IMMUNOLOGICAL AND MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM SPECIES* IN HIV-POSITIVE AND HIV-NEGATIVE DIARRHOEA PATIENTS IN THE NKONKOBE MUNICIPALITY OF THE EASTERN CAPE PROVINCE OF SOUTH AFRICA: A PILOT STUDY.

BY

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DEDICATION

This thesis is dedicated to my late father, my beloved mother, brothers, sisters and all my family members especially my dearest in-law Samson for his continuous encouragement throughout the course of my research. In addition I would like to express my love and gratitude to my husband David for his endless assistance and limitless effort.

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Finally we acknowledged Govan Mbeki Research and Development Centre (GMRDC), University of Fort Hare, and The National Research Foundation (NRF) South Africa for funding this study.

DECLARATION

I, the undersigned, declare that this dissertation submitted to the University of Forth Hare for the degree of Master of science in Biochemistry (Biochemistry and Microbiology Department) in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations; and that this work has not been submitted at any other University in partial or entirely for the award of any degree.

Signature……...

Supervisor's signature……...

Co-supervisor's signature……..

Date……...

LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
AIDS	Acquired Immune Deficiency Syndrome
AMP	Adenosine monophosphate
CDC	Centre of Disease Control and Prevention
CF	Cystic fibrosis
CSA	*Cryptosporidium* Specific Antigen
C. spp	*Cryptosporidium species*
C-src	Proto-oncogene protein pp60
DFA	Direct immunofluorescent antibody test
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbant Assay
FAD	Food and drug administration
FAST-ELISA	Falcon assay screening test enzyme-linked immunosorbent assay
FRET	Fluorescent resonance energy transfer
GMP	Guanosine monophosphate
GMRDC	Govan Mbeki Research Development Centre
qPCR	Quantitative PCR
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidise
IFA	Immunofluorescent Antibody

IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-8	Interleukin-8
mAbs	Monoclonal antibodies
MZN	Modified Ziehl Neelsen
NTZ	Nitrozoxanid
NF-KB	Nuclear factor-kappa B
OD	Optical density
OR	Odds ratio
PCR	Polymerase Chain Reaction
RDT	Recombinant DNA technology
RNA	Ribonucleic acid
RPH	Reverse Passive Haemagglutination
SAS	Statistical Analysis System
SHMT	Serine hydroxymethyl transferase
TAE	Tris, acetic acid, and EDTA
TMB	Tetramethylbenzidine base
UFH	University of Fort Hare

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ABSTRACT

Cryptosporidiosis is an infection caused by *Cryptosporidium*; a protozoan parasite that infects the gastrointestinal tract. The infection is of major public health concern in both developed and developing countries. Faecal samples were collected from 160 in-patient adults, with complaint of diarrhoea, admitted at Victoria hospital in Alice, Nkonkobe Municipality. Twenty apparently healthy subjects were included as controls. All diarrhoea positive patients were interviewed to record socio-demographic information, water supply and animal contact. Initial screening was carried out by microscopy and ELISA to detect positive *Cryptosporidium*. Genomic DNA was extracted from microscopically positive samples and a PCR reaction was perform to amplify the (18S) SSUrRNA gene for further identification and epidemiology of *Cryptosporidium*. Data were analysed using Pearson's $\chi 2$ and Fisher's exact test to assess the univariate association between *Cryptosporidium* infection and the possible risk factors. Of the 180 subjects screened for cryptosporidial infection, *Cryptosporidium* antigen was detected in 122 giving an overall prevalence of 67.8%. In HIV-positive diarrhoea patients, prevalence increased with ages; between 31-43 (mean age 36.5 yr) and 70-82 (mean age 75.8 yr) had a higher prevalence (100%) of the antigen than 18-30 (mean age 23.2 yr) and 83-95 (mean age 88.8 yr) (50.0%) in HIV-positive diarrhoea patients ($P > 0.05$). In HIV-negative diarrhoea patients, prevalence was highest in the 18-30 (mean age 23.2 yr) (87.5%) and least (35.7%) in those aged 83-95 (mean age 88.8 yr) ($P > 0.05$). *Cryptosporidium* antigen was higher in females than in males. Of 115 females (mean age 46.7yr) who participated in the study, antigen was detected in 90 (78.2%) against 32 (71.1%) of 45 males (mean age 42.6yr). None of the 20 apparently healthy control subjects was found to be infected with *Cryptosporidium. Cryptosporidium* was detected in 27 HIV-positive and 97 HIV-negative diarrhoea patients by any one of the techniques. Antigen detection by ELISA

showed the highest positivity 96 (76.8%) in HIV- negative and 26 (74.3%) in HIV- positive diarrhoea patients. PCR detected eighty-nine (71.2%) cases in HIV-negative and 23 (65.7%) in HIV-positive patients with diarrhoea. Only 13 (37.1%) HIV-positive and 34 (27.2%) HIV-negative diarrhoea patients were found positive for *Cryptosporidium* by modified ZN. No significant difference was observed in sensitivity of antigen detection by ELISA and PCR (96.9%) in HIV-negative diarrhoea patients, respectively. Specificity of the staining technique was 88.9% in HIV-positive and 96.6% in HIV-negative diarrhoea patients. No significant difference was found in specificity of antigen detection by ELISA and PCR in HIV-positive and HIV-negative diarrhoea patients, respectively. Positive predictive value of ZN staining in both HIV-positive and HIV-negative diarrhoea patients (92.3 and 96.9%) was statistically higher than ELISA and PCR. No significant difference was observed in negative predictive value of ZN technique for detection of *Cryptosporidium* between HIV-positive and HIV- negative diarrhoea patients. Differences found in prevalence rates due to water source, suggest that the high infection rates of specific groups are associated with their exposure to the contaminated water supply. The results indicate that *Cryptosporidium* infection is highly prevalent in adult faecal specimens in the Nkonkobe Municipality, an indication of active infection that is likely to emerge as major human pathogen in this location due to socioeconomic changes which favour transmission. However, sequencing analysis is required to differentiate between *Cryptosporidium* genotypes in the various outbreaks.

CHAPTER ONE

1.0 Introduction

The credit for the discovery of *Cryptosporidium species* dates back to Ernest Edward Tyzzer, who in 1907, described a cell-associated organism in the gastric mucosa of mice, as well as named two important species: *Cryptosporidium muris* and *Cryptosporidium parvum* (Keusch *et al.*, 1995). He provided the name "*Cryptosporidium*' because of the uncertain taxonomic status of the oocysts. For several decades, *Cryptosporidium* was thought to be a rare opportunistic animal pathogen, but later on it was identified at the Johns Hopkins School of Medicine as the causative agent of human cryptosporidiosis that is now globally distributed worldwide (Flanigan and Soave, 1993).

Cryptosporidiosis, caused by *Cryptosporidium species* remains one of the most important health problems globally and a leading cause of morbidity and mortality in immunocompromised adults, especially in developing countries requiring often hospitalization (WHO, 2006). They are unicellular parasite approximately 3-4 microns in diameter (approximately half the size of a red blood cell). Therefore, it can't be seen with the naked eye.

In humans, the pathogenesis infection mechanism of *Cryptosporidium species* was first recognised in 1976, where the ingested oocyst released sporozoites, which subsequently attached to and invaded the intestinal epithelial cells (Adjei *et al*., 2003). Reported analysis has showed that the organism infiltrates epithelial cells in the small intestine, where it completes its life cycle and causes both water and food-borne outbreaks of cryptosporidiosis (Adjei *et al*., 2003). To date, all the cryptosporidiosis outbreaks occurring worldwide have been caused by *Cryptosporidium hominis* and *Cryptosporidium parvum*, with several being associated with consumption of drinking water or exposure to recreational water

contaminated with *Cryptosporidium* oocysts of zoonotic and anthroponotic origins (Insulander *et al*., 2005). Due to the size and frequency of these outbreaks, cryptosporidiosis became a serious public health issue worldwide and prompted re-evaluation of the microbiological standards for drinking water by health authorities in developed and developing countries, especially in South Africa.

In addition, this incurable parasitic organism has gained much attention in the last 20 years as a clinically important human pathogenic disease that gives rise to a chronic, life threatening condition in immunocompromised individuals, particularly those with Human Immunodeficiency Virus (HIV), acute gastro-enteritis and diarrhoea in healthy people (Pieniazak *et al.,* 1999). In an immunocompetent host, the organism usually produces a short-term and self-limited diarrhoea illness, while in immunodeficient individuals or those undergoing cancer chemotherapy, infection is often prolonged resulting in significant morbidity due to diarrhoea and dehydration, as well as bile duct obstruction with jaundice or pancreatitis (Aboul-Magd *et al*., 2000).

Various therapeutic drugs (anti-infective nitazoxanide (NTZ), Paromomycin, and Highly active antiretroviral therapy (HAART) have been used as an attempt to treat *Cryptosporidium species* (*C. spp*) infection in humans, but are usually unsuccessful with partial limits. Currently, there is no broad effective therapy for *Cryptosporidium parasite* infection (Flanigan *et al*., 2001).

1.1 Problem statement

Cryptosporidium parasites have been estimated to infect up to 500 million people annually in developing countries. In Africa, about 20 to 35% are infected with the organism and 32.5 to 40% harbour this organism in sub-Saharan Africa (Kfir *et al.*, 2000). A prevalence as high as 32% were reported among children in Guatemala, with a significant variation between female (44%) and male (17%) children (Lee *et al*., 2005). In Malawi, molecular epidemiologic studies of cryptosporidiosis in children showed that 41 of 43 were infected with *Cryptosporidium hominis* and only 2 with *Cryptosporidium parvum*, while in Korea, a prevalence of 1% was reported among HIV patients (Lee *et al*., 2005).

In Tanzania a prevalence of about 17.3% was documented amongst HIV patients (Abdel-Messih *et al*., 2005). In Guinea Bissau, *Cryptosporidium parvum* had a prevalence of 7.7% and was the second most common parasite with a marked seasonal variation, with peak prevalence found consistently at the beginning or before the rainy seasons. In the Republic of South Africa, a prevalence rate of 5.6 to 8.5% of Giardia cysts and *Cryptosporidium* oocysts were found in all types of water tested including surface water, sewage or treated effluents respectively (Kfir *et al.*, 2000).

A reported study in Venda, South Africa showed that *Cryptosporidium* infection was the second most common enteric pathogen isolated from children with gastroenteritis, with infection rates varying between 1.2 and 20.9% according to season with the highest prevalence in the summer months (Obi and Bessong, 2002). Relatively few studies have been reported on genotype distribution data for *Cryptosporidium* in South Africa, particularly in the Eastern Cape region. Thus this research is to determine the prevalence and species

distribution of *Cryptosporidium* among diarrhoea and control patients in the South African Nkonkobe Municipality of the Eastern Cape Province.

1.2 Research hypothesis

Cryptosporidium species is prevalent among HIV-positive and HIV-negative diarrhoea patients in Nkonkobe Municipality of the Eastern Cape Province, and may elaborate distinct genotypes from those of other geographical regions.

1.3 Overall objectives

To delineate *Cryptosporidium* prevalence in HIV-positive and HIV-negative diarrhoea patients in the Nkonkobe Municipality of the Eastern Cape Province.

1.4 Specific objectives:

1 To detect *Cryptosporidium* antigens in facal samples obtained from patients with diarrhoea.

2 To determine the prevalence of cryptosporidiosis in HIV-positive and HIV-negative patients attending the Victoria Hospital in Alice between April 2009 and January 2010.

3 To establish if there is a relationship between antigenemia and oocysts presence in the stool sample obtained.

4 To determine the genotypes profile in the study area for the period of May 2010 to July 2010.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Cryptosporidium species*

Cryptosporidium species are an enteric protozoan pathogen that causes a diarrhoea illness called cryptosporidiosis (Fayer *et al.,* 2000). The parasite invades and resides in epithelial cells, most commonly in the small intestines of multiple mammalian species. However, the association of the organism with disease was first described after 48 years in both turkeys and cattle (Panciera *et al*., 1971).

There are fourteen named species of *Cryptosporidium* of which *Cryptosporidium hominis* (previously known as *Cryptosporidium parvum* human genotype or genotype I), is the species that almost exclusively infects humans. *Cryptosporidium parvum* (previously known as the *Cryptosporidium parvum* bovine genotype or genotype II) can infect not only humans but also ruminants and perhaps a few other animals (Sestak *et al*., 2002). Other species that infect human but are less common include *Cryptosporidium andersoni* (cattle), *Cryptosporidium baileyi* (chicken and some other birds), *Cryptosporidium canis* (dogs), *Cryptosporidium felis* (cats), *Cryptosporidium galli* (birds), *Cryptosporidium meleagridis* (birds and humans), *Cryptosporidium molnari* (fish), *Cryptosporidium muris* (rodents and some other mammals), *Cryptosporidium wrairi* (guinea pigs), *Cryptosporidium saurophilum* (lizards and snakes), and *Cryptosporidium serpentis* (snakes and lizards) (Xiao *et al*., 2004). Recent literature has also described *Cryptosporidium parvum* as the most common species isolated from human stools from diarrhoea infected and non-infected individuals (Abdel-Messih *et al*., 2005). There is genetic heterogeneity among *Cryptosporidium species* isolated from humans, thus research surveys suggest that *Cryptosporidium species* undergoes genetic changes following

human infection and/or that there may be distinct pathways of *C. species* transmission from bovine to human (Carryway *et al*., 2001).

2.2 Classification

Cryptosporidium species are classified as eukaryotes in the Phylum Apicomplexa (possessing an apical complex of secretory organelles), Class Sporozoasida (reproduce by asexual and sexual cycles, with oocysts formation), Subclass Coccidiasina (life cycle involving merogony, gametogony and sporogony), Order Eucoccidiida (schizogony occurs), Suborder Eimeriina (independent micro and macrogamy development), Family cryptosporiidae (4 naked sporozoites within oocysts) (Tzipori and Widmer, 2000).

