IMMUNE RESPONSES TO CRYPTOSPORIDIUM SPECIES IN HIV/AIDS INFECTED ADULTS ATTENDING KENYATTA NATIONAL HOSPITAL, NAIROBI, KENYA

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Reg No 156/CE/15624/05

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Immunology) in the School of Pure and Applied Sciences of Kenyatta University

APRIL 2012
DECLARATION

This thesis is my original work and has not been presented for a degree or any other award in any other university.

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Reg No 156/CE/15624/05

Signature…………………… Date…………………………

Supervisors

We confirm that the candidate under our supervision carried out the work reported in this thesis. We have read and approved this thesis for examination.

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Signature…………………… Date…………………………

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Signature Date: April 18th 2012
DEDICATION

This work is dedicated to my entire family.
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**ACRONYMS AND ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
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<tr>
<td>Bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CCC</td>
<td>Comprehensive Care Centre</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of Differentiation 8</td>
</tr>
<tr>
<td>CMR</td>
<td>Centre for Microbiology Research</td>
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<tr>
<td>Cp23</td>
<td>Cryptosporidium antigen 23</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERC</td>
<td>Ethical Review Committee</td>
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<tr>
<td>ETEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>Escherichia coli</em></td>
</tr>
<tr>
<td>E.U</td>
<td>ELISA Units</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
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<td>Gp</td>
<td>Glycoprotein</td>
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<td>IFN γ</td>
<td>Interferon gamma</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>Immunoglobulin G</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IQ</td>
<td>Interquartile</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute.</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KNH</td>
<td>Kenyatta National Hospital</td>
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<tr>
<td>LPS</td>
<td>Lipopolysacharides</td>
</tr>
<tr>
<td>Mgcl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC 1</td>
<td>Major Histocompatibility Complex 1</td>
</tr>
<tr>
<td>MHC 2</td>
<td>Major Histocompatibility Complex 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NASCOP</td>
<td>National AIDS &amp; STI Control Programme</td>
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<tr>
<td>NK cells</td>
<td>Natural Killer cells</td>
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<td>gp 15</td>
<td>Glycoprotein 15</td>
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<tr>
<td>gp 40</td>
<td>Glycoprotein 40</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline.</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate Buffered Saline with Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<tr>
<td>RNA</td>
<td>Ribose Nucleic Acid</td>
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<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
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<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
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<tr>
<td>SSU</td>
<td>Small Sub Unit</td>
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<tr>
<td>TCR</td>
<td>T Cells Receptor</td>
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<tr>
<td>Th</td>
<td>T helper cells</td>
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<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>UN</td>
<td>United Nations</td>
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<td>WHO</td>
<td>World Health Organization</td>
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**ABSTRACT**

*Cryptosporidium* causes significant morbidity and mortality in AIDS patients worldwide particularly in developing countries where ART is not widely available or affordable. In Kenya, approximately 8% of the adult population lives with HIV/AIDS and *Cryptosporidium* has been reported as the leading indicator of death among adult HIV/AIDS patients. In Kenya, approximately 8% of the adult population lives with HIV/AIDS and *Cryptosporidium* has been reported as the leading indicator of death among adult HIV/AIDS patients. There is very little information about the molecular epidemiology of cryptosporidiosis and no documented data on immune responses to *Cryptosporidium* in HIV/AIDS patients in Kenya. A number of *Cryptosporidium* antigens have been found to induce host immune responses in immunocompetent persons, however, their effects in the immunity of immunocompromised individuals are not understood. Glycoprotein 15 (gp15) and *Cryptosporidium* 23 (Cp23) are conserved *Cryptosporidium* antigens that trigger host immune responses and are thus potent agents for vaccine development. This study was aimed at evaluating molecular epidemiology and immune responses against *Cryptosporidium* with an aim of preventing HIV/AIDS disease progression especially in immunocompromised HIV-*Cryptosporidium* co-infected persons. A total of 164 HIV/AIDS patients, 94 asymptomatic (no diarrhea) and 70 symptomatic (with diarrhea) respectively were recruited. Recruited persons provided stool samples for *Cryptosporidium* oocysts microscopic examination and blood for immune responses evaluation. *Cryptosporidium* species were identified through microscopy, and confirmed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) targeting 18S rRNA. Antibodies and IFN-γ responses against *Cryptosporidium* antigens were assessed by enzyme linked immunosorbent assays (ELISA). In this study, *Cryptosporidium* species was found to infect both symptomatic and asymptomatic persons. The species found were *C. hominis*, *C. parvum*, *C. meleagris*, *C. muris* and *C. Canis*, with *C. hominis* being the most prevalent species. On immune responses there was a significant difference between patients with asymptomatic and symptomatic cryptosporidiosis in secretion of serum IgG to Chgp15 ($P = 0.005$) and Cp23 ($P = 0.001$) and fecal IgA to Chgp15 ($P = 0.002$). No significant difference was observed in IgM and IFN-γ levels between patients with asymptomatic and symptomatic cryptosporidiosis. *Cryptosporidium hominis* was found to be associated with majority cases of *Cryptosporidium* infection in HIV/AIDS adult patients and often recurrent/chronic diarrhea in patients, especially those with low CD4 counts. Serum IgG to Chgp15 and Cp23 and fecal IgA to Chgp15 antibodies were also seen to be protective against diarrhea. These findings indicate that serum IgG to Chgp15 and Cp23 and fecal IgA to Chgp15 antibodies may be good candidate antigens for vaccine development against *Cryptosporidium* species. However, study of the roles of CD8+ cells and other cytokines especially Th2 cytokines should be done to establish their effects on the host protective responses against *Cryptosporidium* observed in this study. These study findings indicate that improvement of immune status especially serum IgG and Innate mucosal IgA may be major remedial measures towards reducing effects of cryptosporidiosis which is a predominant infection in HIV/AIDS infected patients in Kenya.
CHAPTER 1: INTRODUCTION

1.1 Cryptosporidium: General overview and classification

*Cryptosporidium* is an apicomplexan parasite in the class Coccidea, order Eucoccidiorida, family Cryptosporidiidae and genus *Cryptosporidium* (Xiao *et al.*, 2004b). All members of the genus *Cryptosporidium* are intracellular parasites. They are eukaryotic protozoa, which mean that most of their DNA is contained within a nucleus surrounded by a double membrane. The genus *Cryptosporidium* is one of over 300 genera that include 4800 named species in the phylum apicomplexan, but there may be many other species yet to be described. Characteristics used to identify and name species of apicomplexa traditionally have been based on host specificity, location of endogenous stages and morphology of endogenous or exogenous stages. Over 150 species of mammals including humans, as well as birds, reptiles, amphibians and fish are parasitized by members of the genus *Cryptosporidium* (Xiao *et al.*, 2004b).

*Cryptosporidium* infects epithelial cells in the microvillus border of the gastrointestinal tract of all classes of vertebrates (Current and Garcia, 1991). Effects of infection vary with the species of *Cryptosporidium*. Some species of *Cryptosporidium* infect many host species, whereas others appear restricted to groups such as rodents or ruminants, and still others are known to infect only one host species. Some species primarily infect the stomach, whereas others primarily infect the intestine. Some infections are acute and self-limiting, whereas others are chronic. The severity and duration of infection with pathogenic species are also affected by the immune status of the infected person or animal. Immunocompetent individuals might suffer mild, moderate or severe
acute illness which is self-limiting, whereas immunocompromised individuals can suffer severe chronic illness and even death (Current and Garcia, 1991).

During much of the 1970s through the 1990s only a single species, Cryptosporidium muris, was thought to parasitize the gastric mucosa of mammals and that was a species with large oocysts, whereas another species, Cryptosporidium parvum, with the small oocysts, was thought to parasitize the intestine of all mammals (Tzipori et al., 1980). During this time, oocysts from many different animal species including humans were identified and published as C. parvum, C. parvum-like or simply Cryptosporidium. Following the onset of HIV/AIDS, reports of associated opportunistic pathogens focused attention on cryptosporidiosis in humans.

1.2 Problem statement

HIV/AIDS is a global pandemic especially in developing countries like Kenya. Cryptosporidium has been associated with many deaths in HIV infected persons especially after continued suppression of the immunity (O’Connor et al., 2011). There exists little information on molecular diversity of Cryptosporidium based on few small scale studies on cryptosporidiosis in Kenya and there is no data on immune responses against Cryptosporidium in Kenya. In Nairobi, 11%-17% of HIV–infected individuals with chronic diarrhea had Cryptosporidium detected by stool microscopy, 41% of Cryptosporidium infected patients died within 4 months and detection of Cryptosporidium was the single most significant indicator associated with the death (Batchelor et al., 1996; Mwachari et al., 1998; Mwachari, 2003). This study therefore provides an insight on the infection of Cryptosporidium in HIV infected adults in Kenya.
1.3 Justification of the study

To date, there are no studies on epidemiology of Cryptosporidium spp infecting HIV/ AIDS patients in Kenya and there are no previous studies on immune responses to Cryptosporidium in this population. Cryptosporidium antigens have been reported to induce immune responses thus enabling control of the infection in immunocompetent individuals and in animals (Khan et al., 2004; Borad and Ward, 2011). Cell mediated immune responses have been shown to be important in protection against cryptosporidiosis and Cryptosporidium serum antibodies could also be involved in protection against recurrent cryptosporidiosis (Riggs, 2002; Borad and ward, 2011). The Cryptosporidium antigens, gp15 and Cp23 are involved in host cell invasion and are in consideration as vaccines candidates (Wanyiri and Ward, 2006). Serum antibodies to gp15 and Cp23 are associated with protection from diarrhea in immunocompetent human volunteers (Riggs, 2002; Borad and ward, 2011). Based on these facts, this study investigated both cellular and humoral immune responses induced by gp15 and Cp23 in the quest for development of effective vaccines and treatment strategies especially in immunocompromised persons.

1.4 Research questions

i. What species of Cryptosporidium infect HIV infected adults with symptomatic or asymptomatic cryptosporidiosis in Kenya?

ii. What types of immune responses are induced by Cryptosporidium antigens in HIV infected adults with symptomatic or asymptomatic cryptosporidiosis?

iii. What is the association between diarrhoea and CD4+ T-cell counts in Cryptosporidium and HIV/AIDS co-infected patients?
1.5 Hypothesis

i. There is no difference between Cryptosporidium species infecting HIV/AIDS infected adults attending Kenyatta National Hospital, Nairobi, Kenya

ii. Immune responses to gp15 and Cp23 are not associated with protection against Cryptosporidium in HIV/AIDS infected adults attending Kenyatta National Hospital, Nairobi, Kenya

1.6 Objectives

1.6.1 General objective

To determine Cryptosporidium species and immune responses in HIV infected adults attending Kenyatta National Hospital, Nairobi, Kenya

1.6.2 Specific objectives

i. To determine the species of Cryptosporidium infecting HIV/AIDS patients attending KNH.

ii. To determine serum IgG, IgM and fecal IgA levels against Cryptosporidium antigens (gp15 and Cp23) in HIV/AIDS patients co-infected with Cryptosporidium.

iii. To determine interferon gamma (IFN-γ) levels in HIV/AIDS patients co-infected with Cryptosporidium.

iv. To determine the association between cryptosporidiosis induced diarrhea and CD4+ T-cell counts in HIV/AIDS patients co-infected with Cryptosporidium.
1.7 Significance of the study

Cryptosporidiosis is a common infection in humans in Kenya and it is an important opportunistic infection in HIV-infected individuals. This study provides a platform to study the molecular epidemiology of Cryptosporidium and to investigate immune responses to putative protective antigens in HIV/AIDS infected persons. The findings of this study will provide recent data on co-infection of HIV and Cryptosporidium and the extent of immune responses in the study population. These findings will therefore provide useful insight on the current trend of Cryptosporidium infection in HIV infected adults in Kenya and thus will enable formulation of disease management and control strategies especially in HIV/AIDS immunocompromised patients.
CHAPTER 2: LITERATURE REVIEW

2.1 Molecular epidemiology and geographical distribution of Cryptosporidium

Cryptosporidium is an apicomplexan parasite that causes diarrhea disease worldwide (Huang and white, 2006; O’Connor et al., 2011). Seroepidemiological studies of particular areas have indicated that the percentage of the population affected at some time in life can vary from under 20% to over 90% (Dillingham et al., 2002). The first human cases of cryptosporidiosis in humans were recorded in the 1970s: one in a young girl with enterocolitis (Nime et al., 1976) and the other in an AIDS patient (Meisel et al., 1976). With the developing AIDS pandemic in the 1980s, more cases of cryptosporidiosis in AIDS patients were identified (Navin and Juranek, 1984; Soave et al., 1984) and Cryptosporidium was also found to be a cause of diarrhea in immunocompetent people too (Blagburn and Current, 1983).

Majority of the cases in immunocompetent hosts are sporadic and can involve either children or adults in both the developed and developing world. Cryptosporidium infection is widespread especially in the developing world with 19-30% of individuals being asymptomatic cyst excretors (Current and Garcia, 1991). The frequency of cryptosporidiosis worldwide often correlates with HIV status. It is considered to be prevalent in HIV-positive patients, particularly those with low CD4+ T-cell count (<200 cells /mm²) (Stark et al., 2009). Cryptosporidium parvum was once considered the only Cryptosporidium species infecting humans. Genotyping tools based on DNA sequences of antigen identified genotypes 1 (the human genotype) and 2 (the bovine genotype) within the umbrella of C. parvum, and these eventually became C. hominis and C. parvum respectively. Both species infect immunocompetent and immunocompromised persons (Xiao and Ryan, 2004a). At the end of the 1990s, small sub unit (SSU) rRNA-based genotyping tools revealed the presence of C. canis, C. felis and C. meleagridis in AIDS patients...
in the United States, Switzerland and Kenya, in addition to the more frequently found *C. hominis* and *C. parvum* (Pieniazek et al., 1999). This observation has been supported by data from France, Portugal, Italy, Thailand and Peru (Caccio et al., 2002; Gatei et al., 2002b). In Peru and Thailand, these three species, *C. canis*, *C. felis* and *C. meleagridis* are responsible for over 20% of *Cryptosporidium* infections in AIDS patients (Cama et al., 2003). Even immunocompetent persons can be infected with zoonotic species other than *C. parvum* (Cama et al., 2007).

