have been at least 23 reported waterborne outbreaks of cryptosporidiosis in the UK and North America (Siddiki and Masuduzzaman, 2009).

The *Cryptosporidium* oocysts may remain viable in water for over 140 days. They are very resistant to commonest disinfectants and therefore, difficult to be destroyed by conventional chlorination treatment (Ramirez *et al.,* 2004). The waterborne transmission through swimming pool or public water parks thus plays a prominent role when it is almost impossible to determine the origin of many individual cases of cryptosporidiosis (Siddiki and Masuduzzaman, 2009).

2.2.5 Pathogenesis of *Cryptosporidium parvum*

After invasion of the enterocytes, the epithelial cells release cytokines. These cytokines activate phagocytes and recruit new leukocytes, which, in turn, release soluble factors (resulting in intestinal secretion of chloride and water) and inhibit absorption.

Enterocyte damage may be a direct consequence of parasite invasion, multiplication, and extrusion. Regardless of the specific mechanism, marked distortion of the villus architecture is accompanied by nutrient malabsorption and osmotic diarrhoea (Mahon and Manuselis, 2000; Hunter *et al.*, 2004).

Examination of biopsy material from symptomatic patients has revealed a variety of changes in intestinal mucosa, including partial villous atrophy, crypt lengthening, low cuboidal surface epithelium, cellular infiltration of the jejunal and ileal lamina propria, and inflammation (Washington Winn *et al.*, 2006).

More severe cases of cryptosporidiosis are observed in patients with AIDS who have very low CD4 counts <50-100/μL (Mahon and Manuselis, 2000).

2.2.6 Virulence of *Cryptosporidium parvum*

Of the 13 identified species of *Cryptosporidium, C. parvum* is the major species responsible for disease in humans and domestic animals such as cattle, horses, sheep, goat and pigs.

Several viru-lence factors that appear to be important in *Cryptosporidium* infections have been described and comprehensively reviewed. It has long been speculated that clinical severity and/or attack rates for *C. parvum* infections might be related to genotype and/or specific virulence differences among isolates. Analysis of genotypic heterogeneity in single and multiple loci has identified 2 major genotypes: genotype 1 (human) which infects primarily humans, and genotype 2 (bovine), which infects both humans and other mammals (Sulaiman *et al.*, 1998; Smith and Ronald, 2001). It is remarkable that although a number of *Cryptosporidium* isolates can replicate and produce oocysts in a variety of mammals, not all of them develop the same symptoms and sometimes remain asymptomatic. Understanding the factors that determine the

infectivity of *C. parvum* and regulate host-specificity is thus very important in the study of the pathogenesis of cryptosporidiosis in humans and other animals, and in providing information to formulate effective therapeutic and con-trol strategies (Siddiki and Masuduzzaman, 2009).

2.2.7 Viability of *Cryptosporidium* oocysts

The oocyst of the *Cryptosporidium sp.* is double layered and can persist in the environment very well. It can survive in water at temperature range of 4−22°C under ambient conditions. Several authors have studied the viability and infectivity of *Cryptosporidium* oocyst in different environmental conditions. A number of chemical and physical methods of oocyst inactivation has been reported where ozone, ultraviolet rays, chlorine, chlorine dioxide, mono-chloramine, ammonia etc has been tried with variable result (Carey *et al.*, 2004). Several investigations are underway using other physical and chemical methods for complete and effective destruction of *Cryptosporidium* oocysts from drinking water sources (Siddiki and Masuduzzaman, 2009).

2.2.8 Incidence of cryptosporidiosis

2.2.8.1 Immunocompromised hosts

Cryptosporidiosis continues to be a serious problem in immunocompromised patients and particularly in undernourished infants and children. The lack of an effective treatment and the propensity of the parasite to survive in and be transmitted through water sources are important public health hazard issues. *Cryptosporidium* has been reported in immunocompromised patients with impaired cell-mediated immunity due to primary states like common variable immunodeficiency, hypogammaglobulinaemia, severe combined immunodeficiency, X-linked hyper-IgM syndrome or gamma interferon deficiency. Other patients with secondary deficiencies

like HIV/AIDS, organ transplantation and treated with immunosuppressive drugs, haematological malignancies and anti-cancer chemotherapy are also vulnerable to severe *Cryptosporidium* infection (Siddiki and Masuduzzaman, 2009). A number of epidemiological studies of cryptosporidiosis in HIV/ AIDS patients have been reported throughout the world and reviewed by several authors. Prevalence of cryptosporidiosis among AIDS patients has been reported as 3−4% in USA and over 50% in Africa and Haiti. The severity and duration of illness depends on the host's immune status and most infections are chronic and debilitating, contributing to severe dehydration, weight loss and malnutrition, extended hospitalizations and mortality. Moreover, in AIDS patients the infection may spread throughout the bowel, extending into the bile ducts, gallbladder, or other mucosal surfaces exposed via vomiting and/or aspiration.

2.2.8.2 Immunocompetent hosts

The prevalence of cryptosporidiosis has ranged from 0.1−27.1% in developed industrialized countries compared to 0.1−31.5% in less developed countries. The epidemiological investigation reveals that almost 1−4% of patients with diarrhoea in developed countries and up to 16% in less developed countries are associated with *Cryptosporidium* infection. It is thought that the higher prevalence of infection in less developed countries is due to poor sanitation, contaminated water supplies, overcrowding or greater contact with domestic animals. Children are more susceptible to infection than adults and a number of reports mentioned higher incidence of cryptosporidiosis in malnourished compared to well-nourished children. Cryptosporidiosis is also reported as a risk factor of childhood death. Malnutrition affects cell-mediated immunity while acute cryptosporidiosis leads to malabsorption and anorexia making the condition further complicated (Siddiki and Masuduzzaman, 2009).

2.2.8.3 Domestic and companion animals

Cryptosporidiosis in animals has been reviewed by different authors (Olson *et al.,* 2003; Ramirez *et al.*, 2004). Infection of domestic and wild animals provides the biggest source of oocysts, which are responsible for environmental contamination. In USA, *Cryptosporidium* is reported to be present in more than 90% of all dairy farms and 50% or more of all dairy calves will shed detectable number of oocysts. Young animals are more susceptible to infection and disease while in adults it is asymptomatic in most cases. Calves are more susceptible shortly after birth and infection has been reported in both dairy and beef calves (At will *et al.,* 1999). The duration of infection is usually short, lasting about two weeks with peak oocysts shedding at second week of infection (O'Handley *et al.,* 1999; Uga *et al.,* 2000). Clinical signs are usually manifested in calves 7−30 days of age with mild to moderate, pale or yellowish diarrhoea, which is accompanied by mucus. The condition can last for two weeks and alongside dehydration, calves become lethargic and anorexic contributing to weight loss. They do not respond to antibiotic therapy and in more severe cases, dehydration and cardiovascular collapse lead to mortality (Olson *et al.,* 2003). However, the healthy calves can be sub-clinically infected which contributes con-tinuous oocyst excretion in faeces (Siddiki and Masuduzzaman, 2009).

The prevalence of cryptosporidiosis in sheep and goats is similar to that in cattle. The clinical signs and pattern of infection are also identical. *Cryptosporidium* is also an important cause of enteric infection in young lambs and goats. Severe outbreaks with high case fatality rates have been reported by several authors (Johnson *et al.,* 1999). Cryptosporidiosis in swine and horses is typically asymptomatic and reported worldwide. Prevalence of swine cryptosporidiosis has been recorded as 5% to 21.9% by several investigators from different geographic regions (Yu and Seo, 2004; Siddiki and Masuduzzaman, 2009).

Cryptosporidiosis has also been reported in dogs, cats and other pets and they are important source of infection for children, elderly and immunocompromised owners (Morgan *et al.,* 2000a, 2000b; Fayer *et al.,* 2001). The prevalence of canine cryptosporidiosis varies between 2% and 44% while puppies are more frequently infected. Cats with or without diarrhoea have been reported to shed *Cryptosporidium* oocysts and there is a high prevalence of *Cryptosporidium felis* in HIV-infected humans (Morgan *et al.,* 2000a). Once infected, cats continue to shed large number of oocysts for over 6 months, which poses a threat of potential zoonoses (Siddiki and Masuduzzaman, 2009).

