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

KANYI HENRY MURIUKI (B.ED SC.)

Reg No I56/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.

KANYI HENRY MURIUKI
Reg No I56/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.

Dr. Michael Gicheru,
Department of Zoological Sciences,
Kenyatta University,

Signature…… Date…….....

Dr. Jane Wanyiri,
Division of Geographic Medicine and Infectious Diseases,
Tufts Medical Center,
Boston, MA, U.S.A.

Signature  Date: April 18th 2012

DEDICATION

This work is dedicated to my entire family.

ACKNOWLEDGMENTS

I extend my deepest appreciation to my supervisors Dr. Michael Gicheru, Chairman, Department of Zoological Sciences, Kenyatta University for his precious guidance, academic advice and his strong support throughout my study period, Dr. Jane Wanyiri of Tufts medical center, Division of Geographic Medicine and Infectious Diseases, Boston, MA, USA for her daily oversight role in the laboratory, facilitation for the funding of the project and daily encouragement in the course of my study. I would further like to acknowledge the enormous input from Prof. Honorine Ward of Tufts Medical Center, Prof. Njeri Wamae of Kenya Methodist University, Mr. Erastus Mulinge and Ms. Tabitha Irungu of Centre of Microbiology Research, KEMRI, Dr. Kimani Gachuhi, Mr. Timothy Kamau and Mr. David Warunge of Centre for Biotechnology Research and Development, KEMRI and Dr Ann Mungai, Mr. Paul Ngugi, Ms. Margaret Okuku, Ms. Zipporah Mworia of Comprehensive Care Centre, Kenyatta National Hospital. I also acknowledge the role played and assistance given by Mr. Sammy Maina of CDC, Kisumu.

I am sincerely grateful to Sr. Carletta Bondi, Principal, St. Theresa Girls Secondary School, Wamba for her unwavering support in difficult moments of my study and the St. Theresa Girls Secondary School community for co-operation and friendship accorded to me during my study. I also extend my heartfelt gratitude to Fogarty International Centre for the financial support awarded to Dr. Jane Wanyiri. Special thanks goes to my mother, Mrs. Susan Njeri, My Grandmother, Mrs. Serah Muthoni and my sisters for their moral support that has enabled me to reach this far. I am also thankful to my friends Mr. Stephen Kuria, Mr. Patrick Kiarie, Mr. Charles Mwangi, Mr. Michael Kahato and my M.Sc. colleague Mr. James Mwangi who supported and encouraged me along the way.

TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGMENTS iv

TABLE OF CONTENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

ACRONYMS AND ABBREVIATIONS x

ABSTRACT xiii

CHAPTER 1: INTRODUCTION 1

1.1 *Cryptosporidium*: General overview and classification 1

1.2 Problem statement 2

1.3 Justification of the study 3

1.4 Research questions 3

1.5 Hypothesis 4

1.6 Objectives 4
1.6.1 General objective 4
1.6.2 Specific objectives 4

1.7 Significance of the study 5

CHAPTER 2: LITERATURE REVIEW 6

2.1 Molecular epidemiology and geographical distribution of *Cryptosporidium* 6

2.2 Lifecycle of *Cryptosporidium* 9

2.3 Pathology and clinical features 11

2.4 Transmission, control and treatment 12

2.5 Immune responses to *Cryptosporidium* 14
2.5.1 Types of immune responses 16

CHAPTER 3: MATERIALS AND METHODS 28

3.1 Study site 28

3.2 Study design 29

3.3 Study population 29

3.4 Recruitment of patients for study 29
3.4.1 Inclusion Criteria 30
3.4.2 Exclusion Criteria 30

3.5 Sample size 31

3.6 Sampling 31
3.6.1 Stool samples 31
3.6.2 Blood samples 39

3.7 $CD4^+$ T cells counts 44

3.8 Ethical clearance 44

3.9 Data analysis 44

CHAPTER 4: RESULTS 45

4.1 General overview 45

4.2 Species of *Cryptosporidium* infecting HIV infected persons attending KNH 45

4.3 Serum IgG, IgM and fecal sIgA levels against *Cryptosporidium* antigens (gp15 and Cp23) in HIV patients infected with *Cryptosporidium* 50

4.4 IFN-γ-mediated cellular immune responses in patients infected with *Cryptosporidium* spp. 54

4.5 Association between diarrhea and CD4 counts in HIV infected patients with Cryptosporidiosis 56

CHAPTER 5: DISCUSSION 58

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS 73

6.1 Conclusions 73

6.2 Recommendations 74

REFERENCES 75

APPENDICES 91

APPENDIX I 91

APPENDIX II 91

APPENDIX III 92

APPENDIX IV 92

APPENDIX V 93

APPENDIX VI 93

APPENDIX VII 94

APPENDIX VIII 94

APPENDIX IX 95

LIST OF TABLES

Table 4.1: General characteristics of the patients studied 45

Table 4.2: Distribution of the species 49

Table 4.3: Summary of CD4 counts and resulting percentage of diarrhea in patients with CD4 count <200 cells/µl 57