Molecular characterization of over 2,000 specimens in the United Kingdom identified 22 cases of *C. meleagridis*, six cases of *C. felis* and one case of *C. canis* (McLauchlin et al., 2000; Leoni et al., 2006). Nineteen cases of *C. meleagridis* infection were identified among 3,100 clinical isolates from England and Wales (Chalmers et al., 2002). Some infected persons were not immunocompromised. HIV-seronegative children in Lima, Peru (Xiao et al., 2001a) and children in Kenya were also infected with *C. canis*, *C. felis* and *C. meleagridis* (Gatei et al., 2006b). The proportion of infections caused by non-*parvum* zoonotic *Cryptosporidium* species, however, was much higher in Peru (about 12%) than in the United Kingdom (Cama et al., 2007). At least 16 other cases of *C. meleagridis* infection have been described in immunocompetent persons in the United Kingdom, France, the Czech Republic, Canada, Japan and Uganda (Xiao and Fayer, 2008). In Peru, where a significant proportion of infections are due to zoonotic *Cryptosporidium*, there was no significant difference between children and HIV positive adults in the distribution of *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis* and *C. canis* (Xiao et al., 2001a; Cama et al., 2003).

*Cryptosporidium muris*-like oocysts were found in two healthy Indonesian girls, but there was no molecular confirmation (Katsumata et al., 2000). A putative *C. muris* infection was reported in
an immunocompromised patient in France based on sequence analysis of a small fragment of the SSU rRNA (Guyot et al., 2001). However, the sequence was more similar to *C. andersonii* than to *C. muris*. Several confirmed *C. muris* infections have been documented in HIV/AIDS patients in Kenya and Peru, both by identified PCR-RFLP and sequencing of the SSU rRNA gene (Gatei et al., 2002a; Gatei et al., 2006b) and a putative human *C. muris* infection has been seen in India (Muthusamy et al., 2006). More human cases have been associated with the *Cryptosporidium cervine* genotype, which has been reported in 10 patients in Canada, three patients in the United States, one patient in Slovenia and one in England (Blackburn et al., 2006; Leoni et al., 2006; Trotz-Williams et al., 2006).

Other *Cryptosporidium* species found in humans include *C. suis* which was identified in a HIV infected patient in Lima, Peru and another patient in England (Xiao et al., 2002; Leoni et al., 2006), a *C. suis*-like parasite identified in two patients in Canada (Ong et al., 2002), a *C. andersonii*-like parasite identified in three patients in England (Leoni et al., 2006) and a W17 (chipmunk) genotype identified in two patients in Wisconsin (Feltus et al., 2006). The *C. hominis* monkey genotype has been found in two persons in the United Kingdom (Mallon et al., 2003). Other new *Cryptosporidium* genotypes will likely be found in humans in future but these parasites account for a very minor proportion of *Cryptosporidium* infections in humans (Chalmers et al., 2002). Some unusual *Cryptosporidium* species may have a broad host range and might emerge as important pathogens in humans when socioeconomic and environmental changes favor transmission. The avian pathogen *C. meleagridis* is increasingly recognized as an important human pathogen and can experimentally infect a wide range of mammals (Akiyoshi et al., 2003; Huang et al., 2003). In Lima, Peru and Bangkok, Thailand, *C. meleagridis* is
responsible for 10 to 20% of human cryptosporidiosis cases. Cryptosporidium hominis, C. parvum, C. meleagris, C. muris, C. canis and C. felis have been identified in HIV-infected patients in Kenya (Peng et al., 2001; Gatei et al., 2002a).

2.2 Lifecycle of Cryptosporidium

A diagrammatic life cycle for Cryptosporidium species is shown in Figure 2.1. The life cycle of Cryptosporidium spp usually takes place within the intestinal epithelial cells and is normally completed within the gastrointestinal tract of the host. Thick walled oocysts excreted from the infected host in fecal material represent the infective stage of the parasite. Ingestion of the oocysts causes the infection of a new host. The infectious dose is as low as 10 oocysts in some strains (Chappell et al., 2006). Once inside the body, the first step towards infection is excystation, the opening of the oocysts wall along a suture at one pole of the oocyst through which the four infectious sporozoites leave the oocyst. After an internalization process, the sporozoite become spherical, attach to enterocytes of the host and is called a trophozoite. The trophozoite invades the epithelial cells and undergoes asexual and sexual multiplication (Deng et al., 2004).

Asexual multiplication called merogony results when the trophozoite nucleus divides. For C. parvum, type I meronts develop six or eight nuclei, each meront becomes incorporated into a merozoite, a stage structurally similar to the sporozoites. Each mature merozoite leaves the meront to infect another host cell and to develop into another type I or into a type II meront. Type II meronts produce four merozoites. It is thought that only merozoites from type II meronts
initiate sexual reproduction upon infecting new host cells by differentiating into either a microgamont (male) or a macrogamont (female). Each microgamont becomes multinucleate and each nucleus is incorporated into a microgamete, a sperm-cell equivalent. Macrogamont remain uninucleate, an ovum. The fertilized macrogamont, or zygote, develops into oocysts that are shed periodically into the intestinal lumen and later to the environment. In the environment the oocysts can survive for months before they infect a new host (Panterburg et al., 2008). The primary site of infection with *C. hominis* and *C. parvum* is the small intestine. *Cryptosporidium* has been found in extra intestinal sites including the gall-bladder, mesenteric lymph nodes, trachea, lung and uterus in animals and in some severely immunocompromised humans (Fleta et al., 1995). Other species such as *C. muris, C. andersoni* and *C. serpentis* favor the gastric mucosa (Xiao and Fayer, 2008).

![Figure 2.1: Life cycle of Cryptosporidium parvum (Adapted from Xiao and Fayer, 2008)](image-url)
2.3 Pathology and clinical features

*Cryptosporidium* is primarily a pathogen of the small bowel causing blunting of the microvilli, sub mucosal edema and mononuclear cell inflammatory infiltration in the lamina propia (Lumadue et al., 1998). In immunocompromised hosts, the impact of the disease is severe and includes respiratory problems, cholecystitis, hepatitis and pancreatitis. *Cryptosporidium* oocysts have been reported in respiratory secretions of patients with diarrhea and respiratory symptoms (Mor et al., 2010). Coexistence with other pathogens and asymptomatic presentation are common in some instances. On the other hand, in immunocompetent persons, infection is considered to be a self limiting disease (Robinson et al., 2001). Although the parasite tends to preferentially infect the jejunum and ileum, the colon can be heavily infected without infection of the small bowel. Multisite involvement has been described and widespread infection of the intestinal tract, including the small and large bowels or localized involvement of the proximal small intestines, has been associated with more severe diarrhea illness (Clayton et al., 1994; Lumadue et al., 1998).

Isolation of *Cryptosporidium* from gastric mucosa has been reported in about a third of patients with AIDS who underwent endoscopy for chronic diarrhea and/or unexplained gastrointestinal symptoms (Rossi et al., 1998). The biliary tract has been a well-known site of cryptosporidial disease in patients with AIDS (Lopez-Velez et al., 1995; Chen and LaRusso, 2002) and rare cases have been reported in bone marrow and solid-organ transplant recipients and immunocompetent children (Campos et al., 2000; Abdo et al., 2003; Dimicoli et al., 2003). *Cryptosporidium* is the most common cause of AIDS-cholangiopathy (Chen and LaRusso,
Biliary involvement in AIDS patients is associated with low CD4+ T-cell counts and increased mortality (Hashmey et al., 1997).

### 2.4 Transmission, control and treatment

As the sole mechanism for transmission, oocysts have evolved to be widely dispersed and to survive in harsh environments for long periods of time. They are highly resistant to natural stresses and many chemical disinfectants (Xiao and Fayer, 2008). Oocysts probably evolved in dispersed mobile populations where there was strong selective pressure for long-term survival, the result being production of massive numbers of oocysts that survived for long periods of time (Blewett, 1989). In contrast, modern times are characterized by concentrated fixed populations of animals and people. The consequence is exposure of both populations to extremely high levels of infective organisms.

To control infections in animal populations, one strategy might be to modify this imbalance by continuously moving animals to clean areas. However, where large numbers of domesticated animals are involved, this is rarely economically possible. For human populations, disinfection procedures are sought to minimize person-to-person transmission in domestic and institutional settings, and to deal effectively with contamination of recreational and drinking water. Because all infections with *Cryptosporidium* are initiated by ingestion or inhalation of the oocysts stage, measures to prevent or limit the spread of infection must be targeted to eliminate or reduce contamination from infectious oocysts in the environment. There are no universally effective drugs for prophylaxis or therapy for humans or animals that will prevent or stop oocyst
production by infected individuals. Hygiene, including disinfection, remains the most effective management tool.

To determine the effectiveness of any anti-oocyst activity, there must be an assay to determine viability and infectivity after treatment. The current principal methods include: (1) animal infectivity using a variety of animal models, especially rodents; (2) *in vitro* cell culture, which utilizes select cell types to support development through a portion of the life cycle and (3) excystation alone or combined with dye techniques, which estimates viability but cannot determine infectivity. There have been great strides in improving the treatment of cryptosporidiosis in humans over the past decade. Despite the paucity of supportive preclinical data, the urgent need to identify effective therapy for this disease in persons with HIV/AIDS has led to the unprecedented administration of a vast array of chemotherapeutic, immunomodulatory, and palliative agents to this population.

In addition to advances in effective therapy, the availability and reproducibility of *in vivo* and *in vitro* methods for screening drugs and conducting preclinical studies has greatly enhanced efforts to identify effective therapy. Progress has been made in developing animal models of the disease, but still little is known of how *Cryptosporidium* species and strain differences impact parasite virulence. The large scientific experience by researchers has thus generated a formidable list of approximately 100 ineffective compounds (Ungar *et al.*, 1986; Soave, 1990). Over the past two decades, controlled treatment trials have provided useful insights for designing effective studies of cryptosporidial therapy. The only US Food and Drug Administration (FDA) approved drug for cryptosporidiosis is Nitazoxanide. However, this drug is not effective in immunocompromised
hosts (Abubakar, 2007) and it is hardly available in developing countries. Currently, there is no vaccine for cryptosporidiosis that is available.

2.5 Immune responses to Cryptosporidium

*Cryptosporidium* reliance on glycoproteins to attach to and invade intestinal epithelial cells. To date, the four *Cryptosporidium* antigens that have been identified as integral to attachment and invasion are all glycoproteins: CSL (Riggs *et al*., 1997), gp900 (Barnes *et al*., 1998), gp40/15 (Cevallos *et al*., 2000) and Cp23 (Perryman *et al*., 1999). Gp15 and Cp23 are under consideration for vaccine development (Wanyiri *et al*., 2007). These relatively conserved, immunodominant antigens, which are consistently identified by sera from infected individuals, have been used to assess antibody responses to *Cryptosporidium* in several studies.

Gp15 is the C-terminal cleavage product of a major surface precursor glycoprotein, gp40/15 (Cevallos *et al*., 2000; Wanyiri *et al*., 2007). It is a surface glycoprotein that has been implicated in mediating infection (Cevallos *et al*., 2000). Gp15 is relatively conserved among isolates and is the most immunodominant *Cryptosporidium* antigen which is consistently recognized by sera from infected people and animals (Frost *et al*., 2000; Frost *et al*., 2003). Monoclonal IgA antibodies to this antigen are partially protective in mice (Tiley *et al*., 1991, Cevallos *et al*., 2000). Serum antibody responses to gp 15 have been reported in HIV–infected adults in developing countries (Sandhu *et al*., 2006). The presence of pre-existing antibodies to gp15 in human volunteers is associated with protection from diarrhea (Moss *et al*., 1998; Frost, 2005). A study in children in Peru reported higher IgG levels against the gp15 antigen in children with
asymptomatic infection, possibly indicating protection against diarrhea (Priest et al., 2006). Another unrelated study reported IFN-γ production in response to C. hominis gp15 in immunocompetent adults with prior cryptosporidial infection (Preidis et al., 2007).

Cp23 is another immunodominant, conserved surface antigen of Cryptosporidium also believed to be involved in host cell invasion. Passively administered mAbs to Cp23 are partially protective in animal models of cryptosporidiosis (Enriquez and Riggs, 1998). Bovine colostrum generated by immunization with recombinant Cp23 partially protected calves following Cryptosporidium challenge (Perryman et al., 1999). The presence of serum antibodies to Cp23 was associated with protection from diarrhea in HIV-infected adults (Frost et al., 2005). Additionally, studies have shown that Cp23 induces a proliferative T cell response in immunocompetent adults (Bonafonte et al., 2000; Smith et al., 2001). Further, DNA encoding Cp23 was shown to elicit protective immune responses in mice (Ehigiator et al., 2007). Like gp15, Cp23 is largely conserved among C. hominis and C. parvum isolates. Both antigens are recognized as potential vaccine candidates (Wanyiri et al., 2007).

In 1980s, as cryptosporidiosis was emerging as an important diarrheal disease of humans and domestic animals, two studies of Cryptosporidium were published which became the hallmark of future studies. The first was the report of mechanisms of protective immunity against the parasite using mice infected with C. parvum (Heine et al., 1984) and the second involved a study of an in vitro technique for growing this parasite in epithelial cells (Current and Haynes, 1984). These in vivo and in vitro infection models have since laid the foundation upon which the development of the Cryptosporidium immunology field has been developed, although neither model is ideal.
Immunological studies involving humans and livestock (mainly cattle and pigs) have obviously been more limited but, generally, most of the findings are in agreement with those obtained in murine studies. These studies indicate that murine animal models could provide a basis for studies of immune responses in humans (Harp et al., 1994).

Advances have been made in the understanding of the host responses to Cryptosporidium, including immune effector mechanisms that control infection, although there are so many questions that still remain unanswered. These studies mainly dwelt on the natural adaptive immunity against Cryptosporidium in the human host. Examination of the adaptive immune responses included assessments of the significance in immunity of CD4+ T-cells and CD8+ T-cells and cell-mediated Th1 versus Th 2 humoral responses. Also, the involvement of B-cells and antibodies in control of infection was evaluated with the prospects for active and passive immunization.