2.2.8.4 Wild animals

Cryptosporidium infection has been reported in a wide range of wild animals including birds, reptiles and fish (Olson *et al.,* 2003). It has been reported that up to one-fifth of oocysts in agricultural drainage is contributed by the wildlife in UK. The most common species *C. parvum* has been reported in 155 species of hosts, mostly wild animals and birds. The zoonotic transmission either by direct exposure to contaminated animal faeces or during waterborne outbreaks may become contributory factors for human infection. On the other hand, exposure of domestic and wild mammals to human sewage may be a source of animal infection (Olson *et al.,* 2003). Wild birds are suspected to be the source of infection in farm animals and may also be responsible for the contamination of surface water. Although wild animals contribute greatly for environmental contamination of *Cryptosporidium* oocysts, not all species are infective for humans and domestic animals. Further molecular genotyping of environmental samples is highly essential to determine the impact of cryptosporidiosis in wild animals. This also will increase our understanding on the water-borne transmission of cryptosporidiosis to other domesticated animals and humans (Siddiki and Masuduzzaman, 2009)..

2.2.9 Host defenses to *Cryptosporidium*

The fact that cryptosporidiosis is self-limiting in normal persons but usually severe and long-lasting in lower animals and immunocompromised humans indicates that host immune mechanisms are probably involved in eliminating the parasite (Washington Winn *et al.*, 2006). Cell-mediated immunity appears to be the primary defense mechanism. Serum antibodies to *Cryptosporidium* have been shown to develop during recovery from infection (Meyer, 1990).

The importance of cryptosporidiosis in immune-compromised patients generally, and in AIDS patients in particular, should not be underestimated. In this group, cryptosporidiosis can cause diarrhoea that is severe, prolonged, and life-threatening. In cryptosporidiosis patients who are immune-suppressed because of drug treatment, the disease can be reversed by withdrawing the drug. Unfortunately, this cure cannot be resorted to with AIDS patients (Navin and Juranek, 1984; Washington Winn *et al.*, 2006).

2.2.10 Diagnosis of Cryptosporidiosis

A number of tests have been developed for the diagnosis of *Cryptosporidium*. These involve direct detection by microscopy of faecal materials after using specialized staining techniques. The modified acid-fast (Ziehl-Neelsen) stain and auramine stain are widely used but have the limitation of relatively low sensitivity with faeces. However, the examination under UV light with a rhodamine filter can increase the sensitivity up to 100 times (Nielsen and Ward, 1999). Use of polyclonal or monoclonal antibodies for detection by immunolabelling was also developed but has proved less sensitive like conventional staining. Recently developed PCR amplification technique has been found very specific and highly sensitive to target different genes encoding oocyst wall protein, the small-subunit rRNA, β-tubulin, thrombospondin-related

adhesive proteins of *Cryptosporidium* 1 and 2, internally transcribed spacer 1, polythreonine repeat, dihydrofolate reductase, un-known DNA sequences, mRNA of heat shock proteins (Sulaiman *et al.,* 1999). The efficacy of this technique for detection of *Cryptosporidium* in environmental and clinical samples was also reported in several studies. Certainly, the use of PCR technique with sequence analysis helps the genetic characterization to identify *Cryptosporidium* at species level which can contributes significantly for epidemiological investigations and assessment of sources and risk factors for zoonoses. The detection of oocysts in environmental water samples is a very important issue for the water industry. Additional re-search is needed for the development of easy, cost-effective, highly sensitive and rapid diagnostic tools for *Cryptosporidium* in clinical and environmental samples (Siddiki and Masuduzzaman, 2009).

2.2.11 Treatment and Control of Cryptosporidiosis

2.2.11.1 Chemotherapeutic agents

Chemotherapy of humans cryptosporidiosis has been reviewed by Coombs and Muller (2002), Coombs (1999) and Gargala (2008). Until now, there is no effective treatment for cryptosporidiosis. Supportive therapy involving oral or intravenous rehydration is the important option to alleviate the clinical signs. Although nitazoxanide was showed to reduce the duration of diarrhoea and oocyst excretion, its safety and efficacy in adult and immunosuppressed hosts are still unknown (Bicart-See *et al.,* 2000; Gilles and Hoffman, 2002; Ramirez *et al.,* 2004). The use of highly active antiretroviral therapy (HAART) in persons with AIDS has reduced the pre-valence of infection with *C. parvum* and the length and severity of its clinical course. HAART has shown to decrease by 90% the incidence of cryptosporidiosis in the USA. Again, there have

been a number of studies evaluating combination therapy. A good response was reported after prolonged use of a wide variety of combinations and dosages of protease inhibitors and/or nucleoside analogs that, along with paromomycin, spiramycin, or azithromycin, were examined in HIV-positive patients with chronic cryptosporidiosis (Maggi *et al.*, 2000; Siddiki and Masuduzzaman, 2009). Development of effective treatment has been limited by lack of a simple *in vitro* cultivation system to study biochemical and metabolic requirements and a good small animal model of screening the efficacy of drug candidates (Siddiki and Masuduzzaman, 2009).

2.2.11.2 Conventional anticoccidial agents

Despite the intensive efforts, treatment of cryptosporidiosis with available anticoccidials is still not satisfactory (Coombs and Muller, 2002). The macrolides, spiramycin and azithromycin have been found ineffective and less tolerable in both immunocompetent and immunodeficient individuals. Diclazuril was tested in humans with unsatisfactory results. In AIDS patients, letrazuril induced clinical improvements in up to 50% patients and some inhibitory activity on oocyst excretion. In an *in vitro* trial, monensin and halofuginone were found to reduce the parasite multiplication by more than 90% while tested *in vitro*. Unfortunately none of the widely used anticoccidials was suitable to treat clinically infected patients while some were suggested for prophylactic use. Still, the search for a new anticoccidial to treat cryptosporidiosis as well as other drug-resistant coccidia continues and the success of the modern drug-discoverer is highly dependent on the unknown, peculiar metabolic features of this important opportunistic parasite. It may offer an explanation to why all anticoccidials are ineffective against *C. parvum*.

2.2.11.3 Immunotherapy

With the paucity of existing therapeutic agents and their resistance, immunotherapy has been thought to be a significant alternative for cryptosporidiosis and has been reviewed by several authors (Ramirez *et al.,* 2004). It has been found that both innate and parasite-specific cell mediated immune responses are involved in immunity to cryptosporidiosis while most of the components of these responses have not yet been identified. Until now, many of the cytokine immune modulators, interferon-γ, interleukin-12 and other modulators like dehydroepiandrosterone, diethyldithiocarbamate, dialyzable leukocyte extract etc. have been experimentally found unsatisfactory against cryptosporidiosis. Passive immunotherapy using bovine hyperimmune serum and hyper-immune bovine colostrum containing antibodies against *C. parvum* surface proteins as well as antisporozoite monoclonal antibodies have also been tested to treat cryptosporidiosis. However, these preparations of antibodies have shown only a limited degree of efficacy both in animals and humans. Possibility for a vaccine to treat cryptosporidiosis is also under consideration (Jenkins, 2001).

2.2.11.4 Novel therapeutic targets

Modern approaches for drug design rely upon identifying possible drug targets. The understanding of distinct features of the parasite metabolism and their metabolic enzymes can be exploited for the design of specific antiparasitic agents. The elucidation of the function and molecular trafficking through the feeder organelle and through the parasitophorus vacuole membrane is also essential to determine the pharmacodynamics of any drug candidate. A number of hypotheses have been reported by several authors to highlight different aspects regarding drug discovery (Coombs and Muller, 2002).

One of the recent concerns for the molecular biologists is whether *C. parvum* possesses membrane bound organelles. Preliminary evidences suggest the absence of plastid structures and the presence of relic mitochondrion with some associated functions (SStejskal *et al.,* 2003; Roberts *et al.,* 2004; Slapeta and Keithly, 2004). The similarity of this organelle of *C. parvum* can be compared with hydrogenosomes of trichomonads (Dyall and Johnson, 2000) and the mitosome of *Entamoeba histolytica* (Tovar *et al.,* 1999). While the function of this organelle or its existence as a relic mitochondrion is not clear, its possible involvement in metabolic events can be exploited in search of potential therapeutic targets.

2.2.12 Prevention of cryptosporidiosis

Prevention is the most effective approach to control cryptosporidiosis. Contamination of water sources is the major source of human infection and thus prevention of environmental spread of oocysts is crucial to check this infection. Water companies should follow the regulatory status and environmental laws regarding safety standards for water for public consumption. Cattle farms should be constructed away from streams and rivers to avoid possible water contamination. Prophylactic measures should be taken to reduce the trans-mission between animals, as they are the main source of zoonotic infection. This involves effective herd management without overcrowding or reducing stocking density, treatment of infected cattle separately, keeping young animals from the adults, minimizing personnel-calves contact etc. (Ramirez *et al.,* 2004). The destruction of oocysts with 5% ammonia solutions with heat is recommended for cleaning houses. Immunosuppressed persons should take special care in avoiding contact with any pets or diarrhoea patients (Siddiki and Masuduzzaman, 2009).