Due to the relative uniform appearance, different genotypes have shown that some are very host specific while others have a broad host range and these have been utilized to classify *Cryptosporidium spp* into 14 valid species that are currently recognised on the basis of oocyst morphology and site of infection (*Cryptosporidium parvum, Cryptosporidium hominis, Cryptosporidium muris, Cryptosporidium meleagridis, Cryptosporidium baileyi, Cryptosporidium serpentis, Cryptosporidium nasorum, Cryptosporidium felis, Cryptosporidium andersoni, Cryptosporidium canis, Cryptosporidium galli, Cryptosporidium molnari, Cryptosporidium wrairi, Cryptosporidium saurophilum*) (Hoepelman and O'Donoghue, 2004). Among these species, only *Cryptosporidium parvum and Cryptosporidium hominis* are responsible for most human cases of infection with some geographical differences (Xiao and Fayer, 2008). While others are known to have less infection in humans.

PCR analysis of the 18S rRNA based on *Cryptosporidium felis* and *Cryptosporidium* dog type has been implicated as an unusual potential causative agent in immunocompetent individuals after morphometric evaluation of oocysts isolated from human faeces in England

(Pedraza-Diaz *et al*., 2000). Additionally, new *Cryptosporidium spp* genotypes such as *Cryptosporidium cervine* have been identified in human stools (Ong *et al.*, 2002). It appears likely that other species may cause occasional zoonotic infections in humans in the future through which the sporozoites exit during excystation.

2.2.1 Taxonomy

The genus *Cryptosporidium spp* is classified taxonomically within the same group as other coccidian parasites such as *Toxoplasma, Eimeria and Isospora spp*. A key difference between *Cryptosporidium spp* and other coccidian parasites is that the organism progresses through a complex life cycle characterised by the host's expulsion of double-walled, sporulated, and immediately infective sporozoites oocysts excyt.

Table 2.0: **Recognized *Cryptosporidium species,* their predominant host specificities and primary site of infection (Egyed *et al.*, 2003)**

Species	**Predominant host**	**Primary site of infection**	**waterborne**	**Reference**
C. hominis	Humans	Small intestine	Yes	Morgan-Ryan *et al*., 2002
C. parvum	Mammals	Small intestine	No	Tyzzer, 1907
C. baileyi	Chickens	Brusa of fabricius, cloaca	No	Ditrich *et al*., 1991
C. meleagridis	Turkey, humans	Small intestine	No	McDougald, 1998
C. felis	Cats	Small intestine	No	Pedraza- Diaz *et al*., 2001
C.canis	Dogs	Small intestine	No	Pedraza-Diaz *et al*., 2001
C. suis	Pigs	Small intestine	No	Xiao *et al*., 2002
C. muris	Rodents, ruminants	Stomach	No	Palmer *et al*., 1990
C. andersoni	Cattle, camel	Abomasum	No	Pieniazak *et al.,* 1999
C. wrairi	Guinea pigs	Small intestine	No	Widmer *et al*., 2000
C. galli	Birds	Stomach	No	Ryan *et al*., 2003
C. serpentis	Snakes	Stomach, small intestine	No	Ryan *et al*., 2003
C. saurophilum	Lizards	Stomach, small intestine	No	Ryan *et al*., 2003
C. molnari	Sea bass, sea bream	Stomach, small intestine	No	Ryan *et al*., 2003

Table 2.1: **Morphometric characterization of oocysts (**Sargent *et al*., 1998**)**

Species	**Measure (µm)**	**Reference**
C. hominis	4.9 x 5.2 (4.4-5.4 x 4.4-5.9)	Morgan-Ryan *et al*., 2002
C. parvum	5.0 x 4.5 (4.5-5.4 x 4.2-5.0)	Upton and Current, 1985
C. baileyi	6.1 ± 0.4 x 4.8 ± 0.2	Ditrich *et al*., 1991
C. meleagridis	4.0 x 4.5	Fayer *et al*., 1998
C. felis	4.0 x 4.6 (3.0-4.0 x 3.2-5.1)	Sargent *et al*, 1998
C. canis	4.7 x 5.0 (3.7-5.9 x 3.7-5.9)	Fayer *et al*., 2001
	4.2 x 4.8 (3.8-4.6 x 4.6-5.1)	Fall *et al*., 2004
	4.2 x 4.6 (4.0-4.3 x 4.4-4.9)	Ryan, 2004
C. suis	5.6 x 7.4 (5.3-6.5 x 6.6-7.9)	Upton and Current, 1985
C. muris	6.1 x 8.1 (5.6-6.4 x 8.0-9.0)	Palmer *et al*., 1990.

2.3 Morphology of the oocyst

The small sizes of *Cryptosporidium* oocysts for the various species are difficult to distinguish from each other during routine fecal examination based solely on morphology by light microscopy (Fall *et al.*, 2003). Oocysts are spherical in appearance, and have many distinct phases. They contain four parallel sporozoites surrounded by a tough protective smooth oocyst wall, measuring 3-6µm in diameter (Fayer *et al*., 2000) (Fig 2.0).

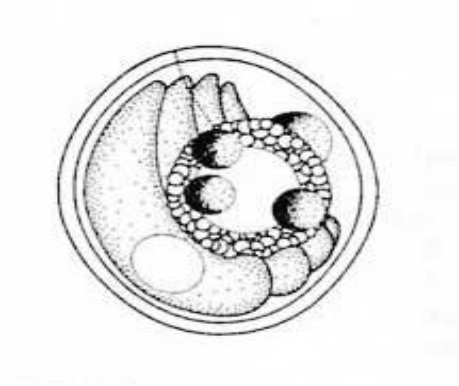


Figure 2.0: ***Cryptosporidium oocyts*** (Upton and Curent, 2002).

In the wall, a faint suture can be seen through which the sporozoites exit during excystation (Morgan- Ryan *et al.*, 2002). Depending on the host species, recovered oocysts can vary in size (3 to 8 pm in width by 3 to 9 pm in length) and they consist of many small granules and a membrane-bound globule with a residuum that is extremely resilient to various inhospitable environmental conditions (Fayer *et al*., 2004).

2.3.1 Life cycle

Cryptosporidium species are reported to have a monoxenous life cycle where all stages of development (asexual and sexual) occur within one host to form oocysts (O'Donoghue, 2002). Each oocyst contains four infective stages termed sporozoites which are fully sporulated when excreted by the host into the environment. The naked nucleus sporozoites chromosomes consisting of 10.1-10.4 million base pairs of DNA with very few introns are released along with parasitized epithelial cells of the *Cryptosporidium* by the infected host through faeces and possibly other routes such as respiratory secretions (Bankier *et al*., 2003). Ingested oocysts excyst in the ileum, releasing sporozoites that are capable of penetrating individual epithelial cells in this region (O'Donoghue, 2002).

Cryptosporidium is capable of completing all stages of its development (asexual and sexual) within a single host as shown in Fig. 2.1 below (Chen *et al.,* 2002). Unlike other coccidian

species, the organism does not require a period of maturation (sporulation) outside the host to become infectious. The thick- walled oocysts are fully sporulated and infectious when excreted in the feces of an infected host (O' Donoghue, 2002).

The life cycle of *Cryptosporidium species* progresses through five phases, where it begins with the ingestion of oocysts by the new host. When these oocyts are ingested, the suture in the oocyst wall opens (excystation), triggered by the body temperature and the interaction with stomach acid, bile salt and pancreatic enzymes. Four infective motile sporozoites are released from the cyst to produce thin-walled and thick-walled oocysts that invade the small intestine of the host and infect the intestinal epithelia cells (enterocytes), mainly in the jejunum and ileum. The freed sporozoites attach to epithelial cells and become enveloped within parasitophorous vacuoles, developing attachment organelles, a stage referred as trophozoites. Trophozoites reside beneath the cell membrane of the epithelial cells but outside the cytoplasm, where they transform into several life stages in an asexual merogony, releasing 8 and 4 merozoites respectively.

The four merozoites released from the second merogony give rise to sexual developmental stages, through gametogony to produce microgamonts (male) and macrogamonts (female). The release of microgametes, and their fusion with macrogametes give rise to a zygote which undergoes further two asexual divisions to form the environmentally robust resistant oocysts containing 4 sporozoites (Smith *et al*., 2004). The majority of oocysts that are produced at this stage are usually thick-walled and are excreted with fecal materials from the host. Thin-walled oocysts can excyst within the same host and start a new life cycle, known as autoinfection (O'Donoghue *et al.*, 2002). This may lead to a heavily infected epithelium of the small intestine, resulting in malabsorptive or secretory diarrhoea characterized by copious volume of fluid feces that may contain blood, mucus, and /or bile (O'Donoghue *et al.*, 2002).

The infectious stage of this disease may persist for several days or months depending on the parasite host.

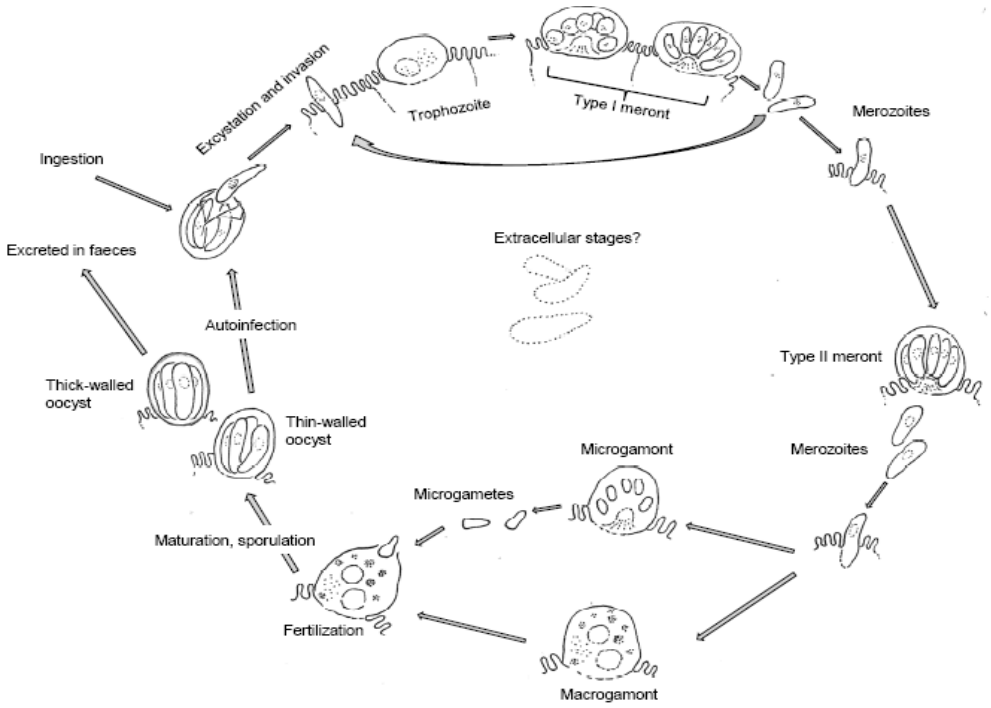


Figure 2.1: Life cycle of *Cryptosporidium* (Chen *et al*., 2002).

2.4 Transmission of infection

Evidence from several investigations suggests that transmission of *Cryptosporidium* is epidemiologically linked via the oocyst, where it is transmitted from an infected host to a susceptible host by the fecal-oral route. The infectious stage of the parasite is the oocyst which is excreted in great numbers along with faeces of infected hosts. The infective dose is not accurately known, but is believed to be very low (Egger *et al*., 2000). However, a median

reported an infectious dose of 132 oocysts in healthy adult volunteers. A mathematical model based on data from the Milwaukee outbreak suggested that some individuals developed cryptosporidiosis following the ingestion of only one oocyst (Mac Kenzie *et al*., 1994; DuPont *et al*., 1995).

During the exogenous stage of the organism, the oocyst is difficult to remove from drinking water supplies because of its resistance to chlorine disinfection and inefficiency in filtering (Xiao *et al.*, 2004). A variety of commercial disinfectants and chemotherapeutic treatment have been used in an attempt to kill sporozoites within oocysts. However, most of these have little or no effect on parasite infectivity because they are resistant to a wild range of environmental conditions even when *Cryptosporidium parvum* oocysts are exposed to Ultra-violet (UV) light for 4 hours (Bankier *et al.*, 2003).

Laboratory studies have shown that oocysts stored in aqueous solutions have remained viable for up to 1 year at ambient temperature of 15 – 20^{o}C and for up to two years when frozen at – 80^{o}C. Infectivity was lost after oocysts were heated to 65^{o}C for at least 30 minutes. Snap freezing has been shown to affect oocysts while slow freezing is less effective. Some oocysts can survive freezing at -22^{o}C for up to one year (O'Donoghue, 2002).

In humans, the pathogenesis infection mechanism was first recognised in 1976, where the ingested oocyst released sporozoites, which subsequently attach to and invade the intestinal epithelial cells (Adjei *et al*., 2003). Reported analysis shown that the organism infiltrates epithelial cells in the small intestine, where it completes its life cycle and causes both water-borne and food-borne outbreaks of cryptosporidiosis (Adjei *et al*., 2003). To date, all the cryptosporidiosis outbreaks occurring worldwide have been caused by *Cryptosporidium*

hominis and *Cryptosporidium parvum*, with several being associated with consumption of drinking water or exposure to recreational water contaminated with *Cryptosporidium* oocysts of zoonotic and anthroponotic origins (Insulander *et al*., 2005). Due to the size and frequency of these outbreaks, cryptosporidiosis became a serious public health issue worldwide and prompted re-evaluation of the microbiological standards for drinking water by health authorities in developed and developing countries, especially in South Africa.

In various individuals with acquired immunodeficiency syndrome (AIDS), the frequency of diarrhoea increases with an increase in impaired immune function. However, cryptosporidiosis may also resolve spontaneously in Human Immunodeficiency Virus (HIV) patients anywhere along the spectrum, thus complicating the interpretation of uncontrolled treatment data (Blagburn *et al*., 2004). For the most part, the disease can be complicated in patients, where it manifests as chronic diarrhoea lasting 4 months or a year. In several cases the infected individuals may produce up to 15 litre/day of fecal matter (Riggs, 2002).