2.5.1 Types of immune responses

2.5.1.1 Innate immunity

Innate immunity serves as an early sensor of infection and also activates antimicrobial killing mechanisms that might curb the reproduction of invading microorganisms until the adaptive immune response becomes functional. The innate immune system includes the activities of inflammatory immune cells such as natural killer (NK) cells that produce cytokines and that might be cytotoxic to infected cells; macrophages and neutrophils that can engulf extracellular
microorganisms and eosinophils. Non immune cells, including epithelial cells, also have immunological functions such as the ability to produce cytokines and antimicrobial peptides. *In vitro* infection of human cholangiocytes has been observed to cause up-regulation of Toll-like receptors (TL) 2 and 4 thus activating the MyD88 pathway and other molecules which terminally activates NF-κB (Chen et al., 2005). An important function of the adaptive immune system when activated by specific antigen recognition is to amplify elements of the innate immune response including antimicrobial killing mechanisms.

Epithelial cells are now known to play a key role in establishing mucosal immune responses to infections. They produce main inflammatory molecules such as chemokines involved in establishing inflammation. They also mediate mechanisms of microbial inactivation that can be activated directly by infection or via inflammation (McDonald et al., 2001). Interaction and synergy between the components of innate and acquired immunity will promote immunity against Cryptosporidium. The interaction of CD40 on antigen presenting cells and CD154 on T cells provides a costimulatory signal which is vital for activating the T cells and for inducing antibody class switching from immunoglobulin M (IgM) to IgG, IgA and IgE in B cells (Grewal et al., 1998).

IFN-γ is one of the major effector cytokine in innate immunity against Cryptosporidium. Studies of *C. parvum* infections in immunocompromised nude mice that are T-cell deficient and with SCID mice that are T- and B-cell deficient but have normal NK cell function indicated that IFN-γ mediated innate immunity plays an important part in the control of the parasite’s reproduction (McDonald et al., 1992a). Adult immunocompromised mice develop chronic infection that is
initially mild but becomes progressive and often fatal (Ungar et al., 1990; Mead et al., 1991; McDonald et al., 1992a). Administration of anti-IFN-γ-neutralizing antibodies to SCID or nude mice exacerbated infection and decreased the time taken for morbidity to occur, indicating the significance of IFN-γ in T-cell-independent immunity (Ungar et al., 1991; Chen et al., 1993; McDonald and Bancroft, 1994). Similarly, IFN-γ-deficient SCID mice developed more intense infections than control SCID mice (Hayward et al., 2000). These studies suggest that IFN-γ is important in checking and controlling extent of immune response. In humans, after in vitro stimulation, lymphocytes from a person who previously suffered from Cryptosporidium produced IFN-γ. However, lymphocytes from HIV infected adults suffering from active cryptosporidiosis did not produce IFN-γ thus indicating that IFN-γ is the main agent of memory response (Gomez-Morales et al., 2004).

A number of studies have shown that the cytokine IFN-γ is important in protection and clearance of infection (Theodos, 1998; Riggs, 2002). IFN-γ expression was detected in jejunal biopsies of Cryptosporidium-infected adult volunteers and was associated with the presence of anti-Cryptosporidium serum antibodies and the absence of oocyst shedding (White et al., 2000). In HIV-infected patients, peripheral blood mononuclear cells (PBMCs), predominantly CD4+ TCRαβ+ cells from Cryptosporidium infected humans were reported to proliferate and to produce IFN-γ in response to crude extract from C. parvum (Gomez Morales et al., 1999) as well as to recombinant and native C. parvum antigens (Gomez Morales et al., 2004).
2.5.1.2 Adaptive immunity

The adaptive immune response induced by specific antigens recognized by T- and B-cells is generally required to eliminate rapidly proliferating or virulent microbial pathogens, and has the added advantage over innate immunity in having immunological memory, which allows prompt reactivation of memory T- and B-cells if re-infection occurs (Seder and Ahmed, 2003; Kalia et al., 2006). T-cells activate B-cells to proliferate and differentiate into plasma cells that produce antibodies (Kalia et al., 2006). Both CD4$^+$ and CD8$^+$ T cells contribute to resistance to and clearance of acute cryptosporidial infection (Panterburg et al., 2008; Borad and ward, 2011). In humans with late-stage HIV infection and low CD4$^+$ T-cell counts, there is increased susceptibility to cryptosporidial infection and the severity of disease is greater (Blanshard et al., 1992; Flanigan et al., 1992). After antiretroviral therapy and recovery of the CD4$^+$ T-cell levels, cryptosporidial infection is readily cleared (Schmidt et al., 2001). It is evident, therefore, that CD4$^+$ T-cells are major effector cells in immunity to cryptosporidial infection. The significance of CD8$^+$ T-cells in immunity is not well understood (Panterburg et al., 2008).

Cryptosporidium-specific antibodies appear in the circulation and in the mucosa during infection of different mammalian hosts, including humans, cattle and sheep (Hill et al., 1990; Peeters et al., 1992). The presence of Cryptosporidium-specific antibodies is indicative of exposure to the parasite and is widely used to estimate seroprevalence (Priest et al., 2006). IgM, IgG and IgA titers measured by ELISA generally increase during infection and decline after recovery, although IgG in serum may persist for several months longer than IgM (Ungar et al., 1986). These infection antibodies have been shown to recognize a wide range of oocyst polypeptides of low to high molecular weights (Hill et al., 1990; Peeters et al., 1992). Secretory IgA (sIgA)
produced by a host as a result of infection can play a major part in protecting the mucosal surface from toxins and microbial pathogens. In *C. parvum*-infected immunocompetent human adults, the level of parasite-specific sIgA was found to be higher in individuals excreting oocysts or with diarrhea (Dann *et al.*, 2000).

AIDS patients with chronic cryptosporidiosis were reported to have high titers of sIgA (Cozon *et al.*, 1994), possibly signifying that additional mechanisms were necessary for control of infection. Investigations with B-cell-deficient mice, however, suggest that antibodies play little part in elimination of *Cryptosporidium*. This is observed in *C. muris* infection in SCID mice reconstituted with spleen depleted of B-cells which followed the same acute pattern as in mice receiving cells that were not depleted of B-cells (McDonald *et al.*, 1992b). In adult volunteers, serum antibody responses coincide with resolution of infection and/or decreased severity of re-infection, suggesting that antibody production is associated with clearance and resistance (Moss *et al.*, 1998; Okhuysen *et al.*, 1999). *Cryptosporidium*-specific fecal IgA antibody responses in human volunteers correlated significantly with the presence of active or recent infection (Dann *et al.*, 2000). These studies suggest that antibody production may be associated with some degree of protection in humans. However, it remains to be determined whether antibodies are themselves protective or are markers of protective responses (Riggs *et al.*, 2002).

The nature of the antigens that the immune system encounters determines whether CD4+ T helper (Th) cells will induce a cell-mediated immune response (Th1) or antibody-mediated response (Th2). These Th responses are determined by a variety of cytokines produced by the T-cells themselves and by the antigen-presenting cells (APCs). In a Th1 response, IL-12 which is
produced by dendritic cells and macrophages induces the T-cells to produce IFN-γ. This response is mainly meant to control and eliminate intracellular infections. In a Th1 response, CD8⁺ cytotoxic T-cells may also be induced with or without involvement of CD4⁺ T cells. These cells kill infected cells in an MHC class I-restricted manner. A Th2 response is associated with production of cytokines IL-4, IL-5, IL-9 and IL-13. Th2 responses are further observed in allergies and asthma while they may also be required to eliminate helminth infections (Wynn, 2003). The Th1 and Th2 pathways can be antagonistic to each other as Th1 responses are inhibited by Th2 cytokines like IL-4 and IL-13 and, similarly, Th2 development is inhibited by Th1 cytokines. Recently, another Th response associated with inflammation has been characterized. Th17 cells are induced by the IL-12-related cytokine IL-23, and they produce IL-6, IL-17 and TNF-α (Weaver et al., 2006). This response occurs in autoimmune diseases, but its involvement in immunity to infection is less clear. Both CD4⁺ and CD8⁺ T cells contribute to resistance to and clearance of acute Cryptosporidium infection (Panterburg et al., 2008; Borad and Ward, 2011).

Th1 cells and their cytokines have been associated with resistance to and control of Cryptosporidium infection whereas Th2 cells and their cytokines have been detected in the resolution phase of Cryptosporidium infection (Gomez-Morales et al., 2004). Th17 cells typically are associated with inflammatory processes and contribute to the recruitment of Th1 cells (Khader et al., 2007; Betteli et al., 2008). Although Th17 cells have not been studied in cryptosporidiosis, they have been implicated in the pathogenesis of leishmaniasis and toxoplasmosis in mice (Boaventure et al., 2010; Guiton et al., 2011). Memory Th17 cells have been described in humans following mycobacterial infection (Scriba et al., 2008), and Th17 cells
are important for vaccine-induced memory immune responses (Lin et al., 2010). Cryptosporidiosis in humans has been associated with intestinal inflammatory responses implicating Th17 cells (Kirkpatrick et al., 2002).

Most studies have indicated that the most effective adaptive immune response to Cryptosporidium infection involves IFN-\(\gamma\) activity. Treatment of immunocompetent mice or SCID mice reconstituted with splenic T cells, with anti-IFN-\(\gamma\)-neutralizing antibodies increased reproduction of C. parvum and C. muris (McDonald et al., 1992b). Infection with C. parvum has been shown to induce IFN-\(\gamma\) mRNA and protein expression in the intestine measured by RT-PCR and ELISA, respectively (Urban et al., 1996; Kapel et al., 1996). It has been observed in neonatal mice that the kinetics of IFN-\(\gamma\) expression reflects the pattern of acute infection, with the levels of IFN-\(\gamma\) increasing as infection approaches its peak level and declining rapidly as recovery gets under way (Kapel et al., 1996; McDonald et al., 2004). Stimulation of spleen cells from previously infected mice with oocysts antigen-induced proliferation of CD4\(^+\) T-cells and production of IFN-\(\gamma\) by these cells (Harp et al., 1994; Tilley et al., 1995). Additional evidence of an association between CD4\(^+\) T cells, IFN-\(\gamma\) and immunity was suggested by the observation that treatment of mice with anti-CD4\(^+\) anti-IFN-\(\gamma\) antibodies produced a greater exacerbation of C. parvum infection than either antibody alone (Ungar et al., 1991).

In human studies, recovery from cryptosporidiosis has been shown to correlate with PBMC producing IFN-\(\gamma\) when stimulated with parasite antigen (Gomez Morales et al., 1999). Severity of chronic cryptosporidiosis in a HIV-negative child was closely associated with a deficiency in IFN-\(\gamma\) production (Gomez Morales et al., 1996). Some studies have measured the therapeutic
effect of IFN-γ treatment on *C. parvum* development. In two of the reported studies which involved mice, no protective effect was observed (McDonald and Bancroft, 1994; Kuhls *et al*., 1994). Alternatively administration of the cytokine to immunosuppressed rats was reported to reduce parasite numbers (Rehg, 1996). Also, remission of chronic cryptosporidiosis in a child was observed after treatment with IFN-γ (Gooi, 1994). It is likely that IFN-γ activity is necessary for early control of infection. However, IFN-γ-independent mechanisms of immunity also exist and in mice might act mainly later during a primary infection.

The early indication of IL-12 protection against *C. parvum* was suggested by two observations in the same study: in the first observation, treatment of immunocompetent neonatal mice with IL-12 prior to oocysts challenges, but not 24 h later, induced resistance to infection; secondly, administration of anti-IL-12-neutralizing antibodies increased chances of susceptibility to infection (Urban *et al*., 1996). An association between IL-12 activity and IFN-γ expression was established by the fact that IL-12 treatment was shown to increase the levels of IFN-γ mRNA in the intestine. A proof that the protective role for IL-12 is closely linked with IFN-γ production was substantiated by the finding that treatment of IFN-γ deficient mice with IL-12 did not enhance immunity to *C. parvum*. Daily administration of IL-12 to these mice exacerbated the infection and reduced the level of parasite antigen-specific responses of spleen cells (Smith *et al*., 2001). IL-12 is expressed while a Th1 response develops during a primary *C. parvum* infection and in mice it plays an important part in inducing IFN-γ expression required for early parasite clearance (Xiao and Fayer, 2008).
IL-2 has been considered as a Th1 cytokine and is also known to play an important role in the induction of NK-cell and T-cell proliferation. PBMCs from *C. parvum*-infected patients and spleen cells from *C. muris*-infected mice produced large quantities of IL-2 as well as IFN-γ when stimulated with oocysts antigen (Tilley *et al*., 1995; Gomez Morales *et al*., 1999). Treatment of mice with anti-IL-2 neutralizing antibodies, however, had no effect on susceptibility to infection (Ungar *et al*., 1991; Enriquez and Sterling, 1993) and treatment of mice with exogenous IL-2 did not influence parasite reproduction (McDonald and Bancroft, 1994). These findings suggest that IL-2 is not an essential cytokine for development of immunity.

Th2 cytokines are also important components of adaptive immune responses. Investigations of the involvement of IL-4 in immunity have produced conflicting conclusions. Reports suggest IL-4 has either no role in immunity, inhibits development of immunity, promotes the protective Th1 response early in infection or is involved late during infection in parasite clearance in an IFN-γ-independent manner. In a study comparing *C. muris* reproduction in MHC congenic strains of mouse, it was found that BALB/b mice (H-2b) had prolonged infections compared with BALB/c mice (H-2d) and the increased susceptibility of the H-2b mice correlated with a high level of production of IL-4 by spleen cells early during infection (Davami *et al*., 1997). BALB/c mice, in contrast, produced IL-4 only late during infection when parasite numbers were in steep decline (Tilley *et al*., 1995; Davami *et al*., 1997).