2.3 Potential Sources of *Cryptosporidim parvum* and *Gardia lamblia*

There are many potential sources of *C. parvum* and *G. lamblia*. These include, domesticand wild animals, daycarecenters, contaminated foods, and contaminated recreationaland drinking waters.

2.3.1 Animals

Cryptosporidium parvum is capable of infecting many species, including dogs, calves, horses, and many herd animals such as deer and elk.

There are other species of *Cryptosporidium* that are found in fish, birds, and reptiles. However, the other species are not likely to pose health threats to humans. *G. lamblia* is capable of infecting humans, dogs, cats, bears, muskrats, some birds, reptiles, llamas, rats, deer, mice, and many other animals. *Giardia* and *Cryptosporidium* may infect the same host simultaneously. The soil in the vicinity of an infected herd of cows, or even individual hosts, can be contaminated with fecal matter. If soil is moist and cool, cysts and oocysts can remain infective for several months.

Dispersed and concentrated areas of animal wastes are potential sources of oocysts and cysts. Farm drainage has been found to contain concentrations of 5,800 oocysts/l. Drainage from slaughterhouses is another potential source of oocysts (Walker *et al*., 1999). Young animals are at high risk for being infected, especially calves.

The probability of finding oocysts in calves on dairy and beef farms is very high in large herds (100-200 cows) and about 80% in small herds (15-100 cows) (Walker *et al*., 1999).

2.3.2 Day Care centers

Fecal-oral contact is one of the most direct routes of infection, because the parasites are spread in large numbers directly from infected hosts. Children's day care centers have frequent outbreaks of cryptosporidiosis and giardiasis. It is estimated that 7-54% of children in childcare settings may be infected with *Giardia* and *Cryptosporidium* (USEPA, 2000).

The spread of the pathogens is highest among children who are not toilet-trained. Toddlers are at a high risk of being infected because of their high level of interaction. Childcare providers are at a high risk if they are involved with changing diapers. Personal hygieneis essential in these settings.

2.3.3 Food

Theoretically, any food could be a potential source of *Cryptosporidium* or *Giardia* if handled by an infected person. The foods with the highest chance of carrying the parasites are salad vegetables fertilized with contaminated manure.

2.3.4 Recreational Water

Recreational water is a very common source associated with outbreaks of *Giardia* and *Cryptosporidium* infection. Between 1999-2000, 23 states reported 59 outbreaks related to recreational water usage (Lee *et al*., 2002). Giardiasis rates are highest among children from 0-5 years of age, due to frequent use of recreational water (Furness *et al*., 2000).

Outbreaks of *Cryptosporidium* and *Giardia* often occur from midsummer to early fall (Furness *et al*., 2000), which corresponds with exposure through recreational swimming. When people are infected and continue to swim, this increases the likelihood of contamination (CDC, 2001). In

the summer of 2000, five outbreaks of cryptosporidiosis were linked to swimming pools. In Delaware County, Ohio,700 clinicalcases of cryptosporidiosis were reported from late June through September (CDC, 2001).

An outbreak in Colorado caused 112 to become ill after attending a private pool party (Lee *et al*., 2002). Another outbreak in mid June 2000, in Nebraska at a private swimming club, also caused many people to become ill (CDC, 2001).

Ten outbreaks were caused by exposure to *Giardia*. In these, 52 people from five states were reported ill (Lee *et al*., 2002).

2.4 Prevention of *Cryptosporidium parvum and Giardia lamblia* infection

Several strategies can be used to minimize the potential for *Giardia* and *Cryptosporidium* contaminations of water supplies. These include eliminating sources, containing and managing wastes and enhancing natural disinfection.

These may be applied in combination with water treatment to ensure that water is safe to drink. Each is discussed below.

2.4.1 Eliminating sources

Many types of mammals, especially young mammals, can be infected by *Cryptosporidium* oocysts, which in turn can initiate infections in humans. The chances of contaminating water supplies can be minimized by eliminating sources of potential contamination-especially in critical areas that may affect water supplies. Examples of this strategy could include transporting sewage effluent out of a watershed and prohibiting livestock and pets in specific areas. If

potential sources are eliminated or prohibited in areas that are most likely to contribute runoff to surfacewater bodies, then the chances of contamination will be reduced significantly.

One of the difficulties of protecting water supplies with this strategy is that it can be extremely expensive to transport sewage effluent out of a watershed, especially if the population is large, and may require changes in zoning or land use codes to keep pets and livestock out of areas where they are currently allowed to go. It is also difficult to eliminate all potential sources of these parasites, because wild animals, such as mice may be infected and excreting cysts and oocysts.

Another practice that is often applied involves capturing stormwater runoff and either treating it prior to releasing it, or allowing natural stresses and processes to eliminate harmful microorganisms. Sand filters can be constructed to clarify stormwater and remove many types of sediments **(**USEPA, 2000).

2.4.2 Containing and Managing Wastes

In many agricultural operations, animal wastes are collected, stored temporarily and used as a soil amendment when weather and land use conditions permit. When no storms threaten, or conditions for runoff due to snow melt are not imminent, stored wastes may be injected or tilled

into soil to add needed plant nutrients. Similarly, pet wastes can be collected by owners and disposed of properly. This means that pet owners carry plastic bags or other suitable containers while exercising pets and that they pick up wastes immediately. The bagged wastes can then be disposed of in collected trash.

One of the most frequently applied methods for encouraging pet owners to collect wastes is education. Education can include posting signs in pet recreation areas and circulating information through internet, print, radio, and television media. Regulations and ordinances are an alternative method for ensuring the pickup of pet waste. However, regulations of this type tend to be difficult to enforce because enforcement requires careful surveillance, inspection and authority to fine. Education can make people aware of the potential effects of pet wastes on public water supplies and may be an effective way of increasing public awareness.

2.4.3 Enhancing Natural Disinfection and Attenuation

Because *Cryptosporidium* and *Giardia* are dormant outside a host, environmental stresses kill cysts and oocysts over time if they are not ingested by a new host. These include temperature stresses (such as freezing and thawing and high temperatures) and dry conditions. When management practices such as grassed buffer or filter strips are put in place to protect water supplies, they lie between potential sources of microorganisms and surface water bodies (USEPA, 1999).

The vegetated strips are designed to capture microorganisms or slow their travel. Research conducted in California with buffer strips showed that 90-99% of *C.parvum* oocysts were removed from surface and shallow subsurface flow per meter of buffer strip (Atwill *et al*., 2000).

When captured or slowed, environmental stresses can reduce numbers of oocysts and cysts. Constructed wetlandscan also reduce numbers of microorganisms in water, though they may be quite difficult to construct and maintain (USEPA, 1999).

Unfortunately, because they serve as ideal habitat for many types of wildlife they may also inadvertently become sources of microbial contamination. Buffers and filter strips must be

maintained to remain effective. This may involve regular inspection and mowing and periodic reconstruction. They also involve setting aside land that may be useful for other purposes. One of the advantages of buffer strips is that they can be applied in areas where no other types of control can be applied. These include areas where wildlife may be the only potential source of cysts and oocysts. Another advantage is that buffer and filter strips have been shown to be effective in removing other types of contaminants, including sediment and chemicals attached to sediment.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in three selected Local Government Areas of Kaduna State, Nigeria. Kaduna State is in the centre of Northern Nigeria and more than 1000km from the Atlantic Ocean. It is located on a Latitude of $11^{O}12`$ N and a Longitude of $07^{O}37`$E. The State is the successor of the old Northern Region of Nigeria, which had its capital at Kaduna. In 1967 this was divided into Six (6), one of which was the North Central State, which was divided to Kaduna and Katsina State in 1987.

The population of Kaduna State is 6,066,562 (NPC, 2006). It is characterized by a tropical climate with two seasons; a rainy season of approximately 210 days (May to October), with a mean annual rainfall of 1092.8mm, and the dry season which spans between November and April. The monthly mean temperature shows a maximum of 39.7^{O}C in June and 13.8^{O}C in December (Ukegbu, 2005).

The State has twenty-three (23) Local Government Areas (LGAs) and three Senatorial Zone. These include the Kaduna north, Kaduna central and Kaduna south Senatorial Zone.