Volunteer transmission studies have shown that cryptosporidiosis in immunocompetent adults normally present itself as an acute self-limiting watery diarrhoea that can persist from a period of several days up to one month with substantial morbidity and mortality among acquired immunodeficiency syndrome (AIDs) patients and infants, thus contributing a decreased lifepan (Blagburn *et al*., 2004). In addition to AIDS, immunological deficiencies and other conditions associated with protracted cryptosporidiosis include congenital hypogammaglobulinemia, concurrent viral infections, malnutrition, and exogenous immunosuppression (Blagburn *et al*., 2004). In childhood, respiratory cryptosporidiosis associated with or without bloody mucous has been increasingly reported, particularly children from 2 to 4years. Pulmonary symptoms are about three-fold more frequent in

children admitted to hospital with cryptosporidal infection than in children with other intestinal pathogens. However, infection by *Crptosporidium parvum* has been reported in six continents and identified in patients aged 3 days to 95 years old (Blagburn *et al*., 2004). Other frequent symptoms and voluminous bowel movements can contribute to rapid weight loss and dehydration, including abdominal cramping (Arrowood and Sterling, 2000).

2.4.1 Routes of transmission

Collectively, the various species of *Cryptosporidium* have a broad host range affecting gastro-intestinal epithelial cells. The durability of oocysts, along with their diminutive size, lend themselves to the incurable transmittable diseases via municipal drinking water, as they can survive many disinfectants, thus having the potential to cause community- wide outbreaks (Robertson and Gjerde, 2007). Outbreaks illustrate different routes of the transmitted diseases namely person-to-person contact with infected patients (family members, health care workers, users of communal swimming pools and travellers), human-animal contact (zoonosis) (veterinarians, farmers), or via indirect transmission through the environment (particularly by water) (Chen *et al*., 2002). The guidelines of world health organisation (WHO) for drinking water, classifies *Cryptosporidium* as a pathogen of significant public health importance (WHO, 2006).

2.4.2 Epidemiological distribution of infection in humans

The prevalence of this infection varies widely from country to country and from one region to another. However, diarrhoea infection is much greater in developing countries than in developed countries, whereby infection is influenced by ethnic background, gender, socioeconomic conditions, different locations and age group (Xiao *et al*., 2004). Research surveys have been conducted to gain some idea about the prevalence of the parasite in

industrialised nations. An average of about 2-3 and 5% of patients hospitalized for diarrhoea in United Kingdom and United States are infected with chronic cryptosporidiosis and 40% in some third world countries (Xiao *et al*., 2004). The reported analysis of 29 storm water samples in the United States revealed the presence of 14 *Cryptosporidium* genotype oocysts each year for every square meter of surface area (Xiao *et al*., 2004).

A longitudinal study of *Cryptosporidium* infections in children in North Eastern Brazil documented a cryptosporidiosis prevalence of 94% (Lee *et al*., 2005). The most common symptoms were persistent diarrhoea (16.5%) and acute diarrhoea (8.4%) (Xiao *et al*., 2004). Analysis of stool specimens from the immunocompetent, adult population with gastrointestinal symptoms revealed a prevalence of infection close to 2% in Germany (Steeb *et al*., 1987). Remarkably, children aged 1-14 years, were found with prevalence close to 2% (Krause *et al*., 1995). As expected, the prevalence of cryptosporidiosis was higher (4-7.7%) among Human Immunodeficiency Virus (HIV) patients (Heise *et al.,* 1988). Cryptosporidiosis in the Netherlands was found to be responsible for 2.2 % of gastroenteritis cases. The prevalence was found to be highest in patients with acute diarrhoea complaints (lasting < 7 days) being 5.4%. In patients with persistent diarrhoea complaints, the prevalence of *Cryptosporidium spp*. was found to be 3.3% (Mank, 1997).

Furthermore, the distribution of infection appears to be age-related, with the highest prevalence of 7.0% found in patients between 0 and 4 years of age whereas the lowest prevalence (1.1%) was found in patients between 45 and 65 years of age. However, cryptosporidiosis in the Netherlands has a seasonal peak and is most frequently found in patients with diarrhoea complaints in the late summer (Mank, 1997; Mank *et al.,* 1998). In

the region of Aragon, *Cryptosporidium* oocysts were identified in 87 (1.93%) patients. (Clavel *et al*., 1996).

A prospective study in Acquired Immune Deficiency Syndrome (AIDS) patients with chronic diarrhoea in the Madrid area, displayed the overall prevalence of intestinal cryptosporidiosis was 15.6%. The prevalence was found be higher in homosexual patients (33.3%) than in intravenous drug abusers (10.6%). Extra-intestinal infection was present in 30% of the patients with known intestinal cryptosporidiosis (Lopez-Velez *et al*., 1995). Eight of the 13 (61.5%) patients with extra- intestinal cryptosporidiosis had *Cryptosporidium* in the bile and 7 of 13 (16.28%) had it in the sputum. Interestingly, of the seven patients with *Cryptosporidium* in the sputum, four had respiratory symptoms and an abnormal chest radiograph (Lopez-Velez *et al*., 1995). *Cryptosporidium* was the only organism detected in induced sputum and bronchoalveolar lavage specimens. Another study performed in the Zaragoza area, found a prevalence of 3% to 8% in immunocompromised HIV-positive adults (Moles *et al.,* 1998).

2.4.2.1 Human-human transmission

The mechanisms of human transmitted infections are usually caused by direct person to person fecal-oral transfer, as illustrated by health care centers. Transmissions have been reported in day care centers, veterinarians, within households, in urban communities and in public institutions where there is poor hygiene or inadequate sanitary conditions leading to a high prevalence of cryptosporidiosis in developing countries (Robertson *et al*., 2000). Another risk factor that is generally found in these studies include sexual practices that imply

oro-anal contact with diarrhoea or HIV/AID patients, which has lead to high yield exposure to *Cryptosporidium* world-wide (Alireza *et al*., 2009).

2.4.2.2 Zoonosis (animal – human)

Over the past 20 years cattle have been identified as a main reservoir host that carries the parasite which then was transmitted to humans (Sulaiman *et al*., 1998). The role of zoonotic infections in human cryptosporidiosis in developing countries appears much more important than in the industrialized world since 60 – 90% of human infections in developing countries results from *Cryptosporidium parvum* (WHO, 2006). Mixed *Cryptosporidium* zoonotic transmission among *Cryptosporidium parvum* and *Cryptosporidium hominis* have improved our knowledge of understanding about the prevalence of zoonotic infections in humans as the leading cause of persistent diarrhoea in developing countries, and this has led to a major threat to the South Africa water supply. However, a review study on *Cryptosporidium spp* suggests that only the cattle genotype of *Cryptosporidium* is capable of zoonotic transmission whereas the roles of companion animals are considered less important (Xiao *et al.*, 2004).

There are also molecular epidemiological studies documenting transmission of *Cryptosporidium* between humans and livestock by direct contact in animal handling (Casemore *et al*., 1997). The high prevalence of infection in cattle and sheep and the high numbers of oocysts shed by infected animals make cattle and sheep important sources of environmental contamination with *Cryptosporidium* oocysts that are able to infect humans. Waterfowl and insects are also known as transport vehicles of infection, where they pick up oocysts from contact with human or cattle feces and may deposit them in water or on food (Graczyk *et al*., 2000; Szostakowska *et al*., 2004). However, the significance of waterfowl in contamination of watersheds with oocysts that may infect humans is unknown.

2.4.2.3 Transmission through water

Previous studies indicate that *Cryptosporidium* is the most frequently recognized cause of recreational water associated outbreaks of gastroenteritis, particularly in treated (disinfected) venues. The risk of waterborne transmission of *Cryptosporidium* is a serious global issue in drinking water safety. Oocysts from this organism are extremely robust, prevalent in source water supplies and capable of surviving in the environment for extended periods of time. The importance of waterborne *Cryptosporidium* transmission is evaluated based on the number of waterborne cases reported and on the various economic, regulatory, recreational, environmental and social aspects of waterborne disease control (Haas and Rose, 1994).

Outbreaks where many people are affected usually occur when surface water becomes contaminated through the discharge of untreated and treated sewage and run-off of manure (Juranek, 1995). Surveys have indicated that *Cryptosporidium* is found in all types of water sources, and their presence is more common in surface water than in groundwater (Glaberman *et al*., 2002). However, the highly environmentally resistant cyst of *Cryptosporidium spp* allows the pathogen to survive during water filtrations and chemical treatment such as chlorination. These levels may increase rapidly through contamination from agricultural and human effluents. In fact, the incidence of human cryptosporidiosis tends to increase during the spring and summer seasons in South Africa (O'Donoghue, 2002). Currently, no curative therapy exists for the complete removal of *Cryptosporidium* from an infected host. Therefore, the presence of this parasite in drinking water represents a serious threat to public health.

The relative importance of both human and agricultural pollution on surface water reserves has yet to be determined, but both sources have great potential for waterborne contamination,

as high numbers of *Cryptosporidium* have been isolated from agricultural and human effluent in many geographical locations (Smith & Rose, 1998).

2.4.2.4 Transmission through food

The consumption of certain foods such as raw milk and meat, farm-made apple cider, fermented milk, salads, raw vegetables has resulted in cases of human cryptosporidiosis (Casemore *et al*., 1997). Food borne transmission, although not a major route of infection is possible if food becomes fecally contaminated. Moreover, infections have been associated with inappropriate food handling and/or using contaminated water to wash food (Quiroz *et al*., 2000). Interestingly, eating of properly washed raw vegetables have been found to be a protecting factor against *Cryptosporidium* in a case-control study in the United Kingdom (UK) (Hunter, 2003). This could serve as a route and build-up of protective immunity.

2.5 Characteristics relating to water borne transmission

Several characteristics of *Cryptosporidium* facilitate waterborne transmission. These are:

2.5.1 Extreme resistance to chemical disinfection

Disinfection with chlorine has always been an important barrier for waterborne pathogens. However, the high resistance of *Cryptosporidium* oocysts against chlorine disinfection renders this process ineffective for oocysts inactivation in drinking water treatment (Ransome *et al*., 1993). Chlorine dioxide is slightly more effective, but still requires a high measure of disinfectant dose. However, exposure of *Cryptosporidium* oocysts to multiple disinfectants has shown to be more effective than was to be expected from both disinfectants alone and synergism between environmental stress and filtration has also been observed (Liyanage *et*

al., 1997). The multiple stresses that oocysts encounter in the environment and during treatment might limit the infectivity of oocysts. A previous study suggests that Ultra-Violet (UV) systems have a limited effect on *Cryptosporidium* viability, probably due to the use of *in vitro* viability assays that over-estimate infectivity (Clancy *et al*., 1998). More successive studies have shown that oocysts are sensitive to low or medium wavelength pressure Ultra-Violet (UV), but this is not proven yet (Craik *et al*., 2001).

2.5.2 Persistence in the environment

Oocysts can survive for months in surface water and estuarine waters (over 12 weeks at 20^{o}C), but less in seawater (Robertson *et al*., 1992). However, under natural conditions, the survival rate of *Cryptosporidium* oocysts in water is very high. In other successive studies, survival was monitored with *in vitro* assays such as excystation or dye exclusion and it was confirmed that the longevity of oocysts in fresh water inactivation rate is 0.095 at 25, 20, 15 and 4^{o}C respectively (King *et al*., 2005). A longer period of survival (120 days) of oocysts in soil has also been reported. Oocysts in soil do not survive well when artificially frozen/thawed in the soil or under field conditions in Norwegian soil (Kato *et al*., 2002).

2.5.3 Oocyst wall

The disulfide bond-rich oocyst wall provides a protective barrier for infective sporozoites (Mitschlere *et al*., 1994). Unlike other coccidians, the oocyst wall of *Cryptosporidium* is bilayered, consisting of a distinct inner and outer layer. This structure perhaps represents a unique feature of *Cryptosporidium spp* oocysts. However, the outer layer of the oocyst wall is comprised of acidic glycoproteins and is at least partially removed by treatment with sodium hypochlorite, an *in vitro* excystation solution (Reduker *et al*., 1985). Previous studies

revealed that the rigidity and elasticity of the oocyst wall is a consequence of the central glycolipid/lipoprotein layer, and the thick inner filamentous layer, which is believed to be composed of glycoproteins (Bonnin *et al*., 1991).

Interestingly, researchers have examined the oocyst wall structure of *Cryptosporidium* and its susceptibility to different treatments. A filamentous array on the inner surface was seen by transmission electron microscopy following thin sectioning and negative staining of isolated oocyst walls. It was seen that this filamentous array can be depleted by digestion with proteinase K and trypsin, but pepsin was less effective. Ultrasonication of untreated oocyst walls produced almost no lysis. Treatment with proteinase K was the most effective in releasing the internal fibrillar layer from the oocyst wall, as compared to treatments with trypsin and pepsin. Chloroform treatment and phenol extraction did not disrupt the oocyst wall (Harris and Petry, 1999).

2.5.4 Small size

Compared to other protozoan parasites, the oocysts of *Cryptosporidium* are very small and are more difficult to remove during soil passage, in bank filtration and in rapid or slow sand filtration in drinking water treatment, thus making the organism a particular challenge to effective water treatment methods. Although, the state in which oocysts in water suspension are attached to particles is important for water treatment through boiling. Little information is available on oocyst attachment to sand particles in batch experiments (Dai and Boll, 2003).

2.5.5 High infectivity

The infectivity of oocysts has shown to be very high, although uncertainty exists concerning the dose required to induce *Cryptosporidium* infection. Studies suggest that very small

inoculums are capable of inducing infection (Chappell *et al*., 1999). A comparative study completed a *Cryptosporidium parvum* human feeding investigation among healthy volunteers which determined that the dose at which 20 percent of the subjects were infected was 30 oocysts while the median infective dose of *Cryptosporidium parvum* in healthy adult volunteers was 132 oocysts (DuPont *et al*., 1995). Based on a mathematical model on data from previous outbreak, some individuals might develop cryptosporidiosis after ingestion of only one oocyst (Haas and Rose, 1999).