Another investigation showed that adult C57BL/6 mice had few intestinal IL-4-producing CD4⁺ T cells early during infection, but large numbers of these cells appeared during the late part of infection when parasites were being eliminated (Aguirre *et al*., 1998). A protective role for the
IL-4-producing cells was implied by the observation that mice treated with anti-IL-4 antibodies had extended infections compared with controls (Aguirre et al., 1998). Also, the IL-4 involvement in immunity appeared to be IFN-γ-independent as anti-IFN-γ-neutralizing antibodies administered late in infection had no effect on the recovery process. However, another group could find no increased susceptibility of adult C57BL/6 IL-41 mice to C. parvum infection (Campbell et al., 2002). In yet another study, when BALB/c neonatal mice were treated with anti-IL-4 antibodies they produced greater numbers of C. parvum oocysts around the time of the peak of infection than control mice (McDonald et al., 2004). Similarly, neonatal BALB/c IL-41 mice were more susceptible to infection than wild-type mice but, significantly, both groups recovered at the same time. IL-4 protein was detected by western blotting in the intestines of wild-type mice 24 h post infection; treatment of wild-type mice with IL-4 prior to infection, but not on day 4, increased resistance to infection (McDonald et al., 2004).

These findings suggested that in neonatal BALB/c mice, IL-4 was required to establish the early control of infection. The source of IL-4 was not determined and its mechanism of action is unclear. However, IL-41 mice had lower levels of intestinal IL-12 and IFN-γ than wild-type mice at the peak of infection, indicating IL-4 could promote the Th1 response. IL-4 has been shown to increase IL-12 production by dendritic cells (Hochrein et al., 2000) and stimulate maturation of IFN-γ-producing T cells (Noble and Kemeny, 1995). IL-4 also acted synergistically with IFN-γ to activate antimicrobial killing by intestinal epithelial cells (Lean et al., 2003). In models of Th1-mediated diseases involving BALB/c mice, treatment with IL-4 exacerbated colitis (Fort et al., 2001), and uveitis (Ramanathan et al., 1996) providing further evidence that IL-4 can enhance IFN-γ-mediated responses.
In studies of IL-4 expression during *C. parvum* infection of humans, it was demonstrated by *in situ* hybridization and immunocytochemistry that IL-4 is expressed in the gut of *C. parvum*-infected persons if they had previously been exposed to the infection (Robinson *et al*., 2001). There was no correlation between symptoms or oocyst excretion levels and expression of the cytokine. In infected cattle, measurement of IL-4 mRNA in intestinal cells showed little expression around the time of recovery (Wyatt *et al*., 2001).

IL-13 has overlapping functions with IL-4, and the two cytokines employ the same receptor, IL-4Rα (Wynn, 2003). BALB/c IL-4Rα1 mice, like the IL-4 mice, were found to have more intense *C. parvum* infection than wild-type mice and the rate of recovery was initially slower than in IL-4 mice (McDonald *et al*., 2004). This suggests, therefore, that IL-13 may have a protective role in this mouse strain. However, unlike IL-4, IL-13 did not increase the capacity of IFN-γ to inhibit *C. parvum* reproduction in an enterocyte cell line (Lean *et al*., 2003). Malnourished Haitian children with persistent cryptosporidiosis were found to have a marked intestinal inflammatory response and expressed IL-13 but not IFN-γ which does not signify a protective role for the cytokine (Kirkpatrick *et al*., 2002).

The first study of the role of Th2 cytokines in control of *Cryptosporidium* infection compared the requirement for IL-4 and IL-5 in immunity by administration of cytokine-neutralizing antibodies to mice infected with *C. parvum* (Enriquez and Sterling, 1993). The most effective antibody for increasing parasite replication was anti-IL-5, whereas a combination of anti-IL-5 and anti-IL-4 antibodies was more effective than anti-IL-5 alone. The results suggested that IL-5 was important, and IL-4 had a protective role in concert with IL-5. It has also been reported that IL-5
is expressed in BALB/c IFN-γ mice that are recovering from cryptosporidial infection (Bonafonte et al., 2000), but no further investigation has been made of the protective role of this cytokine in resistance to infection. Clearly, further studies of IL-4, and other Th2 cytokines, including IL-5 and IL-13, are required to elucidate their contribution to the protective host response against *Cryptosporidium* in different hosts.
CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

The study was carried out at Kenyatta National Hospital (KNH) and Kenya Medical Research Institute (KEMRI) both in Nairobi, Kenya. Kenyatta National Hospital is the largest teaching and research hospital in Kenya with an average of 600,000 outpatient visits and 89,000 inpatients annually. It serves the local population as well as referrals from other parts of Kenya and neighboring countries (Annual report, 2009). In Kenya, between 7.1% and 8.3% of adults live with HIV/AIDS (UNAIDS, 2009). About 200 HIV-positive patients are seen at KNH daily of which 50-80 are new (untreated). Of these, about 10-15 patients are seen at the comprehensive care clinic (CCC) of the respiratory and infectious diseases department, an outpatient facility that is involved in long-term care management of HIV/AIDS patients from all over Kenya as per the Kenya National AIDS/STI Control Programme (NASCOP) guidelines.

The laboratory analyses in this study were carried out at the Centre of Microbiology Research at KEMRI (CMR/KEMRI). KEMRI is the national parastatal responsible for carrying out health-related research in Kenya. CMR has laboratories at the KNH and the KEMRI headquarters on Mbagathi Road. The centre provided good facilities, expertise and environment for the study. It is also at close proximity to sample collection point and thus made it easier for the recruitment of patients to the project and timely sample processing.
The immunological work was carried out at the Centre for Biotechnology Research and Development (CBRD), at KEMRI. The centre has the facilities and equipments that were required for the immunological work.

3.2 Study design

The study was a cross sectional hospital based study where patients were recruited randomly at Comprehensive Care Centre, Kenyatta National Hospital.

3.3 Study population

The study subjects were enrolled into the study at CCC/KNH subject to compliance to inclusion/exclusion criteria. Informed consent was obtained from all participants in the study.

3.4 Recruitment of patients for study

The study subjects were recruited at the comprehensive care centre (CCC) of Kenyatta National Hospital. Prior to being enrolled in to the project, the patients were informed in details about the study and the associated terms. Consequently the patients enrolled in this project made an informed decision and hence signed a consent form given in Kiswahili or English depending with preference of the patient. With the help of the recruiting officer, the patients also completed a standardized questionnaire detailing his/her social demographic background information and medical history. The standardized questionnaire was used to collect data regarding age, gender,
marital status, residence, education, occupation, income, housing, medical history, risk factors for HIV, risk factors for diarrhea, and symptoms including self reported weight loss, vomiting, abdominal pain and fever.

Presence and duration of diarrhea details were also collected using the questionnaire. Diarrhea was defined as three or more watery stools within a 24-hour period. A diarrheal episode was defined as diarrhea for at least 72 hours. The end of a diarrheal episode was defined as absence of diarrhea for 48 hours. Acute diarrhea was defined as a diarrheal episode lasting < 14 days. Persistent diarrhea was defined as a diarrheal episode lasting ≥ 14 days but less than 30 days. Chronic diarrhea was defined as diarrhea lasting more than 30 days. Physical examination was performed by the attending physician at the CCC. At the time of enrollment, stools samples for enteropathogen analysis and blood samples for CD4+ T cell counts and immune response studies were obtained from each patient.

3.4.1 Inclusion Criteria
The study subjects included HIV-infected adults (18 years and older as per the national identification card) who had not previously received HAART, presenting with or without diarrhea and willing to give an informed consent.

3.4.2 Exclusion Criteria
The subjects excluded from the study were those aged less than 18 years, those who had already received HAART and those who declined to give an informed consent.
3.5 Sample size

Sample size was calculated based on previous reports in Kenya where studies have shown that on average 14% of HIV infected adults patients have *Cryptosporidium*. The calculation was based on Dell *et al.*, 2002. A total of 164 HIV infected patients, 94 without diarrhea and 70 with diarrhea were recruited respectively. These enabled recruitment of 20 cryptosporidiosis asymptomatic (no diarrhea) and 20 cryptosporidiosis symptomatic patients (with diarrhea).

3.6 Sampling

3.6.1 Stool samples

Stools samples collected were aliquoted into two samples: one sample was preserved using potassium dichromate while the other sample (NEAT) unpreserved was used for microscopy examination and extraction of *Cryptosporidium* DNA from the oocysts. The samples that were positive for *Cryptosporidium* by microscopy were confirmed by PCR targeting 18s rRNA locus (Xiao *et al.*, 1999; Xiao *et al.*, 2001a). Stool samples from all patients were further, tested by microscopy for ova and other gastrointestinal parasites. The samples from patients with diarrhea were also tested by routine culture for enteric bacteria and by multiplex PCR for pathogenic *Escherichia coli*. 
3.6.1.1 Formol-ether concentration for oocysts

Approximately, 1 g of stool samples was put in a clean 15 ml centrifuge tube containing 7 ml of 10% formalin followed by stirring to mix using an applicator stick. The samples were then filtered through a sieve or gauze into a beaker, and the filtrate poured back into the same tube. Three milliliters of diethyl ether (or ethyl acetate) were then added to the formalinized solution and the tube sealed and the contents mixed vigorously for 30 seconds by vortexing. They were then centrifuged at 1500 rpm for 15 minutes. The suspension was discarded by inverting the tube, allowing only the last one or two drops to fall back into the tube. The remaining suspension was transferred onto a microscope slide, using a Pasteur pipette and allowed to air-dry. Staining for Cryptosporidium spp oocysts was done using the Modified Ziehl-Nielsen Staining protocols (Xiao et al., 1999; Xiao et al., 2001a).

3.6.1.2 Modified ziehl–nielsen staining procedure

Air-dried direct smear was fixed with methanol for 3 minutes; the slide was then immersed in 0.3% carbol-fuchsin and stained for 15 minutes. The slide was then rinsed thoroughly with tap water, followed by decolorizing the slide in 1% HCl (v/v) in methanol for 15 seconds. This was followed by rinsing the slide with tap water. The slides were then counterstained with 0.4% malachite green for 30 seconds and rinsed once again with tap water. The slides were then air-dried and examined using a light microscope at the ×40 objective lens and the presence of oocysts confirmed under the oil immersion objective lens.
3.6.1.3 Extraction of DNA in fecal specimen

Fecal specimens containing *Cryptosporidium* oocysts were stored as follows, unpreserved at 4°C or in 2.5% potassium dichromate solution at 4°C, or frozen at –20°C. DNA was extracted and detected from stool as described by Xiao *et al.* (2002) using a QIAamp stool mini kit (Qiagen, Inc., Valencia, California). During DNA extraction, about 20 g (peanut size) of the fecal specimen was transferred to a 2.0-ml micro centrifuge tube. They were washed thrice with distilled water by centrifugation at 13,200 rpm for 5 min, followed by addition of 66.6 μl of 1-M KOH (potassium hydroxide) and 18.6 μl of 1-M Dithiothreitol (DTT) to the pellet and mixed thoroughly by stirring with a sterile wooden applicator stick. The set up was incubated at 65°C for 15 min. Alkaline was neutralized by addition of 8.6 μl of 25% HCl and 160 μl of 2-M Tris-HCl (pH 8.3), then vortexed continuously for 15 seconds. Two hundred and fifty microliters of phenol, chloroform and isoamyl alcohol solution in the ratio of 25:24:1 was added to the tube and vortexed to mix. The tubes were then centrifuged at 13,200 rpm for 5 minutes. The supernatant was then transferred to a 2 ml micro centrifuge tube. One milliliter of ASL buffer from the QIAamp® DNA Stool Kit (QIAGEN Inc., Valencia, CA) was added to the supernatant and the mixture incubated at 80°C for 5 min. One InhibitEX tablet (from the kit) was added to each specimen and vortexed immediately for 1 minute or until the tablet was completely dissolved. The procedure was then followed as specified in the QIAamp® DNA stool kit manufacturer’s protocol. DNA was then eluted using 60 μl of the AE Elution Buffer and stored at –20°C.
3.6.1.4 *Cryptosporidium* genotyping by PCR-RFLP analysis of the SSU rRNA Gene

Identification of the *Cryptosporidium* species was done by amplifying a highly polymorphic locus of the 18S rRNA gene by nested PCR (Xiao et al., 2001a). Primary PCR cycle amplified about 1325 bp with forward primer 5’-TTCTAGAGCTAATACATGCG-3’ and the reverse primer 5’-CCCTAATCCTTCGAAACAGGA-3’. This was followed by a secondary amplification of an internal fragment with a length of 826-864 bp (depending on the isolate), which was amplified from 2 µl of the primary PCR product using the forward primer 5’-GGAAGGGTGTATTTATAGATAAAG-3’ and the reverse primer 5’-AAGGAGTAAGGAAACACCTCCA-3’ (Xiao et al., 2001a).

**a. Primary PCR**

For each 25 µl PCR reaction, the master mix prepared as follows:

10× PCR buffer 2.5 µl

<table>
<thead>
<tr>
<th>dNTPs (10 nM)</th>
<th>0.5 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 1 (F1) primer (10 mM)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Reverse 1 (R1) primer (10 mM)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>12.6 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Template DNA added to each tube.</td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>
The following PCR program was run:

94°C for 3 min

35 cycles of: 94°C for 45", 55°C for 45", and 72°C for 1 min

72°C for 7 min

4°C hold temperature.

**b. Secondary PCR**

For each 25μl PCR reaction, the following master mix was prepared:

- 10× PCR buffer 2.5 μl
- dNTPs (10 nM) 0.5 μl
- Forward 2 (F2) primer (10 mM) 1.0 μl
- Reverse 2 (R2) primer (10 mM) 1.0 μl
- MgCl₂ (25 mM) 2.0 μl
- Distilled water 15.6 μl
- Taq polymerase 0.4 μl
- Primary PCR product added to each tube. 2.0 μl

The following PCR program was run:

94°C for 3 min

35 cycles of: 94°C for 45", 58°C for 45", and 72°C for 1 min

72°C for 7 min

4°C, hold temperature.
5 μl of the secondary PCR product was run on 1.5% agarose gel containing 0.5 μg/ml of Ethidium bromide, a 100-basepairs DNA ladder (Invitrogen, Carlsbad, CA) was used as the marker. The gel was visualized under UV light and gel photos taken using a digital camera attached to UV Trans illuminator.

c. RFLP Analysis

Master Mix (20 μl) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary PCR product</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>Enzyme (SspI/VspI)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>NEB Buffer 2</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>PCR water</td>
<td>6.0 μl</td>
</tr>
</tbody>
</table>

The samples were incubated in 37°C water bath for 2 hour.