One Local Government was selected from each Senatorial Zone for this study. They are: Zaria for Kaduna north Senatorial Zone, Igabi for Kaduna central Senatorial Zone, and Kachia for Kaduna south Senatorial Zone (Figure 3.1).



Figure 3. 1: Administrative Map of Kaduna showing the study area

(Source: Nigerian muse; www.nigerianmuse.com/maps of various states and local governments.)

3.2 Study Design

This study was a cross-sectional study

3.3 Sampling Method

3.3.1 Determination of Sample Size

The sample size was calculated using the formula for cross-sectional studies:

$$n = \frac{Z^2pq}{d^2}$$

Where,

Z= 1.96 for 95% confidence interval

p= Proportion of positive individuals. Since there were no study conducted concerning the present topic in the area, P was taken as 50% to achieve the maximum sample size.

d= absolute precision and it is taken as 0.05

q= 1-p

n= number of samples

$$n=\frac{1.96^2x0.50x0.50}{0.05^2}$$

= 384 (approximately).

= 1.962x0.50x0.50/.052 =384.

For the purpose of this study, Four hundred and Twenty (420) samples made of 210 Stool and 210 Recreational Water samples were collected at the three selected Local Governments in the three senatorial zones. One hundred forty samples of 70 Stool and 70 Recreational Water was collected at each Local Government.

3.3.2 Sampling Plan

3.3.2.1 Inclusion Criteria

Subjects who use ponds and streams for recreation in the zones of the state and those who gave their consent were included in the study.

3.3.2.2 Exclusion Criteria

Apparently healthy, subjects who are on antimicrobial agent(s) such as Metronidazole, Tetracycline, Erythromycin and Antacids (For example, bismuth or laxative) within a week prior to sample collection were excluded from the study. This is necessary because the aforementioned antimicrobials and antacids prevent the shedding of intestinal parasites in the stool of such individual for several weeks and this may lead to false negative results when such individuals are examined. Also excluded were subjects who do not use ponds and streams for recreation in the zones of the state and who did not give their consent.

3.4 Collection and Processing of Stool Samples

Freshly passed single stool was collected from each subject. All subjects were advised against contaminating their stool with urine, water, dirt and or disinfectant. Samples were collected using clean, dry, wide mouthed, sterile, grease free bottles.

A single fresh stool sample was collected with labeled stool bottles from consulted study subjects (n=210). A portion of the stool was preserved with Sodium-acetate acetic acid Formalin (SAF) (15 g sodium acetate, 20 ml glacial acetic acid, 40 ml formalin and 925 ml distilled water) in a proportion of 1g of stool in 3 ml of SAF. The remaining part was processed using the following methods (Ortega and Adam, 1997).

3.4.1 Sample Analysis

3.4.1.1 Direct Wet Mount Method

A direct wet mount of the stool sample was prepared with normal saline (0.85% NaCl solution) and observed for the presence of motile parasites and trophozoites under light microscope at 10X and 40X magnification. Lugol's iodine staining was used to observe cysts of the parasites.

3.4.1.2 Formol Ether Concentration

Using an applicator stick, about 1 g of preserved stool sample was placed in a clean 15 ml conical centrifuge tube containing 7 ml formalin. The sample was suspended and mixed thoroughly with applicator stick. The resulting suspension was filtered through a sieve (cotton gauze) into a beaker and the filtrate was poured back into the same tube. The debris trapped on the sieve was discarded. After adding 3 ml of diethyl ether to the mixture and hand shaken, the content was centrifuged at 2000 rpm for 3 minutes. The supernatant was poured away and the tube was replaced in its rack. Iodine stain preparation was made from the sediments. Finally, the entire area under the cover slip was systematically examined using x10 and x40 objective lenses and ova or cyst of different parasites was observed under the microscope (Lindo *et al.,* 1998).

3.4.1.3 Modified Ziehl-Neelsen Method

For detection of *Cryptosporidium* Oocyst, direct and concentration smears were prepared. Fresh faecal samples were collected from the subjects and smears were prepared, dried, fixed with methanol for 5 minutes and stained by modified Ziehl-Neelsen techniques and the same procedure was used for smears prepared after concentration. In this technique, the slides was stained with carbol fuchsin for 30 minutes and thereafter, they were washed with tap water. The slides was decolorized in acid alcohol for 1 minute and was counterstained with methylene blue for another 1 minute. Finally, the stained smears was microscopically observed using x100 magnifications (Garcia *et al.*, 1993).

3.5 Recreational Water Sample Collection and Processing

Recreational Water samples were collected, filtered and then transported on ice to the Department of Microbiology laboratory, Ahmadu Bello University,Zaria, Nigeria for processing within 24 hours.

About 10 liters of water was collected from each water body and then transported to the laboratory where it was concentrated, decanted and then filtered through a 2µm pore size polycarbonate filter. The surface of the membrane was scraped with a rubber blade and washed.

The eluent was collected and concentrated via centrifugation. The eluent was then dropped onto a glass slide for microscopy (Ongerth and Stibbs, 1987).

3.6 Identification of Coproantigens of *Giardia lamblia* and *Cryptosporidium parvum* in Stool Samples

Stool samples were homogenized and Enzyme linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions (ProSpecT Microplate Assay). Briefly, microtitre plate wells (provided with the kit) was incubated with 0.1 ml of stool sample (diluted 1:1 in diluent provided with the kit) for 1 h at room temperature. After shaking out the contents of the well and washing three times in the wash solution, 0.2 ml of enzymatic conjugate was added and incubated for 30 min at room temperature. The strips were washed. Stop solution was then added and the strips were read in a microtitre plate reader at 450 nm five times and substrate solution was added and incubated at room temperature for 10 min. Results were interpreted according to the kit instructions as visual readings was considered negative when the reaction is colourless, which indicates that none or undetectable level of *Cryptosporidium* Specific Antigen (CSA) or *Giardia* Specific Antigen (GSA) was present in the sample tested. The positive reaction develops yellow colour of variable intensity and spectrophotometric readings establishing positive tests when optical density (OD) values is $\geq$ 0.05 after the blank of the negative control is subtracted, indicating that the sample tested contains CSA or GSA. The result was considered negative when it presents OD values < 0.05.

3.7 Statistical Analysis

The data obtained were analyzed statistically using Graphpad QuickCals (www.graphpad.com). Chi square test was used to determine the statistical association between the parasites percentage prevalences and factors such as gender, local government and age group. Odd ratio test was used to analyse the risk factors associated with infection by the two parasites. $P \leq 0.05$ was considered statistically significant.