Similar studies employing different *Cryptosporidium spp* isolates recorded doses causing infection in fifty percent of the population from below 100 to 1000 oocysts (Okhuysen *et al*., 1999). Interestingly, an infectious dose did not significantly affect the severity of symptoms, length of the incubation, or number of oocysts shed. The risk of infection following ingestion of one oocyst has been estimated at 0.028 (Okhuysen *et al*., 1999). Significant virulence differences exist between strains of *Cryptosporidium*, but this may not accurately describe the infectivity of other species. However, during acute infection, oocysts can be found in high numbers in the feces of the host, although this is facilitated by auto-infection of the host.

2.5.6 Period of maturation

Unlike other coccidian parasites and helminths, *Cryptosporidium* oocysts do not require a period of maturation of the oocysts after shedding with feces. They are immediately able to infect a new host when excreted by another (Okhuysen *et al*., 1999).

2.5.7 Environmental factors

Cryptosporidium oocysts can persist and survive in the environment for several days or months. In general, aged oocysts are more susceptible to disruption by environmental changes and disinfectants. Temperature has been shown to have an effect on the ability of oocysts to survive in water, including seawater, at temperatures ranging from 4°C to 22°C. Under ambient conditions, the duration of oocyst infectivity decreases as the temperature increased from 4°C to 23°C (Pokorny *et al*., 2002). Warmer temperatures can accelerate oocyst degradation, although oocysts are known to remain infective up to 12 weeks when stored in water at 25°. At extreme temperatures, oocyst viability and infectivity are adversely affected. As the oocyst wall is composed of numerous proteins, their denaturation at elevated temperatures may disrupt oocyst wall integrity and expose the sporozoites to conditions detrimental to their survival (Harris and Petry, 1999).

2.6 Metabolism of *cryptosporidium species*

Analyses of the *Cryptosporidium* genomes have identified some unique metabolic pathways and evidence for the heavy reliance of *Cryptosporidium* on the host for nutrients and glycolysis for energy metabolism. This information might have significant importance in the development of therapeutic agents against *Cryptosporidium* (Abrahamsen *et al*., 2004; Xiao and Ryan, 2008). The molecular and functional studies of proteins and enzymes involved in unique *Cryptosporidium* metabolic pathways would greatly deepen our understanding of the basic metabolism in the parasites. Since *Cryptosporidium* cannot synthesize fatty acids de novo. Instead, it possesses a giant type I fatty acid synthase (CpFAS1) that makes a very-long-chain fatty acids using intermediate- or long-chain fatty acids as precursors (Zhou *et al*.,

2003). The latter type of precursors probably has to be transported from the host cells through the parasitophorous vacuole membrane (PVM).

In recent study, researchers have been able to localized three *Cryptosporidium* proteins to the PVM (Zhu, 2004). These proteins included an unusual fatty acyl coenzyme A binding protein (ACBP), one of the two oxysterol binding protein-related proteins (ORP1), and a long-chain-fatty-acid elongase (LCE). Although the PVM is mainly a host cell-derived membrane structure, it also contains proteins from the parasite. Because all three proteins are known to be involved in fatty acid metabolism, it is very likely that the PVM may play an important role in lipid metabolism and/or remodeling. However, *Cryptosporidium* lacks enzymes for the oxidation of fatty acids, indicating that fatty acids are not an energy source for this parasite.

2.6.1 Amino acid metabolism

Amino acids are the basic building blocks of proteins, C*ryptosporidium* apparently cannot synthesis any of them *de novo*. Despite all amino acids synthetic genes are missing from *Cryptosporidium* genome. Instead, the parasite possesses at least 11 amino acids transporters that scavenge amino acids from host cells and the intestinal lumen. Unlike *P. falciparum*, which only possesses one amino acid transporter (Abrahamsen *et al*., 2004). However, *Cryptosporidium* retains the capacity of intercoverting a limited number of amino acids such that glutamate produced by Guanosine monophosphate (GMP) synthetase can be recycled back to glutamine by glutaine synthetase, serine and glycine may be intercoverted by serine hydroxymethyl transferase (SHMT) within the folate metabolism pathway. Asparagine can be made from aspartate by asparagine synthetase, which might be important in the recycline of NH3 released by Adenosine monophosphate (AMP) deaminase (Madern *et al*., 2004).

2.7 Pathogenesis of *cryptosporidium species*

Cryptosporidium possesses numerous surface glycoproteins thought to play a role in pathogenesis. The mechanism involved in the invasion of *Cryptosporidium* sporozoites into epithelial cells is not clear. Thus far, the few antigens shown to be important for the attachment and invasion of *Cryptosporidium* into the host cell are all mucin-like glycoproteins such as gp60 and gp900 (Xiao *et al*., 2002). GP900 localizes to the apical end of sporozoites and in micronemes of merozoites, where it attaches intimately to the microvillous membrane and causes loss of microvilli and effacement, which results in malabsorption. The organism immediately activates a second-signal pathways, such as the nuclear factor-kappa B (NF-κB) and Proto-oncogene protein pp60 (C-src) systems. Activation of (NF-κB) induces the production of cytokines and chemokines, such as interleukin-8 (IL-8), to trigger an inflammatory reaction and stimulates anti-apoptotic survival signals in directly infected cells (presumably facilitating the organism's ability to survive and propagate). Activation of C-src is associated with host-cell cytoskeletal reorganization and perhaps dysfunction of tight junctions (Cevallos *et al*., 2002). Human immunodeficiency virus (HIV) type 1 infection can further amplify damage by the action of soluble factors, such as tat protein with varying degrees of villous atrophy by an unknown mechanism, resulting in epithelial damage.

Histological findings of severe villous atrophy, crypt hyperplasia and presence of several developmental stages on the mucosa have been found associated with an altered glucose-stimulated Na^+, water absorption, and increase in Cl^- secretion (Argenzio *et al*., 1990). However, glucose stimulated sodium absorption was inhibited, thereby increasing chlorine secretion, and epithelial cell damage. Therefore it is still a thought that diarrhoea results from

a combination of secretory sodium/glucose malabsorption due to villous atrophy and epithelial damage.

Immunological and molecular methods have identified several possible virulence factors which may affect the adhesion of *Cryptosporidium* to the host intestinal epithelial layer such as ATP transport to cause membrane disruption (Riggs *et al*., 1997). These events could ultimately lead to malfunctioning in the small intestine epithelium, induced by *Cryptosporidium parvum*-infected individuals.

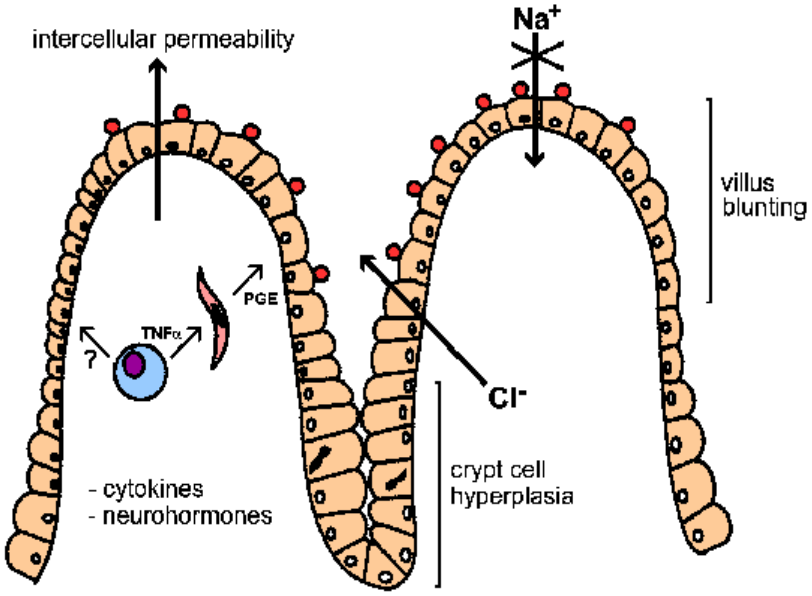


Figure 2.2: Schematic representation of *Cryptosporidium* pathogenesis. (Clark and Sears, 1996).

Villi often are blunt, shorter, and wider than normal and are sometimes fused to other villi, whereas crypts are elongated and hyperplastic. Changes in villous and crypt structures may be accompanied by an inflammatory infiltrate consisting of lymphoid cells, macrophages, and neutrophils in the underlying lamina propria (Xiao *et al*., 2001).

2.7.1 Human immune responses to *cryptosporidium* infection

Knowledge about the human immune response towards *Cryptosporidium* infection is poorly understood (Guk, 2003). In human hosts, cell-mediated immune responses by B and T lymphocytes, which includes the production of IgG, IgM, IgA, and IgE antibodies, are necessary to control cryptosporidiosis (Guk, 2003). Deficiency in any of these immune cells can result in difficulty or inability to conquer Cryptosporidium infection, making cryptosporidiosis a particularly dangerous pathogen for AIDS patients. Although the mechanism by which Cryptosporidium infection induces adaptive immune responses in host cells is not understood completely, information on most immunocompetent animals were found susceptible to *Cryptosporidium* only when very young and then they rapidly developed innate resistance. In contrast, age related susceptibility to *Cryptosporidium* in humans is not clear. Persons of any age may acquire infection (Xiao *et al*., 2001).

2.7.1.1 Clinical features

The symptoms of cryptosporidiosis differ greatly between immunocompetent and immunocompromised hosts. In the immunocompetent patient, the disease is acute and self-limiting, producing diarrhoea illness that typically occurs for one to two weeks. Children are much more susceptible to *Cryptosporidium* infections than other age groups, probably due to their immunological immaturity (Smith and Corcoran, 2004). However, infection takes advantage of the impaired or destroyed immune system, causing a persistent infection that seriously dehydrates the patient as up to 20 litres of excreta per day and ultimately results in a higher mortality rate (Keusch *et al*, 1995). The most common clinical signs of *Cryptosporidium* infection are profuse and watery diarrhoea. Other general signs include crampy abdominal pain, fever, nausea, vomiting and low grade fever (Smith and Corcoran, 2004). A plethora of such signs is often associated with a marked weight loss. Patients can

have chronic diarrhoea that can last for more than two months, shedding oocysts in stool during the entire period, which contributes to severe dehydration, weight loss and malnutrition, extended hospitalizations, and mortality (Shin *et al*., 2001). Thus, the duration and severity of clinical signs reflect the immune status of the patients.

Due to the immunological deficiency, HIV/AIDS patients are at a high risk of contracting cryptosporidiosis. In such patients, infection produces persistent diarrhoea that worsens with time and eventually contributes to death. Infections are not always confined to the small intestine and parasites have been found in the oesophagus, stomach, appendix, colon and rectum (Smith and Corcoran, 2004).

2.7.1.2 Absence of curative therapy

Currently, no drug regimens are known to be effective in preventing the recurrence of cryptosporidiosis infection, despite *in vitro* and *in vivo* testing of hundreds of compounds. One possible explanation for this is that *Cryptosporidium* establishes a compartment within the host cell, which is morphologically different from the setting used by related parasites. This unique parasitophorous vacuole may somehow shelter the parasite from antimicrobial drugs (Griffiths *et al*., 1998).

Regardless of the significance of *Cryptosporidium* infection in childhood health, there has been a lack of serious effort to invest in the development of affordable and effective therapeutics specifically targeting *Cryptosporidium* species. Existing therapeutics for other apicomplexan diseases are ineffective against *Cryptosporidium* infection, perhaps because of the unique intracellular, extracytoplasmic location of *Cryptosporidium* and the poorly understood host-parasite interface. In addition, difficulties in laboratory ideal cell culture methods, have limited drug screening.

Clinical course of cryptosporidiosis depends largely on the immune status of the host, treatment with options vary accordingly (Griffiths *et al*., 1998). Healthy persons with normal immune systems usually get better on their own. The recommended treatment is to drink plenty of fluids and to get extra rest. In immunocompetent adults and children, no specific therapy is indicated, since the disease is self-limiting. Individuals with persistent diarrhoea, an underlying immunodeficiency (HIV, congenital immunodeficiency, etc.) might need special treatment from a health-care provider to replace fluids lost during the illness.

However, some encouraging results following the use of supportive therapy remains the preferred option for the management of symptoms arising from the disease. Several drugs have been tested in the laboratory. An acknowledged report suggests that Paromomycin is an antimicrobial drug that can alleviate cryptosporidiosis symptoms with a reduction of oocyst excretion (Steiner *et al*., 1997). Paromomycin is typically administered at a dose of 500 mg four times daily for 4–8 weeks, with a recommended maintenance dose of 500 mg twice daily following the initial treatment period (Hoepelman, 1996). However, clinical evaluations of these drugs have been disappointing, mainly because they failed to clear the parasite from patients with HIV/AIDS. To date, anti-infective nitazoxanide (NTZ) have shown the most promise against *Cryptosporidium.*

Nitazoxanide, also known as Alinia, is an antiprotozoal drug believed to interfere with the pyruvate ferredoxin oxidoreductase-mediated electron transfer reaction that is essential for anaerobic energy metabolism in the parasite (Herbert and Hoffman, 2002). Presently, nitazoxanide is the only drug approved by the US Food and Drug Administration (FDA) for the treatment of diarrhoea caused by *Cryptosporidium* in children. The drug was found to be effective in AIDS-related cryptosporidiosis during a double-blind placebo-controlled trial of

66 HIV patients in Mexico (Rossignol *et al*., 1998). When administered at a dose of 500 or 1000 mg twice daily for 14 days, nitazoxanide resulted to a cure rate of 63% and 67% of patients, respectively.

Co-administration of both drugs did not further reduce *Cryptosporidium* infectivity (Rossignol *et al*., 1998). A previous study revealed that HAART reduced diarrhoea from both cryptosporidiosis and microsporidiosis in HIV patients (Rossignol *et al*., 1998). As HAART reduces the viral load, CD4 T lymphocyte counts are increased and there was a delay in the onset of HIV-related opportunistic infections, including cryptosporidiosis. However, the therapy is not easily affordable for affected individuals in the developing world.