After incubation, the entire 20 μl of restriction digestion product samples were ran in 2% agarose gel. All gels were visualized using an UV light Trans illuminator and photograph taken using digital camera for analysis. Cryptosporidium species and genotypes were identified based on RFLP banding patterns (Xiao et al., 2001a).

3.6.1.5 Extraction of fecal IgA

The stool sample stored at −80 C ° was thawed on ice and approximately 1 g was weighed and put into 15 ml centrifuge tube. The stool was diluted with 4 ml phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-T) supplemented with 100 μg/ml of soybean trypsin
inhibitor (EMD, Gibbstown, NJ), 0.05 M EDTA and 10 mM phenylmethylsulfonyl fluoride (EMD, Gibbstown, NJ). The suspension was mixed and allowed to stand for 15 minutes at room temperature with intermittent shaking. The mixture was filtered through cheesecloth/gauze to remove particulate matter and centrifuged at 10,000 rpm for 30 minutes. The supernatants (samples) were stored in aliquots at -80°C.

3.6.1.6 ELISA for total fecal IgA

Fecal IgA level to recombinant gp15 and Cp23 were measured by enzyme linked immunosorbent assays (ELISA) as described by Khan et al. (2004). Briefly, 96 well plates were coated with 100 µl/well polyclonal goat anti-human IgA in carbonate buffer pH 9.6 (100 ng/well) and incubated at 4°C overnight. The plate was then washed three times with phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-T). The wells were blocked with 200 µl 5% Fetal Bovine Serum in PBS-T (FBS-PBS-T) at room temperature for 5 hours. The wells were washed again and 100 µl of samples diluted (1/10² to 1/10⁶) were added. A blank was included to represent negative control. Human secretory IgA (AbD serotec, Raleigh, NC) standards (0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125μl) were also added to the plate. The set up was incubated at 4°C overnight (ON). Plates were washed again three times and mouse monoclonal antibody to human IgA (Sigma) diluted to 1:10000 in 5% FBS-PBS-T added and incubated at 37°C for 1 hour. After incubation, the plates were washed three times and 100 µl of goat anti mouse IgG biotin conjugate diluted 1:5000 in 5% FBS-PBS-T added and incubated at 37°C for 1 hour. This was followed by washing three times. Then 100 µl of the streptavidin HRP diluted 1:4000 in 5% FBS-PBS-T was added and incubated again at 37°C for 1 hour after which the plates were
washed three times. The substrate ortho-phylenediamine (1 tablet of OPD (5mg) in 10 ml of citrate phosphate buffer (0.2 M sodium phosphate , 0.1 M citric acid pH 4.6), with 4 µl of 30% hydrogen peroxide (H₂O₂) at room temperature was added, absorbance read at 450 nm after 20 min and ELISA units calculated. Quantitation of total fecal IgA was done by comparison with known amounts of purified human fecal IgA standards.

3.6.1.7 ELISA for fecal IgA

One gram of stool was diluted with four millitres of PBS-T supplemented with 100 µg/ml of soybean trypsin inhibitor (Calbiochem, San Diego, CA), 0.05 M EDTA and 10 mM phenylmethylsulfonyl fluoride (Calbiochem, San Diego, CA). The suspension was mixed and allowed to stand for 15 minutes at room temperature with intermittent shaking. The mixture was filtered through cheesecloth to remove particulate matter and centrifuged at 10,000 rpm for 30 minutes. Total fecal IgA was determined by ELISA using purified human secretory IgA (AbD serotec, Raleigh, NC) as standard. Fecal sIgA levels to each of the antigens were measured by ELISA using a modified protocol (Qadri et al., 1997). Briefly, 96- well plates were coated overnight at 4ºC with 0.5 µg/well of the recombinant protein in 0.015 M Na₂CO₃, 0.035 M NaHCO₃, and pH 9.6. Plates were washed three times with PBS-T and non-specific binding sites blocked with 5% fetal bovine serum (FBS) in PBS-T at room temperature for five hours. Fecal supernatants in 5% FBS in PBS-T were added and the plates incubated at 4ºC overnight. Plates were washed three times with PBS-T and mouse monoclonal antibody to human IgA (Sigma, clone GA-1) diluted in 5% FBS in PBS-T was added and the plate incubated at 37ºC for one hour. After washing three times with PBS-T, biotin-conjugated goat anti-mouse IgA (southern
Biotech, Birmingham, AL) diluted in 5% FBS in PBS-T was added and the plate incubated at 37°C for one hour followed by three washes with PBS-T. Horseradish peroxidase conjugated to streptavidin (Pierce) diluted in 5% FBS in PBS-T was added and plates incubated at 37°C for one hour followed by three washes with PBS-T. The plates were developed with the substrate ortho-phenylenediamine (OPD) (Thermo scientific), containing 5 mg of OPD in 10 mls of 0.2 M sodium phosphate, 0.1 M citric acid pH 4.6, 4 μl of 30% hydrogen peroxide (H₂O₂) at room temperature and absorbance read after 20 minutes at 450 nm and ELISA units calculated.

3.6.2 Blood samples

Five milliliters of blood were collected from patients whose stool samples were positive for Cryptosporidium. Heparinized blood was used within 6 hours for isolation of peripheral blood mononuclear cells (PBMCs) for assessment of cellular mediated immune responses. Serum and cells isolated from the whole blood were aliquotted and stored at -80°C.

3.6.2.1 Isolation of the serum

Serum was separated from the whole blood for the quantification of the antibodies by centrifugation at 1,800 rpm for 30 min. The top serum layer was harvested by gently pipetting off into cryovials using a glass Pasteur pipette and stored at -80°C.
3.6.2.2 Serum enzyme-linked immunosorbent assay (ELISA).

Serum IgG and IgM responses to Chgp15 (type I), Cpgp15 (type II) and Cp23 Cryptosporidium antigens were assessed by ELISA as previously described (Khan et al., 2004; Ajjampur et al., 2011). Gp15 antigen is a diverse and heterogeneous antigen showing variability especially between *C. hominis* and *C. parvum* therefore the antigens are classified into two major categories: gp15 type 1 which is also called Chgp15 which is derived from *C. hominis* and gp15 type 2 which is also called Cpgp15 which is derived from *C. parvum*.

Briefly, 96-well plates were coated overnight at 4 ºC with 0.5 μg/well of recombinant (r) Cp23, rChgp15 and rCpgp15 or control antigens in PBS. Plates were washed three times with phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-T) and blocked with 0.25% bovine serum albumin (BSA) in PBS (BSA/PBS) for two hours at 37 ºC. Patient sera diluted 1:100 in 0.25% BSA/PBS were added to the wells and the plate incubated for one hour at 37ºC. Plates were washed three times with PBS-T, alkaline phosphatase–conjugated goat anti-human IgG or IgM (Southern Biotech, Birmingham, AL) diluted in 0.25% BSA/PBS added and the plates incubated for one hour at 37ºC. After three washes with PBS-T, substrate solution containing *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) at 1 mg/ml in 100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM MgCl2) was added and plates incubated for 30 min at room temperature in the dark. Absorbance at 405 nm (A$_{405nm}$) was measured with a Bio-rad microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA). To control for plate to plate variation, known Cryptosporidium-negative and positive (by ELISA and Western blotting of a *C. parvum* lysate) serum samples were run on each plate. All samples were run in triplicates. A$_{405nm}$
of the test sample for each antigen was subtracted from that for the control protein (to control for non-specific binding to the fusion tags). Plate-to-plate variation was normalized by dividing the \( A_{405\text{nm}} \) of each sample by the \( A_{405\text{nm}} \) of the positive control for that plate and multiplying by 100. Results were expressed as ELISA units (E.U).

### 3.6.2.3 Cell mediated immune (CMI) assay

Freshly collected (within 4 hours) blood (5 mls) was mixed with an equal volume of sterile endotoxin free PBS and carefully layered onto Ficoll-hypaque in a 50 ml tube (Kirti et al., 2009). The tubes were then centrifuged at 400 x g at room temperature. After centrifugation, the tubes were taken to a biological safety hood and the fuzzy lymphocyte layer (middle) was removed into a fresh 50ml tube. The lymphocyte layer was washed with sterile endotoxin free PBS by centrifugation at 200 x g for 10 minutes. To remove RBCs from the pellet, 3 ml ACK Lysis buffer (0.15 M \( \text{NH}_4\text{Cl} \), 10 mM \( \text{KHCO}_3 \), 0.1 mM EDTA), was added to the pellet after removing the supernatant. The tube was centrifuged at 200 x g for 5 min and then washed with PBS. The pellet was resuspended in 10 ml of endotoxin free RPMI -1640 (culture media) containing 10% fetal bovine serum (cRPMI) and viability of the cells determined using trypan blue exclusion.

PBMCs isolated as described above were resuspended at 1 X \( 10^7 \) viable lymphocytes /ml in 12.5% human serum albumin (HSA) (10 ml of stock 25% HSA and 10 ml of sterile incomplete RPMI-1640 medium) in complete RPMI (cRPMI: sterile RPMI-1640 medium supplemented with 10% sterile heat-inactivated FBS and 1% sterile antibiotic/antimycotic) in 50 ml tube at 4
°C. While gently swirling the tube, 2 x freezing media (10 ml of stock 25% human serum albumin, 10 ml of sterile incomplete RPMI-1640 medium and 5 ml of DMSO) was added drop wise to double the volume of the cell suspension. The tube was placed on ice immediately, and the cell suspension slowly removed into a pipette and dispensed into 2 ml cryovials on ice. The cryovials were then placed in a precooled freezing container (“Mr Frosty”) that was filled with 70% isopropanol and the freezing container placed at −80°C and then transferred to liquid nitrogen (De Boer et al., 1981; Bull et al., 2007).

During the process of thawing cells, Cryovials from liquid nitrogen were transferred to a 37 °C water bath and held on the surface of the water bath with occasional flicking. Warm cRPMI (22 °C -37 °C) was added drop wise into the cryovial containing the cell suspension and the diluted cells suspension was transferred to a 50 ml tube containing cRPMI, and centrifuged for 7 minutes at 1200 rpm. The supernatant was decanted and the pellet resuspended in the 1 ml of warm cRPMI. Viability was determined using trypan blue and cells were diluted to the final working solution of 5 x 10⁶ PBMCs/ml in cRPMI at room temperature.

Before lymphocyte proliferation assay, cells were counted and 1 x 10⁶ cells/ml were then plated in 24-well plates. The cells were cultured in RPMI 1640 medium (supplemented with 10% fetal calf serum and antibiotics) in the presence of Cryptosporidium crude soluble antigen (CCA) and the nonspecific antigen Phytohemagglutinin (PHA). The cells were incubated at 37°C in the presence of 10% CO₂. Five microgram per milliliter of antigens and 5 µg/ml PHA were used for
stimulation for 3 days. At the end of the incubation period, the culture supernatant of the cells was collected and used immediately or kept at –80°C for the cytokine assay. IFN-γ levels from peripheral blood mononuclear cells stimulated ex vivo with rCp 23 and rgp 15, as a measure of T cell-mediated immune responses were measured by ELISA assay OptEIA™ (Becton Dickenson), using recombinant gp15 and Cp 23 as described (Preidis et al., 2007). Briefly, 100 µl/well diluted capture antibody (1:250 cap-Ab in coating buffer) was added per well, wrapped tightly with parafilm and incubated overnight at 4°C. The wells are then aspirated and washed 3 times using wash buffer (0.05% PBS-T) followed by blocking with 200 µl/well of assay diluents (10% FBS-PBS) and incubated at room temperature for 1 hour. Three hundred pg/ml stock of standard-IFN-γ (S7) was prepared and diluted serially using assay diluents (S1-S6) while S0 (Assay diluents only) was used as negative control of standards. Samples were diluted 1:10 (sample to assay diluents).

After washing 3 times, standards and samples were added in triplicates, wrapped firmly with parafilm and incubated at room temperature (RT) for 2 hours. Working detector (Detection antibody + SAv-HRP) was prepared 15 minutes before use as follows: (1:250 Detection antibody i.e. biotinylated human IFNγ / assay diluents + 1:250 SAv-HRP/Assay diluents). A 100 µl of working detector was added per well after washing 3 times with wash buffer. The plates were wrapped tightly again with parafilm and incubated at room temperature for 1 hour. The plates were aspirated and washed 7 times using wash buffer. After dispensing wash buffer into wells the plates were allowed to stand for 30 seconds before flicking off the wash buffer. A 100 µl of the substrate solution was added and incubated for 30 minutes at room temperature in the dark.
After 30 min, 50 µl of stop solution (2N H$_2$SO$_4$) was added to each well. The absorbance was read at 450 nm. The test sample results were calculated by interpolation from a standard curve with absorbance values on the vertical axis and cytokine concentrations on the horizontal axis (pg/ml).

### 3.7 CD4$^+$ T cells counts

CD4$^+$ cell counts were determined on whole blood samples, using a CyFlow® SL3, GmbH, Germany) at the Comprehensive Care Clinic at KNH.

### 3.8 Ethical clearance

Approval for this study was obtained from the Kenya Medical Research Institute Institutional Review Board (KEMRI /IRB) and the Kenyatta National Hospital Ethical Review committee (KNH/ERC) (Appendix II) as well as Tufts Medical Center and Tufts University IRB (USA).