CHAPTER FOUR

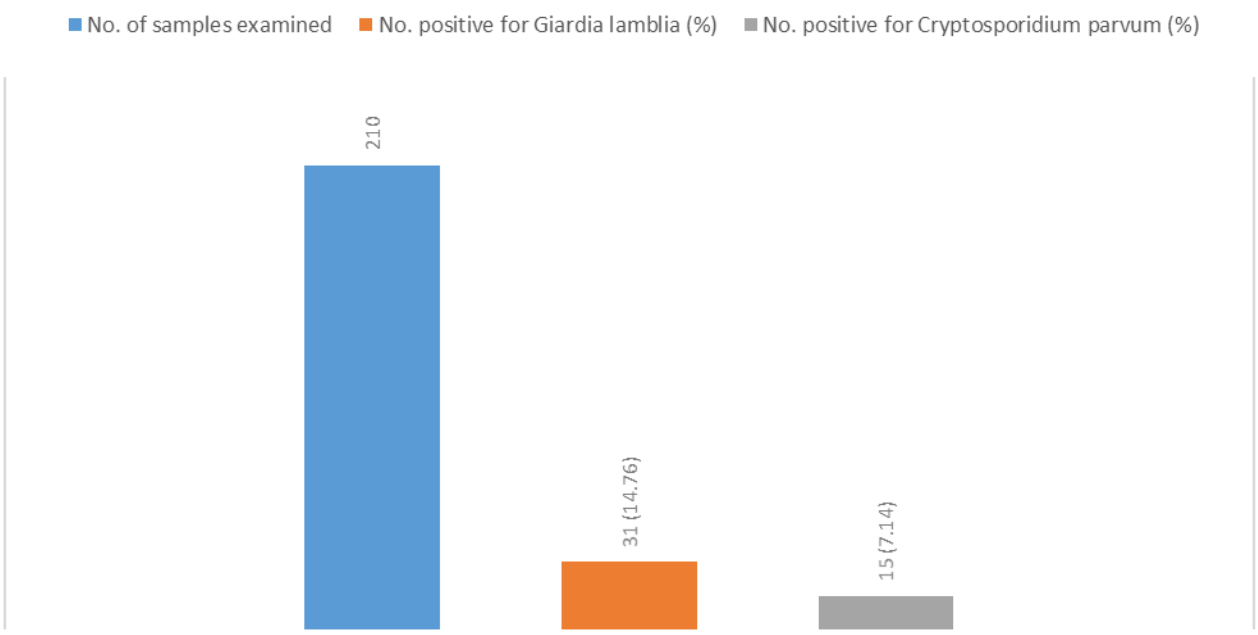
4.0 RESULTS

4.1 Distribution of cysts and oocyst by Gender

The distribution of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts by Gender detected in stool samples by microscopy method is presented in Table 4.1. Of the 210 stool samples screened by microscopy, 201 were from males while the remaining 9 were from females. *Giardia lamblia* cysts were observed in 29 males (14.43%) and 2 females (22.22%) whereas *Cryptosporidium parvum* oocysts were detected in 15 (7.46%) samples from males but none was detected in stool samples from females. The difference observed in the percentages of the two parasites by gender was not statistically significant at $P \leq 0.05$.

4.2 Occurrence of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool samples of subjects using the ponds and streams by Microscopy

The percentages occurrence of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool Samples of subjects using ponds and streams are presented in figure 4.2. *Giardia lamblia* cysts were detected in 31 (14.76%) of the 210 stool samples of the subjects examined while *Cryptosporidium parvum* oocysts were observed in 15 (7.14%) of the 210 stool samples examined by microscopy. The difference observed in the percentages of the two parasites was statistically significant at $P \leq 0.05$



$\chi^2 = 4.095$ df = 1 $p = 0.0430$

Figure 4.1: Percentage occurrence of the microscopic detection of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool samples of subjects

Table 4. 1: Percentage occurrence of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocyst in stool samples detected by Gender

Gender	No. of samples examined	No. positive for *G. lamblia*cysts(%)	No.positive for *C. parvum* oocysts (%)
Male	201	29 (14.43)	15 (7.46)
Female	9	2 (22.22)	0 (0.00)
Total	210	31	15

Giardia lamblia: $\chi^2 = 0.061$ df = 1 $P = 0.8042$

Cryptosporidium parvum: $\chi^2 = 1.271$ df = 1 $P = 0.2596$

4.3 Distribution of parasites cysts and oocysts by Age group.

The Distribution of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool samples detected by microscopy by age group has revealed that out 138 stool samples screened, highest percentage 26 (18.84%) occurrence of *G. lamblia* was observed in the Age group 6-11years followed by age group 12-18 years, (7.14%; 5/70). The parasite was not detected in samples from subjects in the age group ≥ 19 years. The percentage detection of *C. parvum* was observed to be 9.42% (13/138), 2.86% (2/70) and 0% (0/2) for age groups 6-11 years, 12-18 years and ≥ 19 years respectively (Table 4.2). The difference observed in the percentages of the two parasites based on age group is statistically significant at $P \leq 0.05$.

4.4 Prevalence of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in water samples from ponds and streams by microscopy.

Giardia lamblia cysts were detected in 5.24% (11/210) of the water samples collected whereas *Cryptosporidium parvum* oocysts were observed in 2.86% (6/210) of the water samples collected by microscopy (Figure 4.2). The difference observed in the percentages of the two parasites in the water sample is not statistically significant at $P \leq 0.05$.

4.5 Comparison of the distribution of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool samples of subjects using the ponds and streams by microscopy.

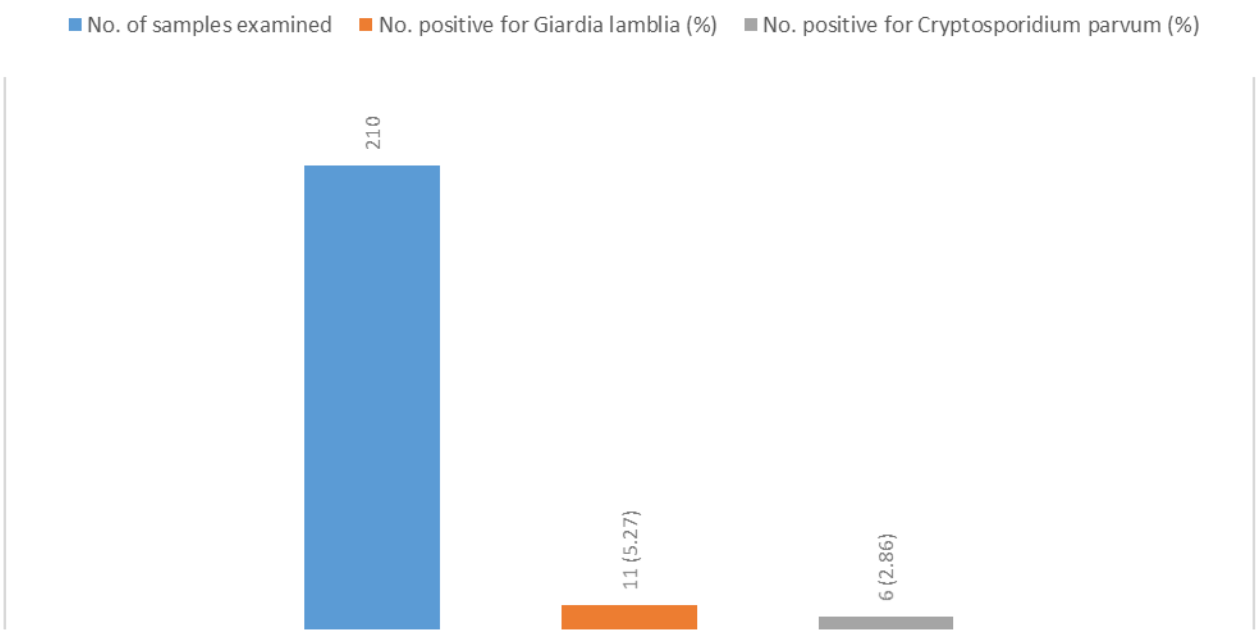
Of the 210 stool samples from the study subjects, 25samples showed the presence of *Giardia lamblia* cysts only, 9samples showed the presence of only *Cryptosporidium parvum* oocysts however the two parasites were found as mixed infection (Figure 4.3).

Table 4. 2: Distribution of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool samples by Age group as determined by Microscopy

Age group (years)	No. of samples examined	No. positive for *G. lamblia* (%)	No. positive for *C. parvum*(%)
6 - 11	138	26 (18.84)	13 (9.42)
12 – 17	65	4(6.15)	2 (3.08)
≥ 18	7	1(14.29)	0 (0.00)
Total	210	31 (14.76)	15(7.14)

Giardia lamblia :$\chi^2 = 10.464$ df = 2 $P = 0.0053$.

Cryptosporidiumparvum : $\chi^2 = 8.715$ df = 2 $P = 0.0128$.



$\chi^2 = 1.533$df = 1 $P = 0.2157$.

Figure 4. 1: Distribution of *Giardia lamblia* cysts and *Cryptosporidiumparvum* oocysts in water samples from ponds and streams by microscopy