2.7.1.3 Prevention

As cryptosporidiosis is initiated by ingestion of infective oocysts, therefore control measures are aimed at reducing or preventing oocyst transmission. In locations such as hospitals, laboratories and day-care centers, contact with potential sources of infection should be minimized. This entails personal hygiene, proper handling and disposal of bio-hazardous wastes, and boiling of water prior to consumption. Individuals with weakened immune system are advised to always use a home micro-straining water filter, capable of removing particles less than 1 micrometer in size. Avoid sexual practices that can result in hand or mouth exposure to stool, avoid direct exposure to cattle and other farm animals. If exposure cannot be avoided, wash hands well immediately after contact (Roberson and Bruno, 1997).

2.8 Laboratory diagnosis

Laboratory techniques are usually applied for identification of *Cryptosporidium*. The need to identify the organism in diarrhoea patients is useful to avoid misuse of antibiotics, to reduce the spread of the disease in a community, as well as to follow the need for control and preventive measures. For many years, microscopy has been the only tool available for the detection of parasites through inspection of blood smears, tissue specimens, feces, lymph node aspirates, bone marrow, and even cerebrospinal fluid (Cruz *et al*., 2006).

Diagnosis of cryptosporidiosis is made microscopically by examination of stool samples for the presence of oocysts. Mature *Cryptosporidium* oocysts recovered from stool can be easily identified using differential staining methods such as safranin-methylene blue stain, modified Kinyoun's acid-fast method, Ziehl-Neelsen and DMSO-carbol fuchsin stain which stain oocysts red and counterstain the background. However, sample preparation for direct observation is time-consuming, labour intensive, and proper diagnosis depends on qualified laboratory technicians (Cruz *et al*., 2006). Because detection of *Cryptosporidium* is difficult, patients may be asked to submit several stool samples for identification. In endemic regions, where resources are limited, this proves to be difficult and misdiagnosis can significantly impact patient care. In reality, all major intestinal helminth infections are still solely dependent on microscopy for diagnosis. As for other parasite infections, many are confirmed by the use of microscopy in conjunction to other methods of diagnosis including serology-based assays and more recently molecular-based assays.

Evaluation of *Cryptosporidium* infection by serology is much more sensitive than detection of oocysts in stool samples, especially in populations chronically exposed to *Cryptosporidium*

through contaminated food or drinking water, and has been widely used for epidemiological studies (Moro and Schantz, 2009). Serology-based assays include the enzyme-linked immunosorbent assay (ELISA), also called enzyme immunoassay (EIA), and all its derived tests such as the Falcon assay screening test ELISA (FAST-ELISA) and the dot-ELISA. Other assays include the hemagglutination test, indirect or direct immunofluorescent antibody (IFA) or Direct immunofluorescent antibody (DFA) tests, complement fixation (CF) test, and immunoblotting and rapid diagnostic tests. Although the ease of use and turn-around times for serologic assays are similar to microscopy. It becomes important for individuals exhibiting low-parasitemia and/or who are asymptomatic (Moro and Schantz, 2009).

Finally, having these tests readily available allows for the monitoring of parasite clearance following therapy. However, cross-reaction leading to false-positives and misdiagnosis is a problem, especially in Africa regions where more than one parasite is endemic (Burbelo *et al*., 2008). To a lesser extent but nonetheless important is the inability of antibody-detection tests to differentiate between past and currently active infection. Furthermore, antibody-detection tests cannot be used in parasitic infections that do not develop a significant antibody response. This has been observed in some individuals carrying *Echinococcus* cysts (Moro and Schantz, 2009). For all these reasons, there is still a need to improve on the current diagnosis approaches available. Since the advent of the polymerase chain reaction (PCR), parasitologists have turned to molecular-based approaches in the hopes to better the existing diagnosis tools (Diez *et al*., 2007).

The many limitations of microscopy and serology-based assays have influenced parasitologists towards the use of gene amplification methods made possible with the advent of polymerase chain reaction. PCR offers many advantages over other techniques for the

clinical and environmental detection of *Cryptosporidium species* and is capable of differentiating the taxonomic membership between *C.* strains that infect humans. Cost-effective and amenable to quantitation, PCR allows for the analysis of multiple samples in a short time frame. Besides the traditional PCR, including nested and multiplexed PCR, the implementation of the quantitative PCR (q-PCR) for the detection of several parasitic infections have also emerged as possible new approaches for the diagnosis of parasitic diseases (Gasser, 2006).

Molecular-based approaches based on nucleic acids have been found to offer greater sensitivity and specificity over the existing diagnostic tests. They permit the detection of infections from very low parasitized samples including those from asymptomatic patient's samples (Zarlenga and Higgins, 2001). Moreover, multiplexed PCR allows for the detection of multiple sequences in the same reaction tube proving useful in the diagnosis of several parasitic infections simultaneously (Gasser, 2006).

q-PCR system unlike conventional PCR, allow for the quantification of the original template's concentration through the use of various fluorescent chemistries, such as Sybergreen, Taqman probes, fluorescence resonance energy transfer (FRET), and Scorpion primers (Muldrew, 2009). This eliminates the need to visualize the amplicons by gel electrophoresis thereby greatly reducing the risk of contamination and the introduction of false-positives. Running the multiplex assay not only reduced the cost per test but also allowed for a rapid turnaround time, in a single reaction tube even in very low parasitized samples and it allows for the high-throughput analysis of different sequences in one single-closed tube reaction. It is a clear advantage over microscopy which is labour intensive and time-consuming with slow turnaround times especially during high-throughput settings.

These examples demonstrate that efficient and early diagnosis can directly impact patients care and that PCR-based approaches have the potential to help in making the right choice for treatment.

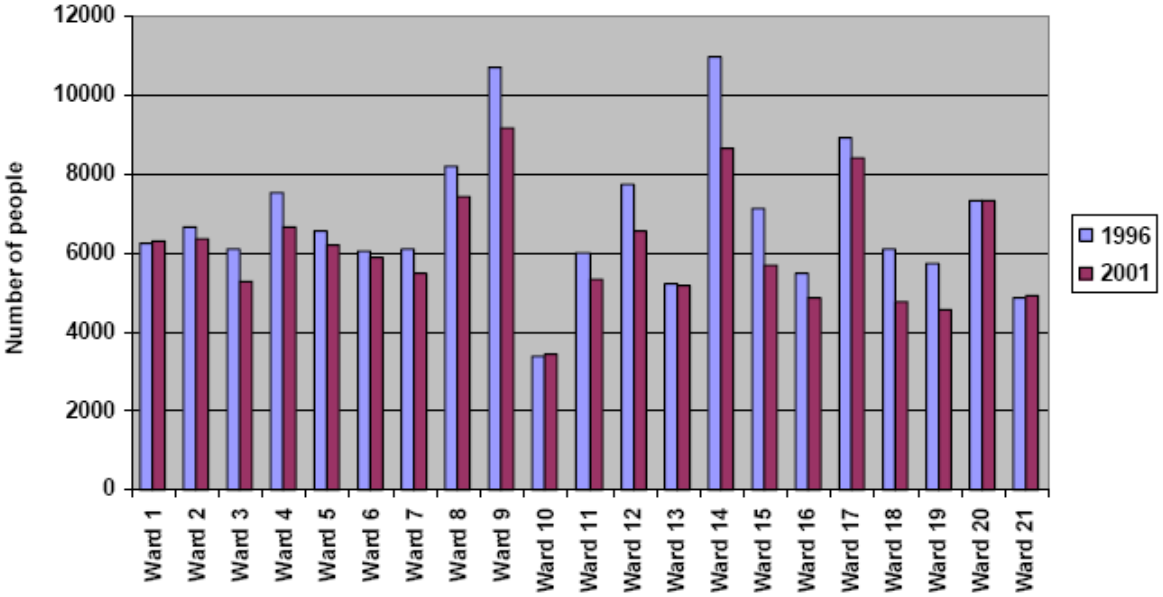
Although DNA-based methods have shown excellent sensitivity and specificity, the introduction of these methods in daily laboratory practice is still uncommon especially in rural endemic regions. In addition, as observed with many serology-based assays, PCR-based methods also suffer by the lack of standardization (Murray and Cappello, 2005). DNA extraction, choice of primer sets, and use of various amplification protocols are all factors that may cause this diversification in results. Adding an automated DNA extraction step would certainly improve PCR assays for use in the diagnosis of parasitic diseases. However, the presence of inhibitors, sample contamination and precipitation of oocyst nucleic acids represent challenges facing the current PCR techniques. A shortcoming of PCR is that it does not directly assess viability (Toze, 1999).

CHAPTER THREE

3.0 Materials and methods

3.1 Study design, site and population

Nkonkobe Municipality is one of the most populated amongst the administrative province of the Eastern Cape. There are 21 wards within the Nkonkobe municipality area. Approximately 74% of people living within the area are indigent. About 20% of the population resides on the farms and scattered locations. Only about 19% of the population of Nkonkobe resides in locations, mostly Alice town (Fig 3.0: Ward 5) which accounts for the smallest population within Nkonkobe area and has a diversity of individuals with different socioeconomic potential and women are in the majority (52.55%). The majority of inhabitants, however, fall in the low socio-economic strata, where they lack proper sanitation, poor water services, high levels of unemployment and poverty (Xuza, 2007).



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suggest that a comprehensive study of the impact of cryptosporidiosis is extremely crucial at the moment (Xuza, 2007). Since the estimated population is known to be 16,000 and using a margin of error of 0.05 and a critical standard score (z) of 1.96, for a 95% confidence interval

by a simple random sampling, the minimum number of respondents needed for the study is 162.2. Victoria Hospital where our samples were collected, serves as a major hospital for the many surrounding villages found in Alice. These surrounding villages from which the study subjects were drawn reside in deprived communities that are relatively undeveloped with poor service delivery (Fig 3.1). This condition may enhance the chances of *Cryptosporidium* transmission.

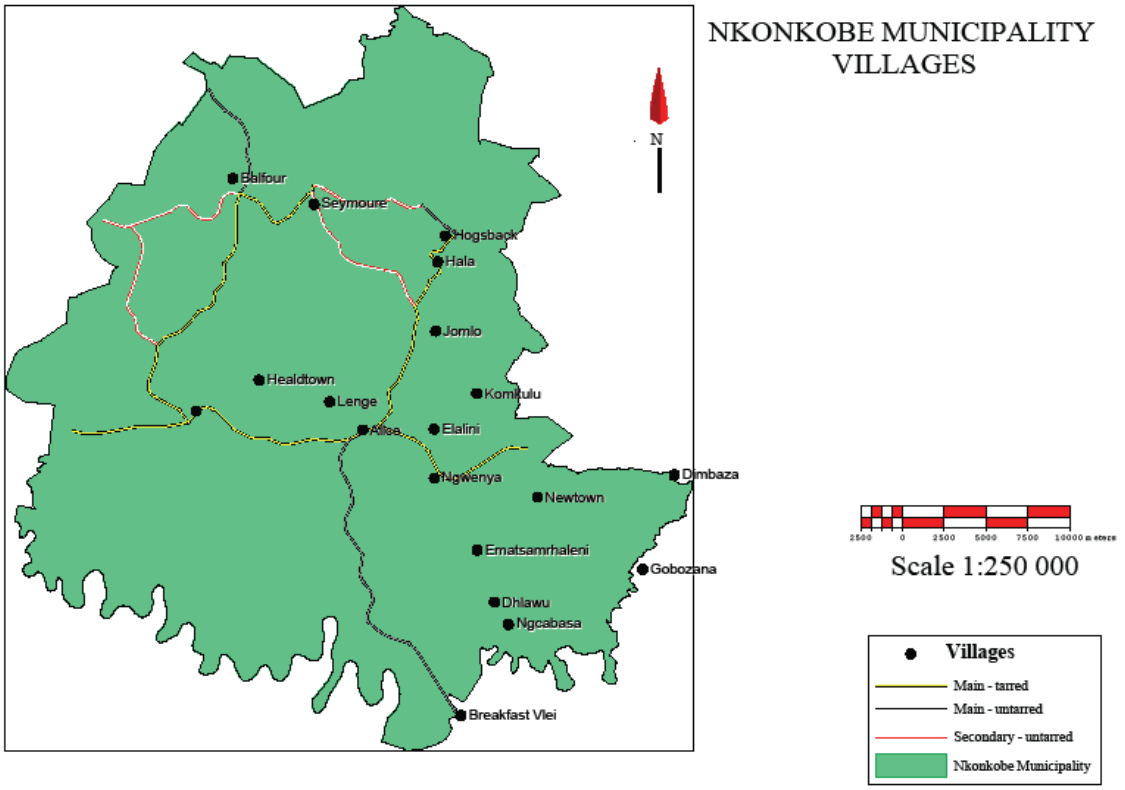


Figure 3.1: Nkonkobe Municipality villages

3.2 Ethical issue and data collection

This study was approved by Institutional Ethics Review Board of University of Fort Hare (UFH) through the Govan Mbeki Research Development Centre (GMRDC). A standardized questionnaire describing demographic characteristics including age, gender, socioeconomic status, route of transmission of infection and the sampling date were given to participants to

fill in. Household characteristics, including toilet facilities, and potable water, were also noted. When required, patients were interviewed in the local dialect by one of our group members before the collection of specimens. The aim of the research was explained to each patient; though their consent to participate was poor. Confidentiality of the voluntary participants was maintained.

3.3 Patients and control

A total of hundred and eighty (180) samples were collected individually from each patient between April 2009 and January 2010 at Victoria Hospital (Alice). Our study population consisted of patients with ages ranging from 18-95 (mean age 41.4 yr). These groups differ with respect to mean age, gender, source of water, contact with any farm animals, availability of sanitary facility and socioeconomic status. A sterile wide mounted plastic container was provided to all participants. The consistency of the stool specimens was graded by categories (watery, soft and hard) while waiting to be processed. Stool specimen comprised of 35 HIV-positive diarrhoea patients, 125 diarrhoea patients without HIV and 20 controls, i.e healthy individuals without any history suggestive of cryptosporidiosis.