### 3.9 Data analysis

Statistical analyses were performed using Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA) and the Statistical Analysis Software package (SAS, Version 9.13). Hypothesis testing was two sided with a type 1 error rate of 0.05. Comparison of the antibody responses in symptomatic and asymptomatic groups of patients was conducted using unpaired T-test for continuous variables that were normally distributed and wilcoxon sign rank test for those that were non–normally distributed respectively.
CHAPTER 4: RESULTS

4.1 General overview

The average CD$^+$ T cells counts for an asymptomatic patients was relatively higher compared to that of the symptomatic patients. Further the value of Interquartile range for asymptomatic patients was higher than that of symptomatic patients. Details of these patients that were studied after recruitment (i.e. those positive for Cryptosporidium) are summarized in the table 4.1:

Table 4.1: General characteristics of the patients studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asymptomatic (n = 20)</th>
<th>Symptomatic (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD$^+$ T cells counts:</td>
<td>Mean</td>
<td>351.6 (±59.7)$^{a}$</td>
</tr>
<tr>
<td></td>
<td>Median CD4 count</td>
<td>304.5 (212.5 - 386.5)$^{b}$</td>
</tr>
<tr>
<td></td>
<td>(IQ range)</td>
<td></td>
</tr>
<tr>
<td>Gender:</td>
<td>Male</td>
<td>10 (50)$^{c}$</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10 (50)$^{c}$</td>
</tr>
<tr>
<td>AGE (mean± SD)</td>
<td>38 (±1.6)$^{d}$</td>
<td>36 (± 2.2)$^{d}$</td>
</tr>
</tbody>
</table>

$^a$CD$^+$ T cells per µl (mean ± SD); $^b$CD4 per µl (Interquartile Range); $^c$number (%); $^d$years (mean ± SD)

4.2 Species of Cryptosporidium infecting HIV infected persons attending KNH

Cryptosporidium oocysts usually appear pink in colour after staining. They are oval in shape. The Figure 4.1 shows a field of view of a light microscope with Cryptosporidium oocysts.
However, due to the fact that microscopy could not identify species, PCR-RFLP was done to identify specific species. After primary and secondary polymerase chain reaction, secondary reaction products were run in 1.5% agarose gel to confirm whether the samples had DNA. Positive samples produced banding patterns that confirmed the presence of Cryptosporidium but which could not be used for establishment of the species and therefore the products were used for restriction fragment length polymorphism (RFLP) digestion. Figure 4.2 shows some Cryptosporidium positive samples ran against a 100 bp marker. The samples have varying banding pattern. The strength of the bands indicates possible concentration and quality of the DNA in the samples.
Figure 4.2: Agarose gel (1.5 %) visualization of Cryptosporidium secondary PCR products. The gel indicates that samples 1-9 ran against a 100bp Molecular marker (M) are positive for Cryptosporidium but cannot be used to identify specific species.

The secondary products were then digested for specific identification. These secondary PCR products were digested using restriction enzyme Ssp 1 and Vsp1 followed by running the samples in 2 % agarose gel. Figure 4.3 shows the distribution of the species in the gels. The products in the upper gels are cut (restricted) using Ssp 1 while those in the lower gels are cut (restricted) using Vsp1 restriction enzymes respectively.
Figure 4.3: Gel Images indicating species of *Cryptosporidium* digested using Ssp 1 and Vsp 1 restriction enzymes. The species are *C. hominis* (Ch) *C. parvum* (Cp), *C. canis*, and *C. meleagridis* (Cmel). Molecular marker (M) is indicated on each gel and the key base pair sizes labeled (100, 300, and 600)

Following Ssp1 and Vsp 1 digestion of the secondary amplicon, they produce banding pattern of diverse sizes. Below are the sizes of the PCR products and the expected sizes of restriction products according to individual *Cryptosporidium* species. *C. hominis*: **851bp**: Ssp1: 450, 267, 111; Vsp1: 561, 115, 104, 71; *C. parvum*: **848bp**: Ssp1: 450, 267, 108; Vsp1: 629, 115, 104; *C. canis*: **843bp**: Ssp1: 417, 267, 107, Vsp1: 624, 115, 104; *C. meleagridis*: **847bp**: Ssp1: 450, 267, 108 and Vsp1: 457, 171, 115, 104. (Xiao and Ryan, 2008).

Species determination by PCR RFLP at the *Cryptosporidium* 18S rRNA locus indicated that *C. hominis* was the commonest species in patients with both symptomatic and asymptomatic cryptosporidiosis, with no difference in prevalence of any species between them (Table 4.2).
Table 4.2: Distribution of the species in patients attending CCC, KNH

<table>
<thead>
<tr>
<th>Species</th>
<th>Asymptomatic n=20 (100%)</th>
<th>Symptomatic n=20(100%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. hominis</em></td>
<td>9 (45%)</td>
<td>11(55%)</td>
<td>0.701¹</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>6 (30%)</td>
<td>4 (20%)</td>
<td>0.656¹</td>
</tr>
<tr>
<td><em>C. canis</em></td>
<td>1 (5%)</td>
<td>2 (10%)</td>
<td>0.715¹</td>
</tr>
<tr>
<td><em>C. meleagris</em></td>
<td>3 (5%)</td>
<td>3 (15%)</td>
<td>0.856¹</td>
</tr>
<tr>
<td><em>C. muris</em></td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0.513¹</td>
</tr>
</tbody>
</table>

¹: Fishers exact test

Further, after examination of the stool samples by microscopy for ova and parasites, wide range of parasitic micro-organisms were detected together with *Cryptosporidium*. They included protozoans like *Isospora belli*, *Cyclospora* spp, *Giardia lamblia*, *Entamoeba histolytica/ dispar*, *Entamoeba coli*, *Chilomastix mensnili*, *Endolimax nana*, *Iodamoeba buetschlii*, and *Microsporidia* spp. Helminthes observed included: *Strongyloides stercoralis*, *Ascaris lumbricoides*, *Schistosoma mansoni*, *Enterobius vermicularis* and *Ancylostoma duodenale* (hook worm).

These parasites were detected in patients with and without diarrhea. Patients with diarrhea were also screened for bacteria pathogens. Enteric bacterial pathogens identified by culture included pathogenic *E.coli (ETEC)*, *Salmonella* spp, *Klebsiella* spp, *Pseudomonas*, *Campylobacter*, *Citrobacter freundii*, *Morganella morgani*, *Aeromonas* spp and *Vibrio cholerae*. 
4.3 Serum IgG, IgM and fecal sIgA levels against Cryptosporidium antigens (gp15 and Cp23) in HIV patients infected with Cryptosporidium

Immunoglobulin G against gp15 type 1 (Chgp 15) was produced by both groups with a mean of 205.5 ± 17.13 E.U for the asymptomatic and 137.7 ± 17.37 E.U in the symptomatic group. There was significant difference in anti-gp15 type 1 IgG production between the asymptomatic and the symptomatic patients ($P < 0.05$; Figure 4.4). In gp15 type 2 (Cpgp 15), the IgG secreted had means of 171.5 ± 28.62 E.U in asymptomatic and 127.8 ± 13.10 E.U in the symptomatic groups.

Overall, there was no significant difference in the anti-gp15 type 2 production ($P > 0.05$; Figure 4.4) between asymptomatic and symptomatic patients. There was a marked higher anti-Cp 23 IgG product in the asymptomatic patients than symptomatic group with a mean of 179.9 ± 20.72 E.U and 93.89 ± 12.2 E.U respectively. The asymptomatic group showed significantly higher anti-CP 23 IgG as compared to the symptomatic group ($P < 0.05$; Figure 4.4). Gp15 type 1 specifically showed the highest IgG response when compared to the other two antigens. The graphs below shows IgG levels against the three antigens where all values indicated consistency in asymptomatic patients secreting more antibodies than symptomatic patients.
A: Chgp15 IgG, B: Cpgp15 IgG, C: Cp23 IgG

Figure 4.4: Serum IgG antibodies to gp15 and Cp23

Serum IgM secretion against gp15 type 2 (Cpgp15) was relatively lower when compared to gp15 type 1 (Chgp15) IgM and Cp23 IgM. Anti-gp15 type 2 (Cpgp 15) IgM had means of 41.38 ± 12.52 E.U in asymptomatic and 55.42 ± 13.72 E.U in symptomatic patients. There was no significant difference between the two (P > 0.05; Figure 4.5). Immunoglobulin M production against gp15 type 1 (Chgp15) was higher in both groups and was almost similar to the response seen in Cp23. The asymptomatic group had a mean of 78.18 ± 7.4 while symptomatic patients
had a mean of 68.36 ± 5.3 E.U. There was no significant difference between the asymptomatic and symptomatic group. Immunoglobulin M production to Cp23 for asymptomatic was 98.11 ± 10.9 E.U while that of symptomatic was 84.29 ± 8.3 E.U with no significant difference being observed between asymptomatic and symptomatic patients ($P > 0.05$; Figure 4.5).

![Graphs showing IgM antibody levels](image)

**Figure 4.5: Serum IgM antibodies to gp15 and Cp23**

Figure 4.5 shows the comparison of IgM means of the different antigens. Gp15 type 2 is unique in that the symptomatic patients had higher means as compared with the asymptomatic patients.
The antigen also had the least values of the secretion of the antibodies. Cp23 which is the most immunodominant antigen indicated highest secretion of antibodies followed by gp15 type 1.

In the case fecal sIgA against gp15 type 1 (Chgp15), the asymptomatic group had a higher mean of 183 ± 11.48 when compared to the symptomatic group which had a mean value of 118 ± 13.85. There was a significant difference in antibody secretion between asymptomatic and symptomatic patients ($P < 0.05$; Figure 4.6). However, there was no significant difference between asymptomatic and symptomatic patients in the fecal IgA production to gp15 type 2 (Cpgp 15) ($P > 0.05$; Figure 4.6). The means were 147.5 ± 21.86 E.U for the asymptomatic patients and 161.6 ± 21.54 E.U for symptomatic patients. In Fecal sIgA response against Cp23, the mean for the asymptomatic patients was 128.5 ± 15.56 E.U while that of the symptomatic was 129.5 ± 17.06 E.U. Similarly to gp15 type 2 antigens, there was no significant difference in fecal IgA response between asymptomatic and symptomatic patients ($P > 0.05$; Figure 4.6). Gp15 type 1 sIgA showed greatest difference between the two groups with asymptomatic having significantly higher IgA levels followed closely by the gp15 type 2. Cp23 indicated almost comparable secretion of IgA by symptomatic and asymptomatic patients (Figure 4.6).
Figure 4.6: Fecal IgA antibodies to gp15 and Cp23.

4.4 IFN-γ-mediated cellular immune responses in patients infected with Cryptosporidium spp.

After stimulation with the gp15 type 1 (Ch gp15) both the asymptomatic and symptomatic group showed production of the IFN-γ. The mean of the asymptomatic was 222.1 ± 18.47 E.U while that of symptomatic was 221.6 ± 23.18. There was no significant difference in IFN-γ levels between asymptomatic and symptomatic patients (P > 0.05; Figure 4.7). Further, there was also production of IFN-γ against Cpgp15 (gp 15 type 2) with the asymptomatic patients having a mean of 151.5 ± 18.7 E.U and 110.2 ± 21.2 E.U for the symptomatic patients. There was no
significant difference between these patients ($P > 0.05$; Figure 4.7). The mean for IFN-$\gamma$ production to Cp23, was $216.7 \pm 26.47$ E.U for the asymptomatic patients and $225 \pm 13.7$ E.U for the symptomatic patients, with no significant difference between the asymptomatic and symptomatic patients ($P > 0.05$; Figure 4.7). The means indicated that gp15 type 1 and Cp23 were almost at par while gp15 type 2 showed lower levels of IFN-$\gamma$ in both symptomatic and asymptomatic patients.

Figure 4.7: IFN-$\gamma$ production by PBMCs
4.5 Association between diarrhea and CD4 counts in HIV infected patients with Cryptosporidiosis

Symptomatic patients had lower CD4 counts when compared to asymptomatic patients. Majority of the symptomatic patients had CD4+ T cells counts of < 200 cells per mm³. Coincidentally majority of the patients in the symptomatic group (8/11) who had acute diarrhea were infected with C. hominis. Figure 4.8 shows that the symptomatic patients CD4+ T cell counts inclined towards <250 cells per mm³ with the majority having between 151 and 200 cells per mm³. Asymptomatic patients CD4+ T cell counts on the other hand had the majority being in the upper categories (>250).

![Figure 4.8: Frequency of CD4+ T cell counts in the symptomatic and asymptomatic patients](image)

Figure 4.8: Frequency of CD4+ T cell counts in the symptomatic and asymptomatic patients
Table 4.3: Summary of CD4⁺ T Cells counts and resulting percentage of diarrhea in patients with CD4 count <200 cells/µl

<table>
<thead>
<tr>
<th>Status</th>
<th>Mean CD4 COUNT</th>
<th>MEDIAN CD4 COUNT</th>
<th>IQ RANGE</th>
<th>% patients with CD4&lt;200 Cells/µl</th>
<th>% of acute diarrhea in the past 1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>351.6</td>
<td>304.5</td>
<td>212.5 - 386.5</td>
<td>15%</td>
<td>10%</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>171.4</td>
<td>158</td>
<td>60.5 - 257.5</td>
<td>65%</td>
<td>55%</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQ: Interquartile

The table above shows the observed risk of diarrhea in patients associated with low CD4⁺ T cells count. In asymptomatic patients, 15 % (3/20) had CD4 count <200 cells/µl and 10% (2/20) of patients who are also in the category <200 cells/µl indicating to have suffered from acute diarrhea for the past one month even though they were asymptomatic for cryptosporidiosis by the time of recruitment into the study. In symptomatic patients, 65 % (13/20) of the patients had CD4 count below 200 cells/µl and 55 % (11/20) who also falls in the category of <200 cells/µl indicated to have suffered from acute or recurrent diarrhea in the past month.
CHAPTER 5: DISCUSSION

Cryptosporidium infects humans and causes diarrheal disease worldwide and is a significant cause of morbidity and mortality in HIV/AIDS patients particularly in developing countries where HAART is not widely available or affordable (O'Connor et al., 2011). Nitazoxanide, approved by US Food and Drug Administration (FDA) for use in treating cryptosporidiosis in immunocompetent patients, has not been effective in treating immunocompromised persons especially in persons such as those infected with HIV (Amadi et al., 2002; Abubakar et al., 2007) and no vaccine is available. In Kenya, Cryptosporidium is a significant cause of HIV/AIDS disease progression and usually leads to death (Batchelor et al., 1996; Mwachari et al., 1998). The use of Highly Active Anti-retroviral Therapy (HAART) has markedly reduced the prevalence of opportunistic parasite infections in developed country (Cello and Day, 2009). However, HAART is not widely available or affordable in developing countries and the emergence of drug resistant HIV variants and failure or discontinuation of HAART is associated with recrudescence to infections with Cryptosporidium infection (Nannini et al., 2002).