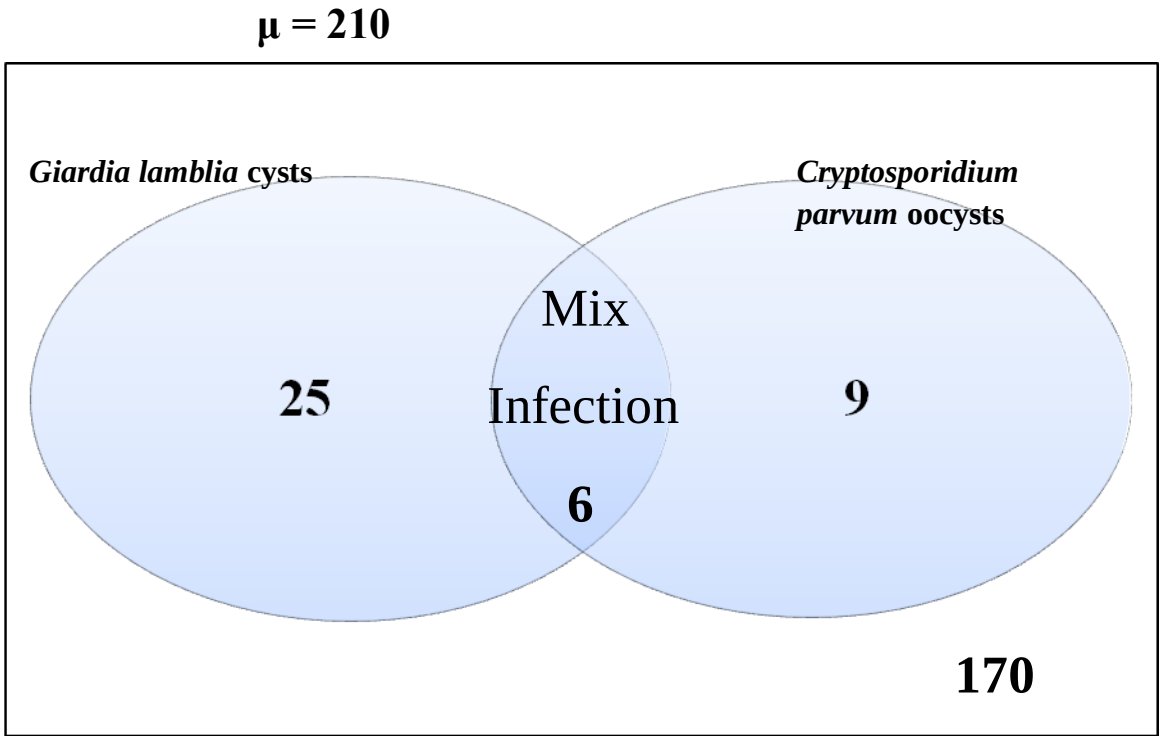


Figure 4. 2: Venn diagram showing the comparison of prevalence of the *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in stool as determined by microscopy

4.6 Comparison of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in water samples from ponds and streams as determined by microscopy

Out of the 210 water samples from the study areas, 7 samples showed the presence of *Giardia lamblia* cysts only, 2 samples showed the presence of only *Cryptosporidium parvum* oocysts However, Four (4) samples had mixed infection.

4.7 Distribution of prevalence of the parasites in water samples by sampling sites.

Prevalence of the parasites in water samples based on sampling site as determined by microscopy has revealed that *Giardia lamblia* cysts were observed in 4 (5.71%) samples while *Cryptosporidium parvum* oocysts were detected in 3 (4.29%) of the 70 water samples collected at Zaria. As for Igabi, *Giardia lamblia* cysts were observed in 4 (5.71%) samples while *Cryptosporidium parvum* oocysts were detected in 1 (1.43%) of the 70 water samples collected. In the case of Kachia, *Giardia lamblia* cysts were observed in 3 (4.29%) samples while *Cryptosporidium parvum* oocysts were detected in 2 (2.86%) of the 70 water samples collected. The difference observed in the percentages of the two parasites in the water sample based on sampling site is not statistically significant at $P \leq 0.05$.

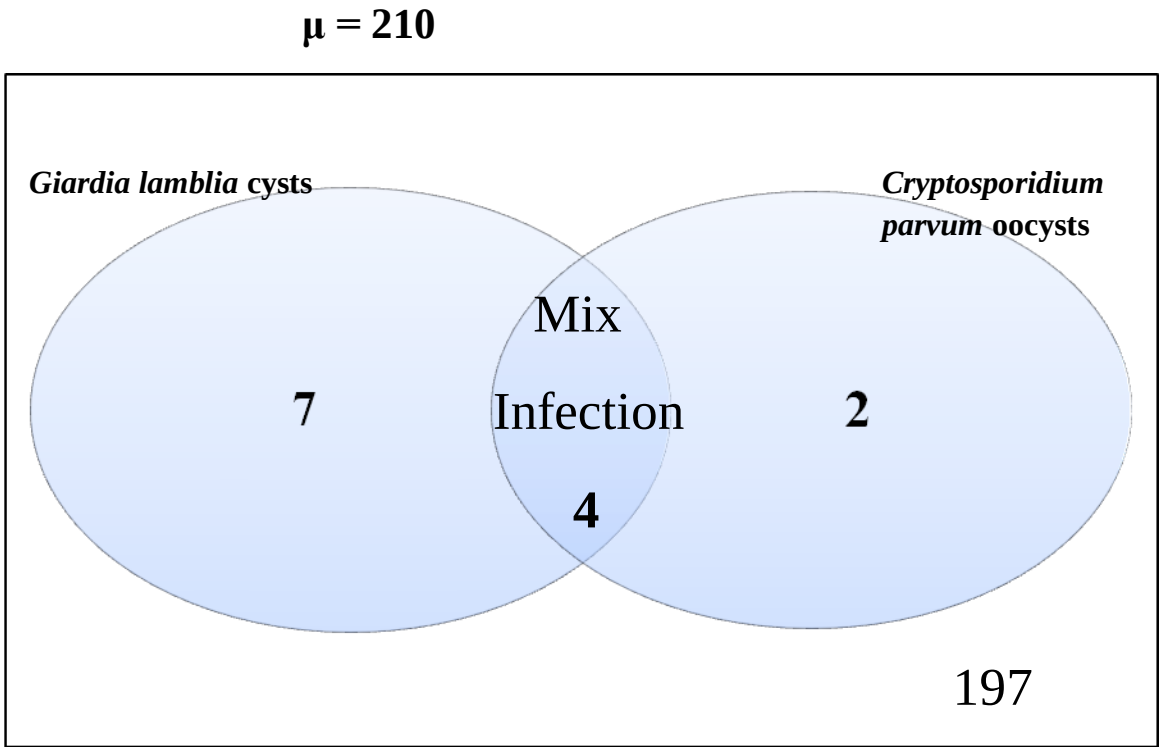


Figure 4. 3: A comparison of the prevalence of the *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in water sample as determined by microscopy.

Table 4. 3: Distribution of *Giardia lamblia* and *Cryptosporidium parvum* in water samples based on sampling site as determined by microscopy

Sampling Site	Organism	Number of Samples Screened	Number positive (%)
Zaria	*Giardia lamblia*	70	4 (5.71)
	Cryptosporidium parvum	70	3 (4.29)
Igabi	*Giardia lamblia*	70	4 (5.71)
	Cryptosporidium parvum	70	1(1.43)
Kachia	*Giardia lamblia*	70	3 (4.29)
	Cryptosporidium parvum	70	2(2.86)

Giardia lambli: $\chi^2 = 0.192$ df = 2 $P = 0.9085$.

Cryptosporidium parvum: $\chi^2 = 1.029$ df = 2 $P = 0.5978$.

Table 4. 4: Prevalence of *Giardia lamblia* and *Cryptosporidium parvum* in water sample based on sampling site as determined by microscopy.

Sampling site	Pond/stream	No. of samples examined	No. positive for *G. lamblia*	No. positive for *C. parvum*
Zaria	A	14	1(7.14)	0(0.00)
	B	14	0(0.00)	17.14)
	C	14	2(14.29)	17.14)
	D	14	17.14)	0(0.00)
	E	14	0(0.00)	17.14)
	Total	70	4(5.71)	3(4.29)
Igabi	A	17	1(5.88)	0(0.00)
	B	18	0(0.00)	0(0.00)
	C	18	1(5.56)	0(0.00)
	D	17	2(11.77)	1(5.88)
	Total	70	4(5.71)	1(1.43)
Kachia	A	23	1(4.35)	0(0.00)
	B	24	11(45.83)	1(4.17)
	C	23	1(4.35)	1(4.35)
	Total	70	3(4.29)	2(2.86)

4.8 Risk factors associated with *Giardia lamblia* and *Cryptosporidium parvum* infections as determined by ELISA detection of coproantigens.