3.4 Stool specimens and processing

Fecal samples from 180 subjects were collected fresh in sterilized, wide mouthed plastic containers and transported to the Microbial Pathogenecity and Molecular Epidemiology Research Laboratory in the Department of Biochemistry and Microbiology, University of Fort Hare on ice and processed directly on arrival or kept refrigerated at -20^{o}C without preservatives until processed.

3.5 Microbiological and immunological characterisation

3.5.1 Parasite diagnosis of *cryptosporidium* oocysts

Microscopic diagnoses of *Cryptosporidium* were performed by the Modified Ziehl-Neelsen (MZN) stain (Casemore *et al*., 2004). Briefly, a drop of each fecal suspension was placed on a glass slide and spread to form a thin smear. The slides were fixed with either acetone or absolute methanol for 30 seconds and then flooded with carbol fuchsin for 1 minute. After washing, slides were decolorized in 3% acid-alcohol for between 15 sec and 1 minute, depending on the film thickness. Slides were washed, counterstained with 3% malachite green for 2 minutes, washed again and dried on a slide warmer at 60°C, for 5minutes and then examined under 40 × objectives and 100 x oil immersion objectives to view oocysts.

Visual oocyst count*:* The number of oocysts was determined by scanning through each slide randomly. This was done by moving three different parts of the slide each at a time across the x10 objective and looking out for the pinkish stained oocyst. Oocysts encountered were confirmed using 200–400× magnification on a Zeiss Axioscop epi-fluorescent microscope to confirm their internal morphology. Oocysts were oval or round shape approximately 4-5 µm in diameter and pink-red coloration (Fig 3.1). These were counted and their numbers recorded. Presence or absence of diarrhoea was recorded. Stained fecal smears on microscope slides were stored at room temperature. In cases where more than four *Cryptosporidium* oocysts were visualized, the sediment of the second tubes were utilized for DNA extraction within 2 weeks of preparation, and subsequent PCR detection.

3.5.2 Enzyme-linked immunosorbent assay

3.5.2.1 Antigen detection

A sandwich-type enzyme immunoassay amplification technology (Amplified IDEIA™ HP StAR™, Oxoid, UK) was used to analyze the fecal samples for detecting *Cryptosporidium* antigens according to the manufacturer's instructions. Briefly, a supernatant of fecal suspension was added to the wells of a microtiter plate coated with fluorescent monoclonal antibodies specific for *Cryptosporidium* antigen. Two hundred microlitres (200 µl) of horse-radish peroxidise (HRP)-labelled mouse immunopure 1gG monoclonal anti-CSA conjugate was added to each well of the plate, covered, and incubated for 60 minutes at 20^{o}C with shaking. The plate was washed with buffer (5 times) to remove unbound antibody conjugate; a colourless single-component enzyme substrate (Tetramethylbenzidine-(TMB) was added, incubated for 10 minutes at 20^{o}C and observed for a colour change. A stop solution was added and the optical density (OD) was read in an ELISA plate reader (Rayto 2100C, CH) at an absorbance of 450 nm. Cryptosporidial antigen was used as a positive control. The value for a positive reaction was calculated to be double the optical density value of the negative control. OD values > 0.05 were considered positive. For comparative evaluation of diagnostic techniques, sensitivity, specificity, positive predictive and negative predictive values and diagnostic efficacy of the two techniques were determined by standard formulae (Galen and Gambino, 1975).

3.6 DNA extraction with zymo-(research) fecal kit

DNA extraction using Zymo-research protocol was performed as per the manufacturers' instruction. Briefly, about 200mg faecal samples were added to a ZR bashing bead lysis tube, and lysed with 750µl lysis buffer. Sample tubes were homogenized and centrifuged at 12,000x*g* for 1minute and 400µl of the supernatant were transferred to each orange top cover

and centrifuged again at 7000rpm for 1minute. The top tube fixed to the collection tubes was discarded and 1,200µl of faecal DNA binding buffer was added to the filtrate in the collection tube. 800µl of each mixture was transferred to a new Zymo-spin 11C column in a collection tube and was centrifuged at 10,000x*g* for 1minute. The step was repeated by discarding the liquid from the collection tube, fixed back to the top tube and another 800µl of the mixture was added and centrifuged for 1minute at the same temperature. Each top tube was placed into a new collection tube and 200µl DNA pre-wash buffer and was added centrifuged for 1 minute at the same temperature. Five hundred microliter (500µl) fecal DNA wash buffer was added to the "11C" tubes, centrifuged for 1minute and transferred to a new clean 1.5ml tube. 100µl DNA elution buffer was added directly to the column matrix, centrifuged for 30sec to elute the DNA. Eluted DNA was transferred to the 'green top' column, fixed in a clean 1.5ml tube. These were centrifuged finally at 8000x*g* for 1 minute. Extracted DNA was run in 2% electrophoresis gel to view bands.

3.7 DNA amplification by PCR

The gene fragment of interest of a 599-bp region of the small sub-unit (18S) rRNA of *Cryptosporidium parvum,* was amplified with the following primers CPF-5' GTGCCAGCAGCCGCGGTAAT-3' together with universal lower eukaryotic reverse primer CPR-(5'- AAGCCGCAGGCTCCACTCCT-3'), which correspond, respectively, to positions 542 to 561 on the coding strand and positions 1,140 to 1,121 on the negative strand of GenBank sequence Af093489. The reactions were performed with Perkin-Elmer thermocycler model 9600 in 0.5 ml thin-wall eppendorf tubes (Sturbaum *et al.*, 2001; Bialek *et al*, 2002). Each 50µl PCR tube reaction contained PCR buffer (10 mM Tris-HCl, 50 mM KCl [pH 8.3]), 1.5 mM $MgCl_2$, 200µM dNTPs, 0.5µM of each specific oligonucleotide

primers, and 2.5 U of *Taq* DNA polymerase (Boehringer), 5µl of purified DNA extract. After a fifteen-minute hot start at 95^{o}C, the reactions went through 35 cycles of denaturation at 94°C for 1minute, annealing at 65°C for 1minute, and extension at 72°C for 1minute, followed by a 72°C incubation for 15 minutes for strand completion. An aliquot of each PCR product was examined by agarose gel electrophoresis. Each run included a negative control, consisting of a reaction mixture with water instead of DNA template.

3.7.1 Detection of amplified products

Following PCR amplification, all reactions were fractionated on a 2.0% agarose gel containing 0.17L 1 x TAE buffer. Gel electrophoresis at 85 volts for 1.5 hours was performed using a using a Subcell tank (BioRad Laboratories, Hercules, Calfornia, USA) and immersing in 1 x TAE buffer. The gel was visualized by 10µg/ml ethidium bromide (Sigma Chemical Co., St. Louis, Missori, USA) under ultra-violet light illumination (FotoPrep R, Fotodyne Inc, New Berlin, Wisconsin, USA) to view the anticipated molecular size.

3.8 Statistics analysis

The statistical package for sciences (SAS Institute Inc. Cary, NC, USA) version 8.2 was used to analyze the data. Univariate association between *Cryptosporidium* infection and possible risk factors were assess using Pearson's $\chi 2$ test and Fisher's exact test. The odds ratio (OR) and the corresponding 95% confidence interval (Cl) were calculated to measure the strength of association. P-values < 0.05 were required for significance.

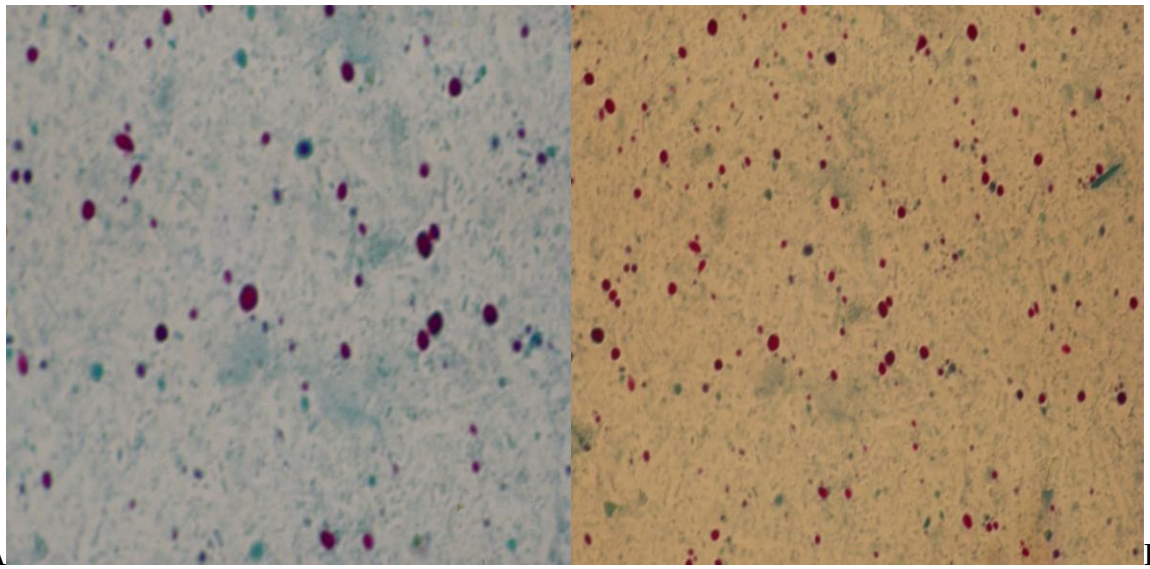
CHAPTER FOUR

4.0 RESULTS

Section A: Symptomatic prevalence studies

4.1 Microscopic detection of *cryptosporidium* oocysts

A total of hundred and eighty (180) samples consisting of 35 HIV positive-diarrhoea patients, 125 HIV-negative-diarrhoea patients and 20 apparently healthy control subjects were examined over a period of one and half ($1^1/_2$) years. *Cryptosporidium* oocysts was detected in 47 (26.1%) of the 180 stool samples by staining with modified Ziehl-Neelsen stain. Oocysts were found in 13 (37.1%) HIV-positive diarrhoea and 34 (27.2%) HIV-negative diarrhoea patient stools. However, because there are marked differences and most oocysts measure 4-6 micrometer, appear nearly spherical, and have obscure internal structures, we were not able to detect any significant morphologic differences between species, although they were statistically significant ($P<0.05$). Consequently, we rely on a combination of data results from the morphometrics, molecular techniques, and host specificity of *cryptosporidium*.



A B

HIV-positive diarrhoea patient HIV- negative diarrhoea patient

Figure 4.0: *Cryptosporidium* oocysts stained pinkish red when examined at 200–400× magnification on a Zeiss Axioscop epi-fluorescent microscope and were observed as thick walled, round structure approximately 4-6µm in diameter.

4.2 Clinical and epidemiological data by ELISA assay

The variables linking epidemiological risk factors and prevalence of the parasite between HIV- positive diarrhoea and HIV-negative diarrhoea patients as analyzed by the univariate analysis are shown in Table 4.0 below.

Table 4.0: Risk factors of *Cryptosporidium* infection associated with prevalence in HIV-positive and HIV-negative diarrhoea patients.

	HIV-Positive					HIV-negative				
Variable	N	Diarrhoea (%)	OR (95% Cl)	$\chi 2$	P- value	N	Diarrhoea (%)	OR (95% Cl)	$\chi 2$	P- value
Age(years)			0.75 (0.34-1.62)	7.233	0.204			0.92 (0.68-1.23)	5.839	0.322
18 -30	2	1 (50.0)				8	7 (87.5)			
31- 43	13	13 (100)				34	27 (79.4)			
44 -56	5	4 (80.0)				11	6 (54.5)			
57- 69	10	8 (80.0)				31	23 (74.2)			
70- 82	3	3 (100)				25	21 (84.0)			
83- 95	2	1 (50.0)				16	6 (37.5)			
Gender			1.39 (0.30-6.23)	0.181	0.670			1.50 (0.63-3.50)	0.852	0.356
Male	11	7 (63.6)				34	25 (73.5)			
Female	24	19(79.2)				91	71 (78.0)			
Water source			1.79 (0.32-9.90)	0.399	0.528			1.24 (0.42-3.69)	1.179	0.271
Tap	17	9 (52.9)				65	55 (84.6)			
River	8	7 (87.5)				15	14(93.3)			
Mixed	10	6 (60.0)				47	41 (87.2)			
Contact with farm animals			0.16 (0.03-0.97)	4.525	0.033			0.51 (0.02-0.14)	45.935	<.0.001
Yes	28	23 (82.1)				92	78 (84.8)			
No	7	3 (42.9)				34	8 (23.5)			
Sanitary facility			10.93 (1.8763.97)	8.426	0.004			4.80 (0.39-58.01)	1.667	0.197
Flush	24	7 (29.2)				90	35 (38.9)			
Pit	11	9 (81.8)				38	27 (71.1)			
Socioeconomic status			0.58 (0.10-0.32)	1.125	0.890			1.69 (0.66-4.28)	19.524	0.034
Low income	21	18 (85.7)				60	50 (83.3)			
Average	8	4 (50.0)				19	11 (57.9)			
High	6	2 (33.3)				45	6 (13.3)			

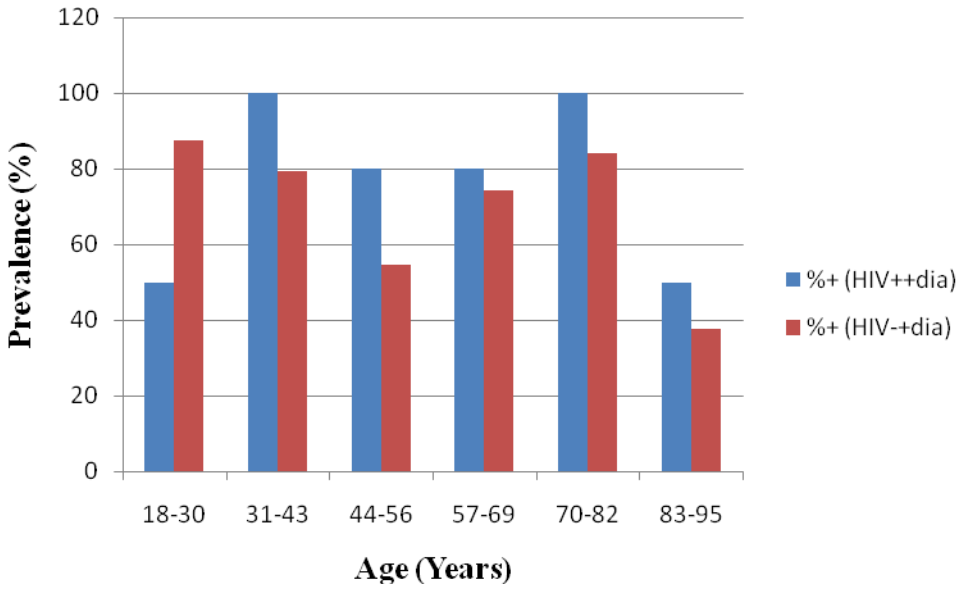
N=Total number of patients, *P*-value for significance; OR, odd ratio; Cl, confidence interval

4.3 Cryptosporidiosis infection in HIV-positive and HIV-negative diarrhoea patients

Cryptosporidium antigen was detected in 26 (74.3%) of 35 HIV-positive and 96 (76.8%) of 125 HIV-negative diarrhoea patients respectively. None of the 20 apparently healthy controls subjects was found to be infected with *Cryptosporidium*. Overall antigen prevalence of 67.8% was detected in 122 of the 180 subjects enrolled.