To date, there have been no studies on the epidemiology of cryptosporidiosis in HIV/ AIDS patients in Kenya and there have been no previous studies on Cryptosporidium species infecting these patients or on immune responses to this parasite in this population. Therefore, elucidating the Cryptosporidium species infecting HIV-infected persons and elucidating ways of enhancing immunity in these patients is the most feasible way of controlling death. The purpose of this study was therefore to describe the epidemiological features of Cryptosporidium species infection in HIV/AIDS infected adults with and without diarrhea in Kenya, to identify the infecting Cryptosporidium spp.
The immune responses against Cp 23 and gp15 antigens (which are vaccine candidates) (Wanyiri and Ward, 2006) were quantified in these patients with a purpose of determining if there was any association between these responses and protection from diarrhea which can be debilitating and even cause death. In this study, most of the HIV infected patients had diarrhea while others confirmed to have suffered from diarrhea in the near past. *Cryptosporidium* was the most common gastrointestinal parasite identified in patients with diarrhea and also in those without diarrhea. The data of this study demonstrate and confirm previous reports that *Cryptosporidium* infection is common among HIV infected adults in Kenya (Batchelor et al., 1996; Mwachari, 1998; Mwachari et al., 2003). Most of these studies however looked at only patients with chronic diarrhea. The prevalence of *Cryptosporidium* in this study is higher than that reported in HIV infected children in Kenya (Gatei et al., 2006b); however in this study we used PCR to detect for *Cryptosporidium* while Gatei et al., 2006b used microscopy.

Our study and another study (Ajjampur et al., 2008) have shown that PCR is much more efficient than microscopy in *Cryptosporidium* detection. Of particular interest in this study is the high rate of asymptomatic cryptosporidiosis (*Cryptosporidium* but no diarrhea). Elsewhere in the developing countries higher rates of asymptomatic cryptosporidiosis have been reported, even among patients with low CD4+ T cell counts (Wanke et al., 1999; Chin et al., 2006). The basis for the high rates of asymptomatic cryptosporidiosis in HIV/AIDS patients despite low CD4+ T cell counts is unknown. The theory that asymptomatic cryptosporidiosis could be due to low parasite loads was ruled out as no significant correlations were found between parasite loads and diarrheal symptoms (Haque et al., 2009). However, in developing countries such as Kenya where cryptosporidiosis is endemic, children are exposed early and frequently to *Cryptosporidium*. Pre-
existing antibody and/or T cell memory immune responses to *Cryptosporidium* infection acquired before HIV infection may protect from subsequent symptomatic cryptosporidiosis in these patients. Given that memory CD4+ T cells are essentially preserved in HIV infection, immune memory that has been acquired prior to *Cryptosporidium* infection may persist despite overall low CD4 counts (Chou et al., 1994).

*Cryptosporidium* indentified in HIV infected adults were *C. hominis*, *C. parvum*, *C. meleagris*, *C. muris* and *C. canis*. The distribution of *Cryptosporidium* species varies from one country to another, even from one region to another (Putignani et al., 1999). Results from this study revealed that the majority of the patients were infected with the *C. hominis* (50%) followed by *C. parvum* (25%) genotypes. In a study carried out on HIV infected children in Kenya, *C. hominis* and *C. parvum* were the main species indentified with prevalence of 87% and 9% (n=175) respectively (Gatei et al., 2006b). Studies elsewhere in developing countries have also reported *C. hominis* and *C. parvum* as the most prevalent species in HIV infected patients (Houpt et al., 2005; Muthusamy et al., 2006; Cama et al., 2007). In Kenya, infection by *C. hominis*, *C. parvum*, *C. meleagris*, *C. canis* and *C. muris* have also been previously reported in HIV-infected patients (Morgan et al., 1999; Gatei et al., 2002a; Gatei et al, 2006b). So far *C. suis* has not been reported in Kenya but has been reported in HIV infected adults in Peru (Cama et al., 2007). This study did not identify any *C. felis* which was previously identified in HIV infected patients in Kenya (Gatei et al., 2002a; Gatei et al., 2006b). The presence of *C. hominis* is attributed to direct human-to-human transmission while the presence of *C. parvum*, *C. meleagris*, *C. canis*, and *C. suis* in humans is indicative of zoonotic transmission (Leav et al., 2002; Xiao et al., 2004a).
Previous studies have reported that *C. hominis* infection is associated with a higher rate of asymptomatic infection and a lower CD4 cell count in patients with HIV when compared to *C. parvum* infection (Houpt *et al*., 2005). Our results are in agreement with another study that identified *C. hominis, C. parvum, C. meleagridis, C. muris* and *C. canis* in HIV-infected Kenya children though in that study *C. felis* was also identified (Gatei *et al*., 2003). These two studies are also in agreement with the finding that the two major species infecting humans are *C. hominis*, the anthroponotic species (previously called *C. parvum* genotype 1) and *C. parvum* (previously called genotype II). *Cryptosporidium hominis* infects almost exclusively humans while *C. parvum*, the zoonotic species infects human and animals (Leav *et al*., 2002; Xiao *et al*., 2004a; Tumwine *et al*., 2005; Gatei *et al*., 2006a; Samie *et al*., 2006). Humans infection with other zoonotic species such as *C. meleagridis, C. muris, C. canis, C. suis* and *C. felis* have also been reported elsewhere (Gatei *et al*., 2003; Cama *et al*., 2007).

In clinical manifestations, the symptomatic patients were mostly associated with *C. hominis* species which comprised 55% (11/20) of the cases followed by *C. parvum* which comprised 20% (4/20). The symptomatic patients had relatively low mean CD4⁺ counts of 171.4 ±24.4 cells per µl of blood with minimum of 16 and maximum of 362. Asymptomatic patients on the contrary had mean CD4⁺ count of 351.6 ±59.7 with minimum of 68 and maximum of 1278. Therefore, *C. hominis* could be associated with higher risk of development of symptoms in HIV infected persons compared to other species according to this study. This study also indicates that the presence of clinical symptoms is due and/or associated with low CD4⁺ T cells counts which has been implicated to be a marker of low immunity.
In a study carried out in Tanzania amongst the HIV-infected persons, infection with *C. hominis* was associated with longer periods of symptoms and a lower CD4+ counts compared to *C. parvum* (Houpt et al., 2005). These findings further support our findings that asymptomatic patients were mainly infected by *C. hominis* and *C. parvum* at 45% (9/20) and 30% (6/20) respectively. In India, children infected with *C. hominis* had more severe diarrhea than those infected with the *C. parvum* (Ajampur et al., 2007) which possibly could support the finding that most symptomatic patients were infected with *C. hominis*. In addition, in Lima, Peru a study involving HIV-infected persons showed that although *Cryptosporidium* infections was associated with diarrhea, only infections with *C. canis*, *C. felis* and *C. hominis* were associated with diarrhea while infection with *C. parvum* was associated with chronic diarrhea and vomiting (Cama et al., 2007).

Protozoan parasites co infecting with *Cryptosporidium* that were detected in this study include *Isospora belli*, *Cyclospora*, *Entamoeba histolytica/dispar*, and *Giardia lamblia*. Bacteria co infecting with *Cryptosporidium* included *Salmonella* spp, *Klebsiella* spp, *EPEC* and *EAEC*. Co-infecting helminthes included *Ascaris lumbricoides*, *Schistosoma mansoni*, and Hookworm. *Isospora belli*, *Giardia lamblia*, *Entamoeba histolytica /dispar*, and *Microsporidia* were significantly higher in patients with diarrhea indicating that they may be significant causes of diarrhea in HIV infected patients (Stark et al., 2009). All protozoa, helminthes, and bacteria pathogens identified in this study have been reported previously in HIV infected patients in Kenya and elsewhere in Africa (Mwachari et al., 1998; Gassama et al., 2001; Samie et al., 2006). The presence of multiple enteric pathogens was observed in both patients with or without diarrhea. Recovery of multiple enteric pathogens has been reported in HIV infected patients
(Germani et al., 1998; Weber et al., 1999; Carcamo et al., 2005). On review of medical records, both asymptomatic and symptomatic patients were found to have had suffered other opportunistic infections that included candidiasis, cryptococcal meningitis, tuberculosis, recurrent pneumonia, boils, herpes zoster and herpes simplex though their numbers were not statistically significant.

In this study, Cryptosporidium specific serum antibodies (IgG and IgM) and fecal antibodies (IgA) response of 20 symptomatic patients and 20 asymptomatic patients were assessed. Comparison of antibody response in symptomatic or asymptomatic subjects showed that asymptomatic patients had significantly higher serum IgG levels to Chgp15, and Cp23, and fecal IgA levels to Chgp15 than symptomatic patients. These suggests that Cryptosporidium specific serum IgG and mucosal IgA may be associated with protection from symptoms. In humans, the presence of pre existing antibodies to specific antigens correlates with resolution of infection and protection from subsequent challenge (Moss et al., 1998; Chappell et al., 1999; Frost et al., 2005). Serum antibody responses to gp 15 (15-17 kDa antigen) have been reported in HIV – infected adults in developing countries (Sandhu et al., 2006). The presence of pre existing antibodies to gp 15 correlated with protection from diarrheal symptoms in infected adult humans (Moss et al., 1998; Frost et al., 2005).

A study of HIV–infected patients showed that patients with higher antibody responses to Cp23 (27KDa antigen) tended to be asymptomatic (Frost et al., 2005). The same study showed that there was a significant difference in antibody responses to recombinant C. hominis antigens (Chrgp15) between symptomatic and asymptomatic cryptosporidiosis but not the C. parvum
antigens. Although a few polymorphisms have been identified in the p27 gene that encodes Cp23, this protein is relatively conserved among different isolates (Sturbaum et al., 2003). Increased response to C. hominis antigens may reflect more frequent exposures to C. hominis (White et al., 2005). It may also be that C. hominis may be more antigenic. Cryptosporidium hominis but not C. parvum gp 15 has been reported to induce interferon gamma mediated cellular immune responses in adult volunteers with serological evidence of prior Cryptosporidium infection (Preidis et al., 2007).

The immune responses to Cryptosporidium antigens Cp23, Chgp15 (Type 1) and Cp gp15 (Type 2) were diverse. All the patients produced IgG antibodies to the antigens. However, the levels of antibodies varied among patients samples. The levels of anti-Chgp15 IgG production was higher in asymptomatic than in symptomatic patients. The fact that there was more antibody production to C. hominis gp15 when compared to C. parvum gp 15 in asymptomatic patients could possibly be due to intensive sensitization by C. hominis given that they are still immunocompetent. Antibodies production against gp15 type II showed a threshold of response with no significant difference between asymptomatic and symptomatic patients. This possibly suggests that gp15 is not a major antigen in challenging human immune system or has less immunological significance and protection. Generally, symptomatic patients showed minimal secretion in responses to both antigens which could be an indication of the impaired humoral immune system hence the inability to respond effectively towards the antigen.

Previous studies by Ungar et al., 1986., have indicated that there is considerable secretion of anti-gp15 IgG against the Cryptosporidium specific antigens in both HIV infected and
immunocompetent patients. Another study indicated that IgG is produced prior and during infection against 15-kDa protein (gp15 type 1 or Chgp15) and asymptomatic patients produced significantly higher levels of IgG against Cp23 compared to symptomatic patients suggesting that it could also be protective (Moss et al., 1998). In a separate study, the presence of IgG to Cp23 antigens was associated with low levels of oocysts shedding (Frost et al., 2005) and therefore this could be an indication that asymptomatic patients were able to prevent parasite multiplication. A study done in immunosuppressed individuals indicated that a strong serological responses to Cp23 were associated with low risk of developing diarrhea (Sang-Mee et al., 2008).

A unique finding was observed in study comparing IgG levels to Cp23 and gp15 in humans where these antibodies against gp15 antigens reduced rapidly whereas IgG antibodies against Cp23 remained above the detectable threshold for at least 2 years after infection (Ong et al., 2005). These observations are clear pointers to the suitability of Cp23 as a candidate for vaccine development due to the presence of immune response long time after infection hence can protect against re-infection.

There was no significant difference in the IgM levels to any of the Cryptosporidium antigens tested between symptomatic and asymptomatic patients. Though there was production of the IgM antibodies, quantities were relatively less when compared with other IgG antibodies. This may be an indicator of the lesser role played by the IgM production on its own in the immune responses against Cryptosporidium. A stronger response was observed against Cp 23 (27-kDa) followed by gp15 type 1 (15-kDa) while the gp15 type 2 (17-kDa) had the least response. In another study, the immunological significance of IgM antibodies during recovery from Cryptosporidium appeared to be of less importance (Frost et al., 2005). In another study
involving HIV seropositive *Cryptosporidium* positive persons, only 18.2% of studied persons showed secretion of the IgM hence putting into doubt its significance in persons with active Cryptosporidiosis (Kirti *et al.*, 2009). In another finding, the IgM *Cryptosporidium* antibodies declined with persistence of diarrhea (Khan *et al.*, 2004) thus reducing its dependability especially in symptomatic patients.

Secretory fecal IgA (sIgA) secretion is extensively perceived to be a marker of the mucosal immune response especially on the intestinal surface. AIDS patients with chronic cryptosporidiosis were reported to have high titers of sIgA (Cozon *et al.*, 1994), possibly signifying that additional mechanisms are necessary for control of infection. Production of IgA was considerably higher compared to IgM antibodies. In the current study, patients without diarrhea had significantly higher fecal IgA levels of anti-Chgp15 IgA (*P* < 0.05) but there was no significant difference in IgA level to either *Cp*gp15 or *Cp*23 among symptomatic or asymptomatic patients. This clearly underscores the importance of Chgp 15 antigen to the host immune response and also consistent with the fact that *C. hominis* is the dominant species in this study. The IgA production to 17-kDa Proteins (gp15 type 2) has previously been reported (Moss *et al.*, 1998). Our study concurs with this study suggesting that gp 15 may be a good candidate for mucosal immune vaccine development. Furthermore, AIDS patients with cryptosporidiosis often have elevated levels of duodenal lamina propria IgA cells and specific fecal IgA levels (Benhamou *et al.*, 1995). Symptomatic patients especially those with low CD4 counts showed lower levels of antibodies to all the tested antigens.
It was also observed in a different study that as the number of CD4+ T cells decreases in HIV infected individuals, mucosal antibody responses may deviate from the usual trend and there may be an increase in the levels of polyreactive T-cell independent antibodies. These polyreactive T-cell antibodies may not be sufficient to clear infection (Janoff et al., 1990). It is therefore possible that strong immunity before infection with Cryptosporidium would enable control of the infection without adverse effects.