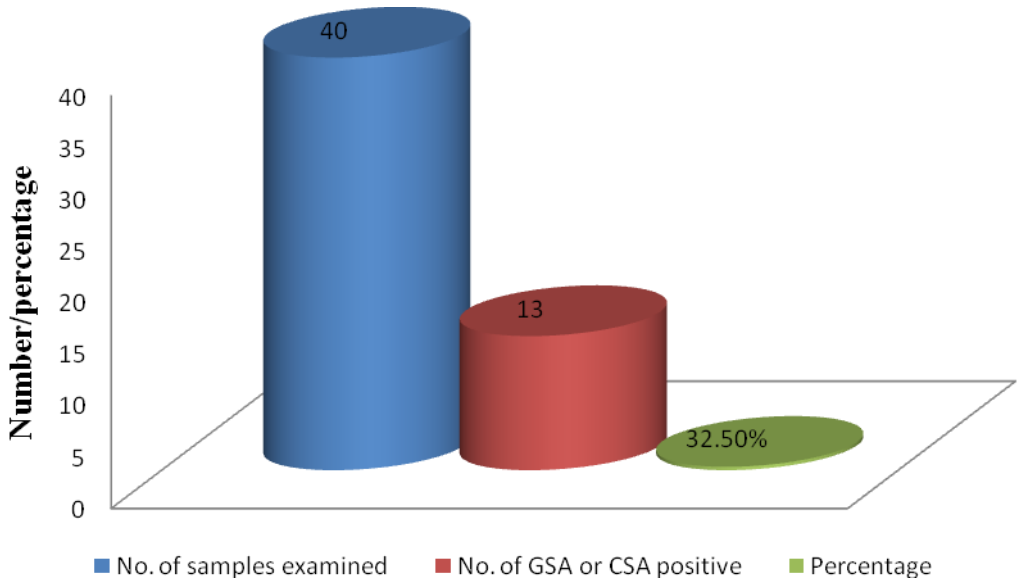
The values of the risk factors associated with Giardiasis and Cryptosporidiosis among the study subjects, the Odds ratio, 95% confidence interval as well as the *P* value for each of the risk factors examined are showed in table 4.5. The differences observed in terms of the knowledge about the infections, educational status, type of water and type of treatment were not statistically significant. However, the differences observed in terms of the hand washing after using the toilet, hand washing with soap before eating and previous treatment were statistically significant at $P \leq 0.05$.

4.9 Detection of coproantigens of *Giardia lamblia* and *Cryptosporidium parvum* in stool samples of the subjects positive by microscopy using Enzyme Linked Immunosorbent Assay (ELISA).

The result of the enzyme linked immunosorbent assay detection of *Giardia* Specific Antigen and/or *Cryptosporidium*specific Antigen in the samples positive by microscopy shows that 13 (32.50%) were positive for the parasites' coproantigents (Figure 4.5; Appendix I).

Table 4. 5: Risk factors associated with *Giardia lamblia* and *Cryptosporidium parvum* infections as determined by use of Questionnaires

Variable	No. examined	No. positive by ELISA (%)	OR (95% CI)	P – value
Educational status				
Nursery	1	1 (100.0)	0.3 (0.06-1.51)	0.1425
Primary	197	10 (05.08)		
Secondary	12	2 (16.67)		
Type of water				
Pond	104	6 (5.77)	0.9 (0.28-2.67)	0.8020
Stream	106	7 (6.60)		
Hand washing habit				
After toilet use with soap	100	1 (1.00)	0.009 (0.002-0.04)	<0.0001
After toilet use without soap	97	3 (3.09)		
No hand washing	13	9 (69.23)		
Eating with:				
hand after washing with soap	10	1 (10.00)	0.3 (0.08-0.94)	0.0391
hand without washing soap	109	2 (1.83)		
hand without washing	85	9 (10.59)		
cutleries	6	1 (16.67)		
Previous treatment for Giardiasis/Cryptosporidiosis				
Yes	95	2 (2.11)	0.2 (0.04-0.94)	0.0416
No	115	11 (9.57)		
Type of treatment				
Traditional	180	2 (25.00)	0.99 (0.05-21.58)	0.9935
Orthodox	20	0 (0.00)		



Key: GSA = *Giardia* specific antigen, CSA = *Cryptosporidium*specific antigen, No. = Number.

Figure 4. 4: Detection of coproantigens of *Giardialamblia* (GSA) and *Cryptosporidiumparvum* (CSA) in stool samples

CHAPTER FIVE

5.0 DISCUSSION

The results of the current study demonstrate that infection by *Giardia lamblia* and *Cryptosporidium parvum* are common among the subjects using pond and streams in the study area. *Giardia lamblia* and *Cryptosporidium parvum* were detected in the stool samples of subjects using pond and streams for recreation in parts of Kaduna state at prevalence of 14.76% and 7.14% respectively as detected by microscopy.

This prevalence is high considering the fact that single stool sample was collected per subject. The high rate of infections might be due to poor hygienic practices as revealed by the study. It may also be due to low socioeconomic status, poor personal hygiene practices and life style of the study subjects, since intestinal parasite infections are usually considered as poverty-related diseases.

Lower prevalence of 10.5% for *Giardia lamblia* and 4.6% for *Cryptosporidium parvum* were reported by Jombo *et al.* (2011) in Jos and Gambo *et al.* (2014) in Zaria respectively. However higher prevalence of 15.6% to 19.6% were reported by Molloy *et al.* (2010) for *Cryptosporidium* oocysts over one year period in four semi-urban villages in Ile-Ife, Osun State, Nigeria. So also Ayinmode *et al.* (2012) in a study carried out in Ibadan, Oyo State reported a prevalence of 11.6% for *Cryptosporidium parvum.*

Higher percentages of 35.3% for giardiasis and 12.2% for cryptosporidiosis were reported by Ayalew *et al.* (2008) in Lege Dini, Ethiopia. Also Fisseha *et al.* (1998) reported a higher percentage (25.9%) of *Cryptosporidium* infection among AIDS patients. The percentage of *C. parvum* (7.14%) in this study is similar to the percentage range of *C. parvum* from 5-10% among

children reported by Iqbal *et al.* (1999). The percentage is also higher than 5.6% and 3.3% reported by Assefa *et al.* (1996) and Gebru and Girma (2000) respectively.

Similarly, the result of the current study shows a higher prevalence of *G. lamblia* (14.76%) among the study subjects compared to *C. parvum* (7.14%). The observed high prevalence suggests that *G. lamblia* is more common among the people in the study area. The variation could be an indication of the development of giardiasis at early in life and high rate of carriage in all age groups and possibly of re-infection in the community. This is in line with the results of Scheelbeek, (2005) and Ayalew *et al.* (2008).

Based on gender, the prevalence of *Giardia lamblia* was slightly higher among females (22.22%) compared to male (14.43%). The possible explanation for this finding could be the increased chance of exposure of females to contaminated waters as they are usually engaged in fetching water for the family. However, *Cryptosporidium parvum* was detected at the rate of 7.46% in male while it was not detected in females. Though most of the subjects are males, it is likely that the source of infection may be also through other means such as swimming. This is contrary to the study conducted by Gambo *et al.* (2014) in Zaria, where they reported that the distribution of *Cryptosporidium* oocysts with respect to gender was slightly higher in females than in males. Generally, parasitic infections are not gender dependent since the route of transmission in both gender are same. This is not in line with the result of Anejo-Okopi (2016) who also reported about the same percentage of *Cryptosporidium* among the gender.

The prevalence of the two parasites in this study decreases with increase in age. As such the higher prevalence was observed among subjects within the age group 6-11 years. The fact that younger children are particularly susceptible to infection by *Giardia lamblia* and

Cryptosporidium parvum supports the higher the higher observed in younger children. Low immune status and poor hygiene of the younger children might be responsible for the prevalence observed. The established trend of giardiasis is that its prevalence increases with age and with development of immunity in older individuals, prevalence of giardiasis becomes reduced.

Similarly, it may be because older hosts have developed adaptive immunity which results in less cyst excretion; however, they also can be considered as a source of infection (carriers).

This is in agreement with the study of Alexander *et al.* (2012)who reported positive samples for *Cryptosporidium* in only younger children and none in older children and adults. However, they reported a broad distribution of *Giardia* among age groups.

Giardia lamblia and *Cryptosporidium parvum* were detected in the water samples collected from ponds and streams at the rate of 5.24% and 2.86% respectively. This result also shows that *Giardia lamblia* is more common in the study area compared to *Cryptosporidium parvum.*

Giardia lamblia was detected in 4 (5.71%) of the 70 water samples each from Zaria and Igabi while it was detected in 3 (4.29%) of the 70 water samples from Kachia. As for *Cryptosporidium parvum,* it was detected in 3 (4.29%), 1 (1.43%) and 2 (2.86%) of the 70 water samples each from Zaria, Igabi and Kachia respectively. The fact that there was significant differences in the geographical factors and life style between the local governments study may account for the similar percentages observed.

One of the risk factors associated with *Giardia* contamination of the environment is that *Giardia* cysts are readily infectious to the new host once they are excreted in the environment and do not require sporulation. The cysts are hard and can survive in surface water and soil for 2-3 months, but the cysts do not persist well in cold environments. Another factor is that a large number of

cysts are shed into the environment from infected animals. The prolonged excretion period of infectious cysts to the environment can be another factor that go hand in hand with the high excretion rate from infected individuals that contribute to a significant environmental contamination (Thigeel, 2016).