4.4 Demographic data and socio-economic risk factors

The ages between 31-43 (mean age 36.5 yr) and 70-82 (mean age 75.8 yr) had a higher prevalence (100%) of the antigen than 18-30 (mean age 23.2 yr) and 83-95 (mean age 88.8 yr) (50.0%) in HIV-positive diarrhoea patients (P > 0.05; OR = 0.75, 95% Cl: 0.34-1.62). In HIV-negative diarrhoea patients, the prevalence was highest in the 18-30 (mean age 23.2 yr) (87.5%) and least (35.7%) in those aged 83-95 (mean age 88.8 yr) (P > 0.05; OR = 0.92, 95% Cl: 0.68-1.23).



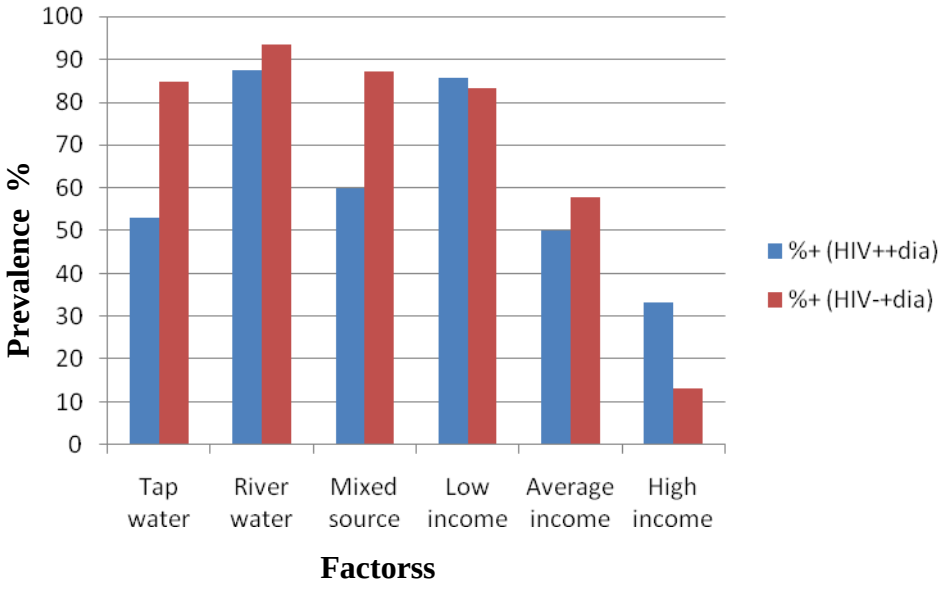
Keys: % + (HIV++dia) = HIV-positive diarrhoea patient

% + (HIV-+ dia) = HIV-negative diarrhoea patient

Figure 4.1: Prevalence of cryptosporidiosis in HIV-positive diarrhoea and HIV-negative diarrhoea patients.

The prevalence of *Cryptosporidium* antigen was higher in females than in males with a ratio of approximately 1:4. Of 115 female (mean age 46.7yr) who participated in the study, the antigen was detected in 90 (78.2%) against 32 (71.1%) of 45 males (mean age 42.6yr). There was no statistically significant association between HIV-positive male and female diarrhoea patients ($P > 0.05$; OR = 1.39, 95%Cl: 0.30-6.23) and HIV-negative male and female diarrhoea patients (OR=1.50, 95% CI: 0.63-3.650).

Cryptosporidium antigen was higher in those who used river and mixed water sources than in those who used tap water. A significantly higher ($P < 0.05$) prevalence of antigen was observed in HIV-negative diarrhoea than HIV-positive patients considering contact with farm animals as a risk factor. Table 4.0. Prevalence of antigen was significantly higher ($P < 0.05$) among HIV-negative diarrhoea patients than in HIV-positive diarrhoea patients as per socio-economic status. Prevalence peak was detected in low income (85.7%) of HIV-positive diarrhoea patients than high income (32%) of same category of patients.



Keys: % + (HIV++dia) = HIV-positive diarrhoea patient

% + (HIV-+ dia) = HIV-negative diarrhoea patient

Figure 4.2: Correlation of independent factors associated with disease prevalence.

4.5 DNA amplification by direct PCR

Cryptosporidium positive samples yielded products of the expected size about 599bp in the PCR analysis of the SSUrRNA gene. This was less sensitive than ELISA. Of the 180 subjects samples amplified, *Cryptosporidium species* was detected in 112 (62.2%). Amplification yielded band fragments which indicated the presence of *Cryptosporidium species* (Fig 4.3). No amplification of DNA was detected in the negative-controls.

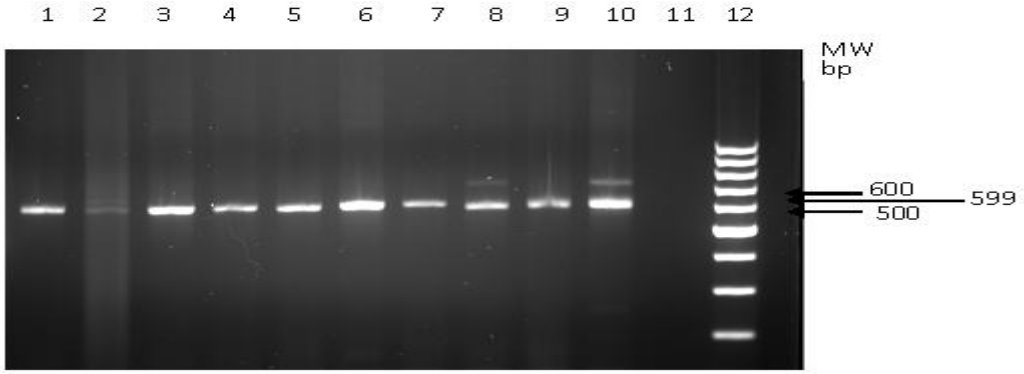


Figure 4.3: Amplification products were confirmed using PCR from DNA. The 599 bp amplicons were visualized on 2·0 % agarose electrophoresis gels. Lane 12 was loaded with a 100 bp standard; lane 11; negative control containing al1 reaction products and no DNA; lane 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 are HIV-negative diarrhoea results showing *C. species.*

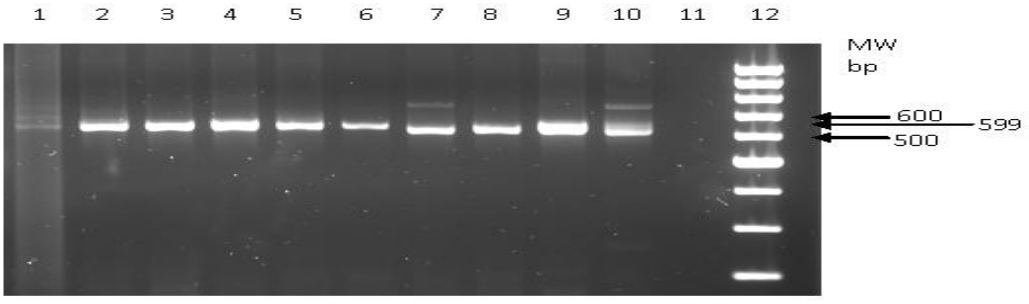


Figure 4.4: Amplification products from the PCR reaction of the 18S (rRNA) region of *Cryptosporidium isolates* observed on 2.0% agarose electrophoresis gels stained with ethidium bromide. 12, 100bp DNA ladder; lane 11, negative control containing al1 reaction products and no DNA; lane 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 are HIV- positive diarrhoea results showing *C. species.*

Section B: Evaluation of three different techniques used for *Cryptosporidium species* detection

4.6 Comparative evaluation of techniques for *cryptosporidium* detection

Cryptosporidium was detected in 27 HIV-positive and 97 HIV-negative diarrhoea patients by any one of the techniques. Antigen detection by ELISA showed the highest positivity 96 (76.8%) in HIV- negative diarrhoea and 26 (74.3%) in HIV- positive diarrhoea patients, followed by PCR. Eighty-nine (71.2%) HIV-negative and 23 (65.7%) HIV-positive patients with diarrhoea were found positive for *Cryptosporidium*. PCR inhibitors were not detected in any of these samples. Crossover contaminations were ruled out, because all water and reaction mix controls were negative. The sensitivity of the PCR assay and the antigen ELISA was identical (96.5%, 95% CI: 89% to 99.5%). However, the PCR assay did not disclose more cases of cryptosporidiosis in addition to those already detected by the ELISA assay; only 13 (37.1%) HIV-positive diarrhoea and 34 (27.2%) HIV-negative diarrhoea patients were found positive for *Cryptosporidium* by modified ZN.

Table 4.1: *Cryptosporidium* positivity by one or more techniques

mZN	Antigen detection by ELISA	PCR	HIV-positive diarrhoea (n = 35)	HIV-negative diarrhoea (n = 125)	Healthy controls (n = 20)
+	+	+	12 (34.3%)	31 (24.8%)	Nil
+	-	+	1 (2.9%)	Nil	Nil
-	+	+	2 (5.7%)	Nil	Nil
-	+	-	4 (11.4%)	8 (6.4%)	Nil
-	+	+	8 (22.9%)	57 (45.6%)	Nil
+	-	+	Nil	1 (0.8%)	Nil
-	-	-	8 (22.9%)	28 (22.4%)	Nil

mZN = Modified Ziehl-Neelsen staining, ELISA = Enzyme linked-immunosorbent assay, PCR = Polymerase chain reaction, + = High *Cryptosporidium* detection, - = Negative detection.

Number of samples positive for *Cryptosporidium* by one or more techniques is shown in Table 4.1. Twelve (34.3%) and 31 (24.8%) patients were found positive by all the three techniques in HIV-positive and HIV-negative diarrhoea patients, respectively; 4(11.4%) HIV-positive and 8 (6.4%) HIV-negative diarrhoea patients were found positive by only antigen detection. None of the patients was found positive by only staining or PCR techniques in both HIV-positive and HIV-negative diarrhoea groups. One patient each in the HIV-positive and HIV-negative diarrhoea group was found negative by ZN staining and positive by either ELISA or PCR. None of the 20 healthy controls was found positive for *Cryptosporidium* by any of the techniques.

4.7 Overall correlation

Performance of overall correlation values based on sensitivity, specificity, positive predictive value, and negative predictive value of the techniques were assessed to define the best possible criteria of true positive for detecting *Cryptosporidium species*. Table 4.2.

Table 4.2: Sensitivity, specificity, positive predictive value and negative predictive value of the techniques.

Techniques	HIV status	Patients' positive (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
ZN	Positive	13 (37.1%)	46.2	88.9	92.3	36.4
	Negative	34 (27.2%)	32.3	96.6	96.9	30.1
Antigen detection ELISA	Positive	26 (74.3%)	92.3	36.4	46.2	88.9
	Negative	96 (76.8)	96.9	30.1	32.3	96.9
PCR	Positive	23(65.7%)	75.0	42.1	52.2	66.7
	Negative	89 (71.2%)	96.9	32.6	79.5	77.8

On comparison based on criteria of true positive, the sensitivity of ZN staining technique was 46.2% in HIV-positive diarrhoea and 32.3% in HIV-negative diarrhoea patients, respectively. No

significant difference was observed in sensitivity of antigen detection ELISA and PCR (96.9%) in HIV-negative diarrhoea patients, respectively. Specificity of the staining technique was 88.9% in HIV-positive diarrhoea and 96.6% in HIV-negative diarrhoea patients. No significant difference ($P > 0.05$) was found in specificity of antigen detection ELISA and PCR in HIV-positive and HIV-negative diarrhoea patients, respectively. The positive predictive value of ZN staining in HIV-positive and HIV-negative diarrhoea patients was 92.3 and 96.9% respectively, which was statistically higher than the ELISA and PCR results. No significant difference was observed in the negative predictive value of ZN technique for the detection of *Cryptosporidium* between HIV-positive and HIV- negative diarrhoea patients.

CHAPTER FIVE

5.0 Discussion

The present study was stimulated by an interest in health concerns of the population living in the Alice settlement of the Eastern Cape Province. *Cryptosporidium* diseases attributable to environmental contamination occur more commonly in other regions of Limpopo Province (Moodley *et al.,* 1991; Obi and Bessong, 2002). However, no information exists regarding infection in the Eastern Cape Province. *Cryptosporidium* infection in residents of the Alice districts is of particular interest because these districts are populated by families of lower socioeconomic status, and typically have a substandard water supply and limited sanitation facilities (Xuza, 2007).