Previously, it has been shown that in immunocompetent human adults, the level of parasite-specific sIgA was higher in individuals excreting oocysts or with diarrhea (Dann et al., 2000). In addition, another study showed that AIDS patients with a strong serological response to a 27-kDa (Cp23) parasite antigen had a reduced risk of cryptosporidial diarrhea (Frost et al., 2005). Immunization with Cp23 expressed antigens in mice showed production of secretory IgA though the titres were not as high as the titres produced in mice orally infected with the antigen which appears to emphasize the significance of innate mucosal immunity against Cryptosporidium. These studies indicate that establishment of a mucosal response appears to be essential for providing resistance and protection to Cryptosporidium since the parasites life cycle takes place in the gut (Chatfield, 1995).

IFN-γ production against the Chgp15 and Cp23 showed higher production of the cytokine compared to production exhibited by Cpgp15. However, there was no significant difference between asymptomatic and symptomatic patients in all the antigens. A number of studies have shown that the cytokine IFN-γ is important in protection and clearance of infection (Riggs, 2002; Theodos, 1998). In human studies, recovery from cryptosporidiosis has been shown to correlate
with PBMCs producing IFN-γ when stimulated with parasite antigens (Gomez Morales et al., 1999). Severity of chronic cryptosporidiosis in an HIV-negative child was closely associated with a deficiency in IFN-γ production (Gomez Morales et al., 1996).

Some studies have measured the therapeutic effect of IFN-γ treatment on *C. parvum* development. In two of the reported studies which involved mice, no protective effect was observed (Kuhls et al., 1994; McDonald and Bancroft, 1994). Alternatively, administration of the cytokine to immunosuppressed rats was reported to reduce parasite numbers (Rehg, 1996). Also, remission of chronic cryptosporidiosis in a child was observed after treatment with IFN-γ (Gooi, 1994). It is likely that IFN-γ activity is necessary for early control of infection, therefore, but IFN-γ-independent mechanisms of immunity also exist and in mice might act mainly later during a primary infection. The ability of both asymptomatic and symptomatic patients to produce IFN-γ indicates that this cytokine is a potent factor in mounting an immune response towards *Cryptosporidium* parasites. Generally, CD4+ T-cells and IFN-γ are critical in protection from *Cryptosporidium* infections in human. The susceptibility of HIV/AIDS patients to *Cryptosporidium* and the resolution of cryptosporidiosis following the immune restitution and rise in CD4+ T counts underscore the importance of CD4+ T cells (Schmidt et al., 2001). This is in agreement with the finding of our present study that symptomatic persons had lower CD4+ T cells counts.

Elsewhere it has been reported that the patients reporting recovery from HIV/AIDS *Cryptosporidium* associated diarrhea and symptoms were associated with increased CD4+ T counts (Moss et al., 1998). Peripheral blood mononuclear cells (PBMCs), predominantly
CD4⁺TCRαβ⁺ cells from Cryptosporidium infected humans were reported to proliferate and produce IFN-γ in response to crude extract from C. parvum as well as to recombinant and native C. parvum antigens (Gomez-morales et al., 1995). In murine models experiment, IFN-γ is a critical mediator of the innate and acquired immune responses controlling cryptosporidiosis (Leav et al., 2003). Studies have also indicated IL-15 expression to be inversely correlated with parasite clearance and IFN-γ associated with T-cell memory immune response to Cryptosporidium, thus hypothesizing that the separate memory (associated with IFN-γ) and innate (associated with IL-15) immune responses are involved in clearance of human cryptosporidiosis (Panterburg et al., 2008). This could possibly indicate that despite importance of IFN-γ and CD4⁺ T cells in recovery against Cryptosporidium, they do not work in isolation but involve other components like cytokines.

There was a strong association between the cryptosporidiosis associated diarrhea and CD4⁺ T counts with 55% (11 / 13) of the symptomatic patients and 10% (2/3) in asymptomatic persons whose CD4 counts were less than <200 reporting acute diarrhea in the past one month. These details were gathered using the questionnaire. This therefore led to inclusion of asymptomatic persons though they did not have diarrhea during recruitment time but they indicated to have suffered from diarrhea in the past one month. This clearly indicates the predisposition or increased probability of a patient suffering from diarrhea when the CD4 counts falls below 200 Cells/μl. The asymptomatic group had fewer numbers of patients possibly indicating their increased ability in terms of immunity to withstand diarrhea. Association between diarrhea and CD4⁺ count has in the past been documented by many studies. Some studies indicate that
diarrhea is a significant cause of death in immunocompromised persons (Tzipori et al., 2002; Cama et al., 2007).

In humans with late-stage HIV infection and low CD4+ T-cell counts, there was an increased susceptibility to cryptosporidial infection and the severity of disease was greater (Blanshard et al., 1992; Flanigan et al., 1992). After antiretroviral therapy and recovery of the CD4+ T-cell levels, cryptosporidial infection was readily cleared (Schmidt et al., 2001). It is evident, therefore, that CD4+ T-cells are major effector cells in immunity to cryptosporidial infection.

Socio-economic status based on income, property and education showed that only about 8% were from high socio-economic group, 15% were from a middle income group and the majority of the cases 78% came from low socio-economic status group. People of low socio-economic status are at risk of infections due to compromised food and water qualities, poor nutrition, poor access to good medical attention and poor living standards of hygiene. Amongst the studied persons, 28% came rural areas, 60% from slums and 12% from semi rural (peri urban) areas. Rural and urban environments might be expected to differentially facilitate Cryptosporidium transmission. This is because of disparities in exposure to animals, access to safe water and sanitation, and population density (Siobhan and Tzipori, 2008).

There was seasonal pattern in the study with Cryptosporidium being detected at higher rate in the months of June/July and November/December that are characterised with long rains and short rains respectively. It has been shown that Cryptosporidium seasonal infection patterns may results of several factors which could be environmental, parasite related, and host related factors.
(Siobhan and Tzipori, 2008). Storm water run off during rainy seasons increases environmental transport of feaces while wet and humid conditions favor parasite survival. Replenished surface waters also increases transmission especially in children by serving as playing areas and also water holes for animals bringing them to close proximity (Morse et al., 2007). In contrast, studies in Kenya and Rwanda have indicated peak of cryptosporidiosis being in dry season perhaps this may be because of the use of alternative use of unsafe water during these months (Siobhan and Tzipori, 2008).

Water sources for home use can also be a factor in Cryptosporidium transmission and spread. In rural areas the main source of water was reported to be boreholes, rivers and some from taps. In urban areas, most patients were reported to use tap water or buying it from vendors. These sources are potentially capable of transmitting Cryptosporidium bearing in mind its resistant to conventional water treatment such as treatment with chlorine. Poor sanitations also characterises the urban areas especially the slum areas with many people sharing toilets amenities while others having none at all. Poor disposal of human waste and also poorly drained environments form disease reservoirs in densely populated areas. Some patients came from peri urban interface of Nairobi such as Riruta and Dagoretti where they have ended up migrating to urban areas without “moving” due to expansion of city. The peri urban area being originally agricultural land, causes interaction of humans with animals. Animal wastes often used as manure for vegetables and animal products like milk predisposed the people to infection with Cryptosporidium and could be major factors toward zoonotic transmission amongst the residents. This is in contrast to transmission of Cryptosporidium spp in industrialized countries which is mainly associated with the drinking (Sopwith et al., 2005) or recreational water (Yoder and Beach, 2007).
There were some limitations to this study. First, due to financial constraints we were not able to:
- Sequence the samples to establish genetic variability at species level in Cryptosporidium, isolate and identify viruses and look at bacterial pathogens in patients without diarrhea. For the pathogenic parasites, we were not able to perform sensitive test like PCR for confirmation of parasites such as Microsporidia. We did not obtain follow up information regarding diarrhea and the effectiveness of HAART.

In conclusion, this study confirms that Cryptosporidium is an important cause of diarrhea in HIV infected people in Kenya. The study reveals that among those infected with Cryptosporidium some remain asymptomatic despite low CD4+T cell counts suggesting that other factors could contribute to protection from symptomatic disease. We also report that antibody responses and IFN-γ responses to three Cryptosporidium antigens that are vaccine candidates were higher in asymptomatic than symptomatic patients suggesting that they could be protective. Currently improvement of hygiene remains the main method of control from infection.
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

a. *Cryptosporidium hominis* is the most prevalent *Cryptosporidium* species infecting HIV/AIDS patients attending Kenyatta National Hospital.

b. Patients with asymptomatic cryptosporidiosis had higher levels of serum IgG to Chgp15 and Cp23 and fecal IgA to Chgp15 than symptomatic patients suggesting that these antibodies could be protective against diarrhea.

c. The finding that serum IgG and fecal IgA responses to Chgp15 were significantly higher in asymptomatic patients than symptomatic cryptosporidiosis patients is consistent with the finding that most patients were infected with *C. hominis* and that the patients are immunocompetent.

d. No significant difference was observed in IFN-γ levels between patients with asymptomatic or symptomatic cryptosporidiosis, suggesting that IFN-γ mediated cellular responses may not be involved in protection from symptoms but rather support the assertion that it is mainly involved in memory and requires involvement of other cytokines in control of cryptosporidiosis.

e. *Cryptosporidium hominis* is associated with majority of the diarrhea cases in HIV/AIDS adult patients and often low CD4⁺ counts in HIV patients with cryptosporidiosis.
6.2 Recommendations

a. Monitoring and appropriate interventions targeting CD4$^+$ T cells, Cp23 and Chgp15 antigen immune response parameters should be the hallmark of management of HIV/AIDS cryptosporidiosis co-infection to arrest disease progression.

b. Study of the roles of other cytokines especially Th2 cytokines to elucidate their contribution to the protective host response against *Cryptosporidium* in human hosts is recommended.

c. Roles of CD8$^+$ cells in the immune responses against cryptosporidiosis in both asymptomatic and symptomatic HIV infected persons should be studied.
REFERENCES


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APPENDICES

APPENDIX I

Recipe for making 6x gel loading dye

i. Dissolve 25 mg bromophenol blue and 25 mg xylene cyanol FF in 5ml distilled water

ii. Add 3 ml glycerol.

iii. Make up volume to 10 ml with distilled water.

iv. Store at 4°C.

Recipe for making 500μl of 100 bp ladder.

i. 84μl 6x gel loading dye

ii. 50μl 10x PCR buffer

iii. 316 μl sterile water

APPENDIX II

Recipe for making 500 milliliters of 0.5M EDTA buffer.

i. Weigh 93.05g EDTA disodium salt (Fw=372.2)

ii. Dissolve in 400ml of deionized water.

iii. Adjust pH using NaOH to about 8.0

iv. Make up the volume to 500ml
APPENDIX III

Recipe for making 1 litre of 50x TAE buffer

i. Weigh 242g of Tris Base and put it in a 1L beaker,

ii. Add 57.1 ml of glacial acetic acid.

iii. Add 100ml of 0.5M EDTA (pH 8.0)

iv. Add 800 ml of distilled water and stir to dissolve the solids,

v. Adjust the pH to 7.6-7.8 with HCl.

Make up the volume to 1 liter with distilled water.

vi. Store at room temperature.

APPENDIX IV

Recipe for making 1 liter of 10x PBS buffer.

Weigh the following components:

80g of NaCl

2.0g of KCL

14.4g of Na₂HPO₄

2.4g of KH₂PO₄

Dissolve in 800ml of distilled water by stirring
Adjust the final volume to 1000ml.

Store at room temperature.

**APPENDIX V**

Recipe for making 100 ml of Coating buffer (pH 9.6)

i. 0.015M Na₂CO₃ i.e. 0.16g

ii. 0.035M NaHCO₃ i.e. 0.29 g

Dissolve in 70 ml of distilled water by stirring.

Adjust the pH to 9.6 with HCL

Make up the volume to 100ml using distilled water.

Store at 4 °C for a maximum of one week.

**APPENDIX VI**

Recipe for making AP buffer

First prepare the following stock solutions

1M Tris: Dissolve 60.6 g Tris Base in 400 ml of distilled water, adjust pH to 7.5 using concentrated HCl. Make up the volume to 500ml

3M NaCl: 87.8g of NaCl in 500ml distilled water

1M MgCl: 101. 65 g MgCl₃-6H₂O in 500ml distilled water
To make 100 ml of AP buffer, add the following components in a 50 ml centrifuge tube or a bottle

i. 10 ml of 1M Tris (pH 7.5)

ii. 3.3 ml of 3M NaCl

iii. 0.5 ml of 1M MgCl₂

Add distilled water up to 100mls

Mix well and store at 4 °C.

APPENDIX VII

Recipe for making 250 ml of 0.25% BSA-PBS (Blocking buffer)

i. Weigh 0.625g of Bovine Serum Albumin (BSA)

ii. Dissolved in 250 ml of PBS by stirring

iii. Stored at 4 °C.

APPENDIX VIII

Recipe for making 2 liters of Wash Buffer (0.05% Tween-20-PBS)

Add 1 ml of Tween-20 in 2 litres of 1X PBS.

Store at 4 °C and use within 3 days.
APPENDIX IX

Recipe for making Citrate Phosphate Buffer (pH4.6)

First prepare the following stock solutions

0.2M Sodium Phosphate: 2.8 g in 100ml

0.1M Citric Acid: 2.1g in 100ml

To make 100ml of Citrate Phosphate Buffer (pH4.6) mix the flowing components

23.3ml of 0.2M Sodium Phosphate

26.7ml of 0.1M Citric Acid

Make up to 100 ml