LIST OF FIGURES

Figure 2.1: Life cycle of *Cryptosporidium parvum* 10

Figure 4.1: *Cryptosporidium spp* oocysts 46

Figure 4.2: Agarose gel (1.5 %) visualization of *Cryptosporidium* secondary PCR products. 47

Figure 4.3: Gel Images indicating species of *Cryptosporidium*. 48

Figure 4.4: Serum IgG antibodies to gp15 and Cp23 . 51

Figure 4.5: Serum IgM antibodies to gp15 and Cp23. 52

Figure 4.6: Fecal IgA antibodies to gp15 and Cp23. 54

Figure 4.7: IFN-γ production by PBMCs. 55

Figure 4.8: Frequency of $CD4^+$ T cell counts in the symptomatic and asymptomatic patients. 56

ACRONYMS AND ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cells
ART	Antiretroviral Therapy
Bp	Base Pairs
°C	Degree Celsius
CCC	Comprehensive Care Centre
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CMR	Centre for Microbiology Research
Cp23	*Cryptosporidium* antigen 23
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme Linked Immunosorbent Assay
ERC	Ethical Review Committee
ETEC	Enteropathogenic *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
E.U	ELISA Units
FBS	Fetal Bovine Serum
HAART	Highly Active Antiretroviral Therapy

HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
Gp	Glycoprotein
IFN γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IQ	Interquartile
KEMRI	Kenya Medical Research Institute.
Kb	Kilobase
KNH	Kenyatta National Hospital
LPS	Lipopolysacharides
$Mgcl_2$	Magnesium chloride
MHC 1	Major Histocompatibility Complex 1
MHC 2	Major Histocompatibility Complex 2
MRNA	Messenger RNA
NASCOP	National AIDS & STI Control Programme
NK cells	Natural Killer cells
gp 15	Glycoprotein 15
gp 40	Glycoprotein 40
PBMC	Peripheral Blood Mononuclear Cells

PBS	Phosphate Buffered Saline.
PBS-T	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribose Nucleic Acid
Rpm	Revolutions per minute
SCID	Severe Combined Immunodeficiency
sIgA	Secretory IgA
SSU	Small Sub Unit
TCR	T Cells Receptor
Th	T helper cells
µl	Microlitre
UN	United Nations
WHO	World Health Organization

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. 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. meleagridis, 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 s*pp 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 /mm^2) (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 indentified in two patients in Canada (Ong *et al*., 2002), a *C. andersoni*-like parasite indentified in three patients in England (Leoni *et al*., 2006) and a W17 (chipmunk) genotype indentified 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. meleagridis*, *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).

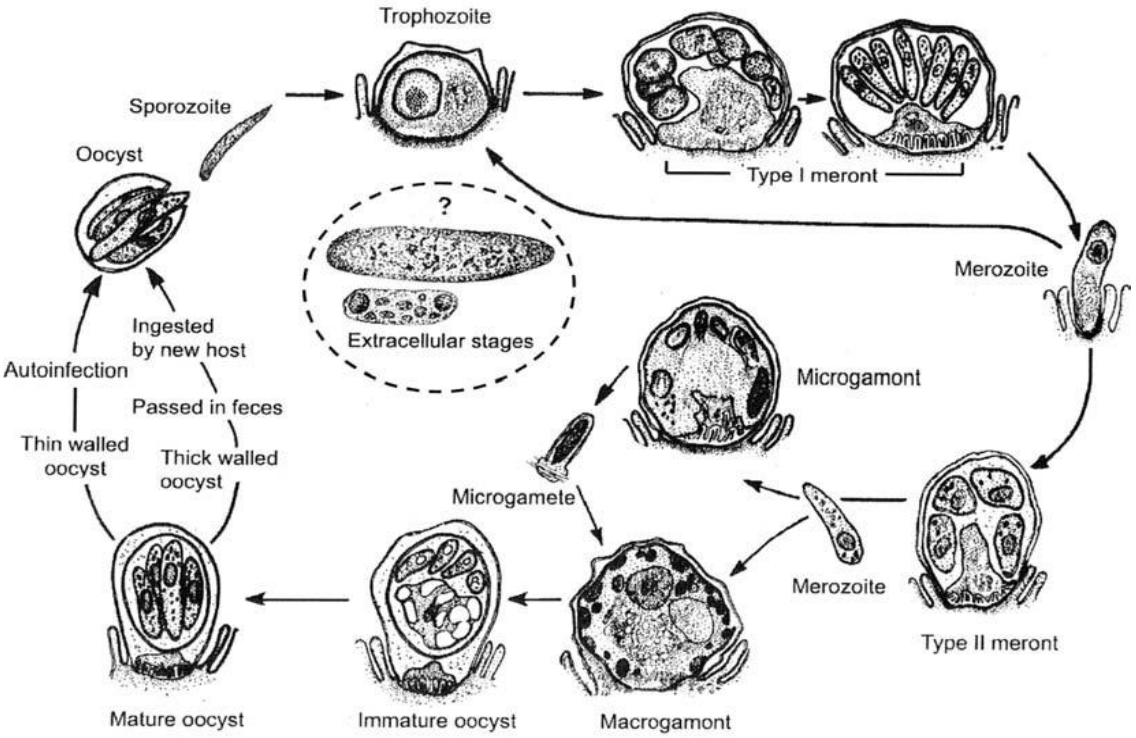


Figure 2.1: Life cycle of *Cryptosporidium parvum* (Adapted from Xiao and Fayer, 2008)