Cryptosporidium is a well established cause of diarrhoea among HIV infected patients worldwide with prevalence of infection ranging from 3% in developed countries to 50% in developing countries (Framm and Soave, 1997). The prevalence of cryptosporidiosis in Alice patients as revealed in this study was 67.8% (122 of 180). This is higher than the 25 – 35% reported in other studies conducted in Varanasi, India (Attili *et al*., 2006). However, a study along the Mexican/US border reported a prevalence of 86.0% cryptosporidiosis in both immunocompromised and immunocompetent patients (Redlinger *et al.*, 2002). Our study supports these findings because prevalence rate was 16% lower than Mexico study cryptosporidiosis in both immunocompromised and immunocompetent patients.

Symptomatic cryptosporidiosis has been found more severe in HIV infected persons (Carcamo *et al*., 2005). Our present study showed a relatively high prevalence rate of 74.3% among HIV

infected diarrhoea patients, higher than the rates reported from other African studies. A study conducted in Tanzania, reported a prevalence of 17.3% amongst HIV-infected patients (Smith and Corcoran, 2004), and in Ethiopia, a prevalence of 29% was documented in HIV patients with chronic diarrhoea (Tadesse and Kassu, 2005). These differences may be linked to the varied study populations used in the various studies.

Cryptosporidium antigen levels was higher in females (78.3%) than in males (71.1%), however this was not statistical significance (P >0.05). This result differs from the findings of previous reports in Brazil (Okafor *et al*., 1994; Mahgoub *et al*., 2004; Gatei *et al*., 2006), in which a higher prevalence rate was reported in males than in females. However, our observations are in conformity with a study in Nigeria where an increased prevalence was found in females (Salsirisampant *et al*., 2002). About 156 of Thai HIV-positive patients showed a prevalence of 12.8% cryptosporidiosis (10.0% in male and 19.1% in females) (Doyle, 1995). In Brazil, 9.33% of samples were positive for *Cryptosporidium*, with higher frequency of cases in male patients from 20 to 50 years old (Mahgoub *et al*., 2004). Moreover, in rural communities of developing countries, females may be more susceptible to diarrhoea disease as they are the primary caregivers for children, and are therefore more frequently in contact with their stools samples diapers and lack of clean water. This could potentially lead to infection as a result of poor hygiene. Females are also fed less, given less nutritious food, provided with less health care and given more work (Garvey and McKeown, 2006).

Our study on *Cryptosporidium* patients is a pilot in a rural country of the Eastern Cape Province. The prevalence rate of 67.8% (122 of 160) reported among HIV-positive and HIV-negative diarrhoea patients is similar to rates reported in the Ireland (Inuugu *et al*., 2000).This indicates

that cryptosporidiosis is an important opportunistic parasitic disease causing diarrhoea among South Africans in our study population.

The highest prevalence of antigenamia (100%) in our study occurred in young adults (31-43yr), 100% in old people (66-80) among HIV-positive diarrhoea patients, while in HIV-negative diarrhoea patients, prevalence was higher in 18-30 yr (87.5%) of age to 84.0% in adults aged 70-82 yr, but less (54.5%) in adults aged 34-56 to patient aged 83-95yr. This pattern is consistent with similar observations by other investigators (Naumove *et al*., 2003; Florez *et al*., 2003; Steinberg *et al*., 2004). These findings, therefore suggest that *Cryptosporidium* detection rate can vary remarkably depending on the age of the patients. Elderly people appear to be at a greater risk of severe disease due to *Cryptosporidium* infection, as they are inactive and always with children who might be infected with cryptosporidiosis, by trying to change their diapers and as a result, they are at a high risk of secondary person-to-person transmission (Florez *et al*., 2003). Similar studies in Kajang, India indicated a risk of 15%. Thus diarrhoea illnesses in elderly people are on the rise.

Access to potable water and good toilet patterns remains a factor that plays a major role of acquiring cryptosporidiosis in this region. The prevalence was higher in those who used river water in both HIV-positive and HIV-negative diarrhoea patients (87.5%) and (93.3%) respectively, than in those who used tap water, but this was not statistically significant ($P > 0.05$; OR = 1.79). Similarly, those who used a pit toilet had a prevalence of (81.8%) and (71.1%) in both HIV-positive and HIV-negative diarrhoea patients respectively (OR =10.93), than those who used flush toilet in both HIV-positive (29.2%) and HIV-negative diarrhoea patients

(38.9%), but this was not statistically significant (P > 0.05) (OR = 4.80).The odd ratios indicated, however, that those who use river water and pit toilets are more likely to be infected by the parasite. Significant difference (P<0.034) was observed more in the low socioeconomic group among HIV-negative diarrhoea patients than in those of HIV-positive diarrhoea patients (P < 0.05).

The importance of zoonotic transmission as a wide spread cause of diarrhoea is well recognized. We observed a prevalence of 82.1% and 84.8% in both HIV-positive and HIV-negative diarrhoea human contact with domestic animals. This result was in agreement with prior studies from North-Eastern Spain pet ownership, especially dogs and farm animals. *C. meleagridis* and *C. felis* were detected in both immunocompetent and immunocompromised patients (Causape *et al*., 2002; Hunter and Thompson, 2005; Chalmers *et al.*, 2006). However, animal-human mixing patterns and access to potable drinking water are all factors that play a major role in the risk of acquiring cryptosporidiosis and are different from region to region.

The second part of the present study was an attempt to evaluate three different diagnostic techniques on stool specimens of HIV-positive and HIV-negative diarrhoea individuals living in the study area. Laboratory diagnosis of cryptosporidiosis relies on the recognition by conventional light microscopy of morphological features specific to the parasite oocyst. However, this technique is laborious, and can be insensitive and prone to error when performed by inexperienced staff (Bialek *et al*., 2002). Studies comprising three different techniques, i.e. modified ZN staining, antigen detection by ELISA and PCR for the detection of cryptosporidiosis is necessary so as to find out the best possible diagnostic marker for the diagnosis of cryptosporidiosis.

Our findings showed a high incidence of cryptosporidiosis in the 160 adults whose stools we examined. The highest positivity was shown by antigen detection (76.8% in HIV- negative and 74.3% in HIV- positive diarrhoea patients), followed by PCR (71.2% in HIV-negative and 65.7% in HIV-positive diarrhoea) and ZN staining (37.1% in HIV-positive and 27.2% in HIV-negative diarrhoea patients).

Earlier reports from Chennai using ZN staining detected 12% *Cryptosporidium* from patients with HIV-positive and HIV-negative diarrhoea stool samples (Kumar *et al*., 2002), 3.4% from India (Nagamani *et al*., 2007), 2.7% from Tunisia (Rym *et al*., 2007) and 29.6% from Peru (Vitaliano *et al*., 2008). Also, other reports documented from India revealed 0.06% *Cryptosporidium* in adults from Chandigarh (Sethi *et al*., 1999), and 1.5% from Pondicherry (Parija *et al*., 2003). Reports from France indicated 37.3% *Cryptosporidium* in stool samples of HIV-positive diarrheoa patients (Chintu *et al*., 1995), 21.5% from Zambia (Brandonisio *et al*., 1999), 50% from South Italy (Oguntibeju, 2006) and 2.2% from Australia (Stark *et al*., 2007). In our study, *Cryptosporidium* was detected in 34 (27.2%) HIV-negative diarrhoea patients by ZN staining.

In the present study, ZN staining showed a sensitivity of 46.2% and in HIV-positive and 32.3% in HIV-negative diarrhoea patients, respectively. Studies have reported a sensitivity of 83.7% for microscopy; however, the HIV status of the patients was not mentioned (Morgan *et al*., 1998). *Cryptosporidium* oocyst is very small in size and can easily be mistaken in stool debris for artefacts. Also, it is easy to confuse with other oocysts, such as those of *Cyclospora species* and cells, especially yeast cells, which resemble *Cryptosporidium* oocysts in size and morphology

(Connelly *et al*., 2008). Using this method we could not differentiate between *Cryptosporidium species* oocysts which take a red to pink colour, and other faecal components.

In the present study, the antigen detection method (ELISA) gave the highest number of positive samples, 96 (76.8%) in HIV-negative and 26 (74.3%) in HIV-positive diarrhoea patients. This is in accordance with earlier reports from the Mexican/US border indicating an ELISA positivity of 86.0% cryptosporidiosis in both immunocompromised and immunocompetent patients (Redlinger *et al*., 2002). This is the first time the antigen detection (ELISA) test is being used in the Nkonkobe Municipality of the Eastern Cape Province. Our prevalence is higher than that previously reported in the Limpopo province 13% (Samie *et al*., 2006). This procedure revealed a clear *Cryptosporidium* antigen; other parasites could not be detected by this method. In the healthy human controls, no antigen was found. Cryptosporidial infection in this case could be asymptomatic, according to Casemore (2004).

In comparison with other methods, this method showed a high sensitivity of 92.3% only in HIV-positive diarrhea stools than other methods. So we were able to detect *Cryptosporidium species* even when present in low numbers in the samples and large numbers of samples could be screened. Our findings support and agree with other studies which reported that using fluorescent monoclonal reagents increased the sensitivity and specificity of the detection of *Cryptosporidium* oocysts. It provides an excellent screening method and offers a useful technique for epidemiological studies, and hence, control of the parasite (Xiao and Herd, 1993; Alles, 1995).

No significant difference ($P > 0.05$) was observed in sensitivity and specificity of antigen detection by ELISA and PCR in HIV-negative diarrhoea patients, respectively, which is in agreement with an earlier study in Bangladesh whereby sensitivity, specificity, positive and

negative predictive value had no significant differences (Bialek *et al*., 2002; Weitzel *et al*., 2007). Ubiquitous PCR inhibitors in faecal specimens have been found to cause significant problems, therefore, in this study, a commercial kit (Zymo-research faecal kit) was used that guarantees reproducibility and simplicity; although in PCR, there is a greater chance of contamination especially in a clinical microbiology laboratory handling infectious diseases. There is need to take extreme precaution to avoid contamination and therefore, we used negative controls with every PCR to check the contamination.

In our study, comparative analysis of three techniques revealed that *Cryptosporidium* could be detected by either ZN, antigen detection (ELISA) or PCR respectively. The number of *Cryptosporidium* positive patients by any one or more techniques was 34.3% and 24.8% in HIV-positive and HIV-negative diarrhoea patients respectively. The prevalence of *Cryptosporidium* in HIV-positive patients with diarrhoea was significantly different as compared to those in HIV-negative patients without diarrhoea ($p < 0.05$). An earlier study from Vellore, India reported that *Cryptosporidium* was significantly higher in HIV-positive patients with diarrhoea (22.6%) as compared to 0.5% in patients without diarrhoea by ZN staining (Mukhopadhya *et al*., 1999; Mohandas *et al*., 2002). *Cryptosporidium* positivity was 11.4% in HIV-positive and 6.4% in HIV-negative diarrhoea patients by only antigen detection (ELISA). None of the patients were found positive by only staining or PCR techniques in both HIV-positive and HIV-negative diarrhoea groups. One patient each in HIV-positive and HIV-negative diarrhoea group was found negative by ZN staining and positive by either ELISA or PCR. None of the 20 healthy controls were found positive for *Cryptosporidium* by any of the techniques.

5.1 Conclusion

From the results of this study, the following conclusions can be drawn:

1. *Cryptosporidium* antigen was detection in faeces using ELISA with a prevalence of 96 (76.8%) in HIV- negative and 26 (74.3%) in HIV- positive diarrhoea patients. The antigen was higher in females (78.3%) than in males (71.1%), but were not statistical significance (P >0.05).

2. The prevalence of cryptosporidiosis in the Nkonkobe Municipality was 67.8% (122 of 180). The highest prevalence (100%) was found in young adults aged 31-43yr among HIV-positive diarrhoea patients, while in HIV-negative diarrhoea patients, prevalence was highest in the 18-30 yrs (87.5%) age group.

3. To the best of our knowledge there was no relationship between antigenenamia and oocysts presence in stool. However, comparing the two techniques (mZN and ELISA), the modified Ziehl-Neelsen (mZN) staining was less sensitive for *Cryptosporidium* detection (37.1%) in HIV-positive and HIV-negative (27.2%) patients with diarrhoea and this suggests that cases of cryptosporidiosis may be missed in patients who have diarrhoea if only mZN staining technique is employed. ELISA was more sensitive in detecting *Cryptosporidium* antigen in both HIV-positive (74.3%) and HIV-negative (76.8%) diarrhoea patients.

4. Preliminary analysis was conducted by PCR to further confirm the presence of *Cryptosporidium* in stool samples of selected subjects. Identification of genotypes circulating in the study area could not be done in this particular study due to time and financial constraints. However, we are focusing on further studies to elaborate on the genetically distinct genotypes by

sequencing the amplified PCR products targeting *Cryptosporidium* gene at GenBank accession no. AF093489.

5.2 Recommendation

Further studies based on sequencing should focus on the different genotypes and phenotypes of *Cryptosporidium species* to fully delineate the situation. These will be achieved using an ABI Prism Dye Terminator Cycle Sequencing kit. Genetic characterization of the epidemiology of cryptosporidiosis may identify several novel genotypes/species of *Cryptosporidium* as well as expanding the host range of species in order to better understand the epidemiology and potential human health risks of this ubiquitous parasite. To achieve this, the taxonomy of this genus will need further evaluation.

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Appendix

a) Publication.

Omoruyi, B., Matongo, F., Mkwetshana, N.T., Green, E., Clark, A.M and Ndip, R.N (2010). Environmental and demographic risk factors associated with prevalence of *Cryptosporidium* infection in the Alice rural settlements of the Eastern Cape Province of South Africa: a pilot study. *Rev. Enviro Health*. In press.

b) Manuscript in preparation.

Clinical evaluation of three different diagnostic techniques on disease prevalence in HIV-positive and HIV-negative diarrhoea patients in the Eastern Cape Province of South Africa.