Personal risk factors such as poor personal hygiene, poor hand washing habit, immune status, nutritional status and age of the host can be potential risk factors for acquiring the infection. Also, environment-related factors can be responsible for differences in the severity of infection.

Giardia specific antigen and/or *Cryptosporidium*specific antigen were detected in 13 (32.50%) of the 40 microscopy positive stool samples for either *Giardia lamblia* or *Cryptosporidium parvum* or both by enzyme linked immunosorbent assay. Definitive diagnosis requires the microscopic identification of *G. lamlia* cysts or *C. parvum* oocysts in stool samples. The lower detection rate by the ELISA kit may be as a result of false negative result. Over the past years, ELISA kits for the detection of *Cryptosporidium* and *Giardia* antigens in stool have been reported to have problems with specificity and sensitivity, resulting in significant numbers of false-positive and false-negative results (Johnston *et al.,* 2003).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

The distribution of the infection is mainly due to human activities on the source of infection

Low immune status and poor hygiene of the younger children might be responsible for the prevalence observed in the current study

Age group has no significant effect on the distribution of the disease.

Proper hygiene practice and early treatment of infectious giardiasis and cryptosporidiosis are suggestive of protective factors against the disease.

6.2 CONCLUSION

Giardia lamblia and *Cryptosporidium parvum* were detected in this study at the rate of 14.76% and 7.14% respectively in the stool samples collected. Whereas in the water samples examined, the prevalences of *Giardia lamblia* and *Cryptosporidium parvum* were 5.24% and 2.86% respectively.

Higher prevalence of *Giardia lamblia* was observed compared to *Cryptosporidium parvum.*

Male had lower prevalence of *Giardia lamblia*, with higher prevalence of *Cryptosporidium parvum*, and vice versa.

ELISA detected 13 positive samples for *Giardia* Specific Antigen and *Cryptosporidium* Specific Antigen in samples that were microscopically positive. Lack of knowledge about the parasites

was found to be a risk factor while previous treatment, hand washing before eating, and after using the toilet were found to be protective factors against infections by these parasites.

6.3 RECOMMENDATIONS

1. Awareness programs to enlighten the people about giardiasis and cryptosporidiosis.

2. Measures should be taken to prevent and control infections caused by these parasites.

3. personal hygiene should be encouraged and enforce in children and adults.

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APPENDICES

Appendix I: Structured Questionnaire

A questionnaire addressing risk factors for Giardiasis and Cryptosporidiosis was given to the Subjects. Information on the subject's sex, age, literacy level (not of school age, nursery, primary, secondary, tertiary, illiteracy) and related risk/socio-demographic factors including source of water (pond or stream), hand washing habit (after toilet with soap, after toilet without soap, after handling diarrhoea patient, no hand washing), Eating habit (eat with hands after washing with soap, eat with hands after washing without soap, eat with hands without washing, eat communally with hands, eat with cutleries), and Drug administration using a structured questionnaire were obtained.

Risk Factor Questionnaire

Dear respondent,

I am a student of the above named department conducting a survey on risk factors associated with *Giardia lamblia* and *Cryptosporidium parvum* infections among Subjects using ponds and streams for recreation in some parts of Kaduna State, Nigeria. Any information provided is strictly for this purpose and will be treated with utmost confidentiality. Kindly fill this questionnaire appropriately and accurately.

SECTION A: BIO DATA

Age: 6yrs-11yrs ( ) 12-17yrs ( ) 18yrs and above ( )

Sex: Male ( ) Female ( )

Local Government Area of residence: Zaria ( ) Igabi ( ) Kachia ( )

SECTION B: KNOWLEDGE BASED QUESTIONS

1. Education Status: Nursery ( ) Primary ( ) Secondary ( ) Tertiary ( ) Illiterate ( )

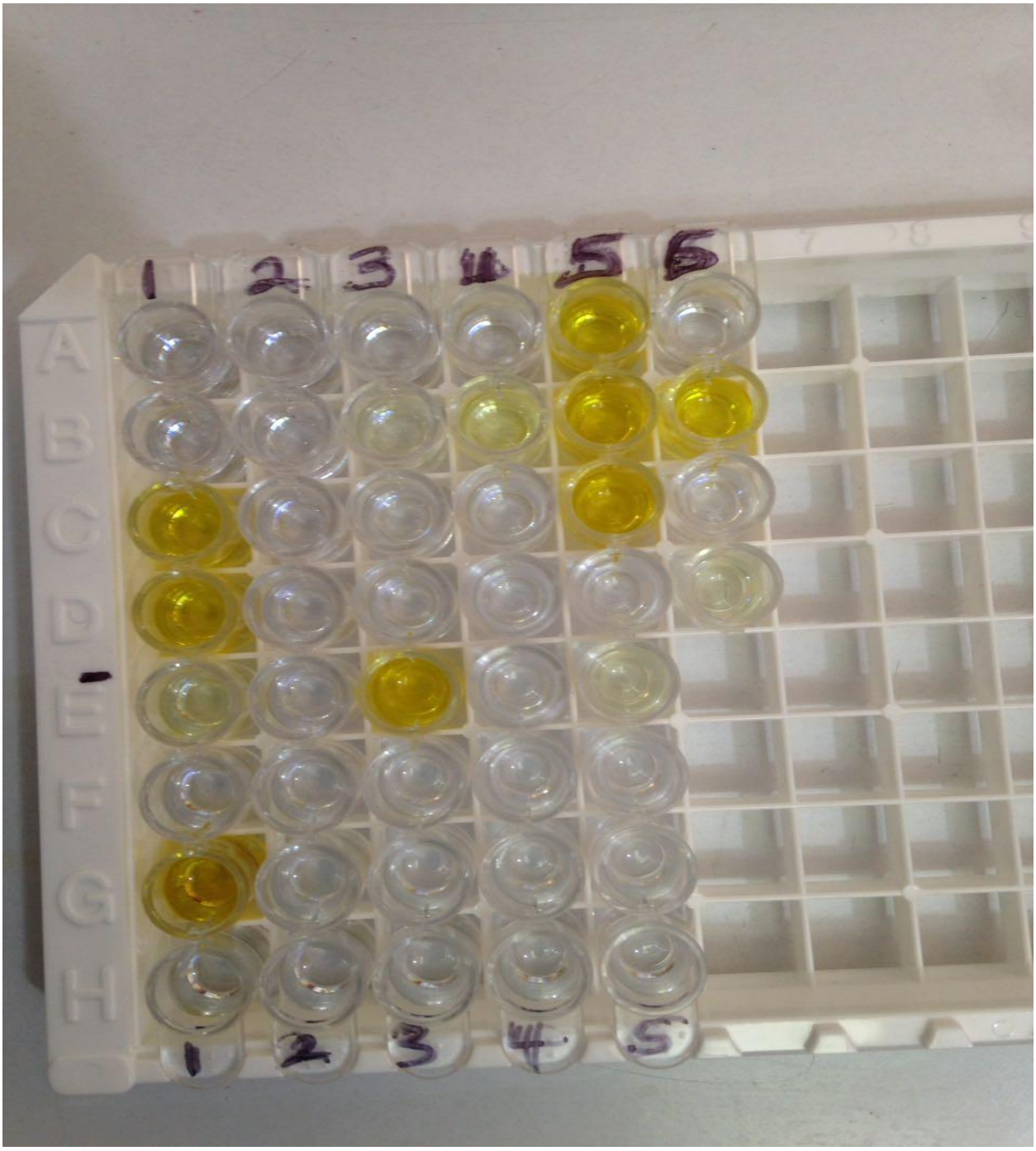
2. Source of water: Pond ( ) Stream ( )

3. Hand washing habit: after toilet with soap ( ) after toilet without soap ( ) after handling diarrhoea patient ( ) no hand washing ( )

4. Eating habit: eat with hands after washing with soap ( ) eat with hands after washing without soap ( )eat with hands without washing ( ) eat communally with hands ( ) eat with cutleries ( )

5. Have you ever taken drug for Giardiasis/Cryptosporidiosis? Yes ( ) No ( )

6. What kind of drug? Traditional ( ) Orthodox ( )



Appendix II: Enzyme linked immunosorbent assay result showing samples that positive (yellow) and those that are negative (white) for the GSA and CSA.