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,

2002). 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 relies 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-γ activity. Treatment of immunocompetent mice or SCID mice reconstituted with splenic T cells, with anti-IFN-γ-neutralizing antibodies increased reproduction of *C. parvum* and *C. muris* (McDonald *et al*., 1992b). Infection with *C. parvum* has been shown to induce IFN-γ 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-γ expression reflects the pattern of acute infection, with the levels of IFN-γ 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-γ by these cells (Harp *et al*., 1994; Tilley *et al*., 1995). Additional evidence of an association between $CD4^+$ T cells, IFN-γ and immunity was suggested by the observation that treatment of mice with anti-$CD4^+$ anti-IFN-γ 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-γ 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-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 (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 $\geq$ 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'-GGAAGGGTTGTATTTATTAGATAAAG-3' and the reverse primer 5'-AAGGAGTAAGGAACAACCTCCA-3'(Xiao *et al*., 2001a).

a. Primary PCR

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

10× PCR buffer	2.5 µl
dNTPs (10 nM)	0.5 µl
Forward 1 (F1) primer (10 mM)	1.0 µl
Reverse 1 (R1) primer (10 mM)	1.0 µl
$MgCl_2$ (25 mM)	2.0 µl
Distilled water	12.6 µl
Taq polymerase	0.4 µl
Template DNA added to each tube.	5.0 µl

The following PCR program was run:

94° for 3 min

35 cycles of: 94ºC 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
$MgCl_2$ (25 mM)	2.0 µl
Distilled water	15.6 µl
Taq polymerase	0.4 µl
Primary PCR product added to each tube.	2.0µl

The following PCR program was run:

94°C for 3 min

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

72ºC for 7 min

4ºC, hold temperature.

5 µl of the secondary PCR product was run on 1.5% agarose gel containing 0.5 ug/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:

Secondary PCR product	10.0 µl
Enzyme (*Ssp*I/*Vsp*I)	2.0 µl
NEB Buffer 2	2.0 µl
PCR water	6.0 µl.

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 l00 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 ($1/10^2$ to $1/10^6$) 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 (H_2O_2) 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 Na_2CO_3, 0.035 M $NaHCO_3$, 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 4°C 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 37°C 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 (H_2O_2) 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 aliquoted 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 °C 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 °C. Patient sera diluted 1:100 in 0.25% BSA/PBS were added to the wells and the plate incubated for one hour at 37°C. 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 37°C. 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_{405nm} of each sample by the A_{405nm} 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 NH_4Cl, 10 mM $KHCO_3$, 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^{O}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 5×10^6 PBMCs/ml in cRPMI at room temperature.

Before lymphocyte proliferation assay, cells were counted and 1×10^6 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% CO_2. 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^{TM} (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_2SO_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^+4 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

Characteristics		**Asymptomatic (n = 20)**	**Symptomatic (n = 20)**
CD^+4 T cells counts:	**Mean**	351.6 (±59.7)[a]	171.4 (±24.4)[a]
	Median CD4 count (IQ range)	304.5 (212.5 - 386.5)[b]	158.0 (60.5 - 257.5)[b]
Gender:	**Male**	10 (50)[c]	11 (55)[c]
	Female	10 (50)[c]	9 (45)[c]
AGE (mean± SD)		38 (±1.6)[d]	36 (± 2.2)[d]

[a]CD^+4 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.

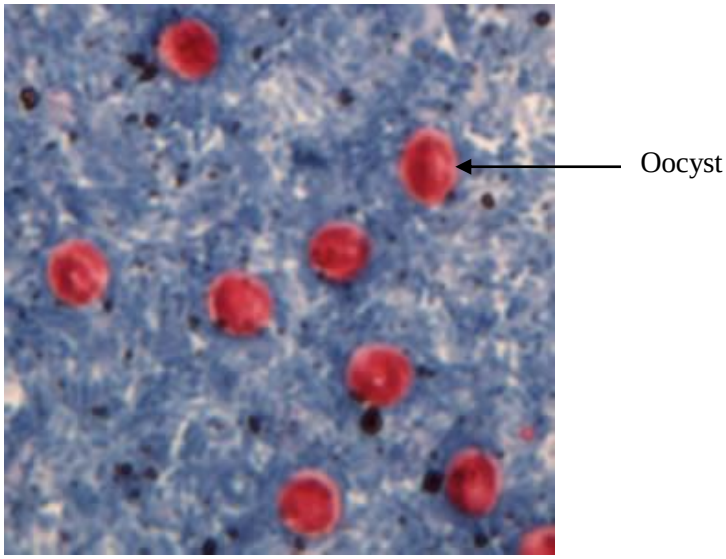


Figure 4.1: *Cryptosporidium spp* oocysts

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.

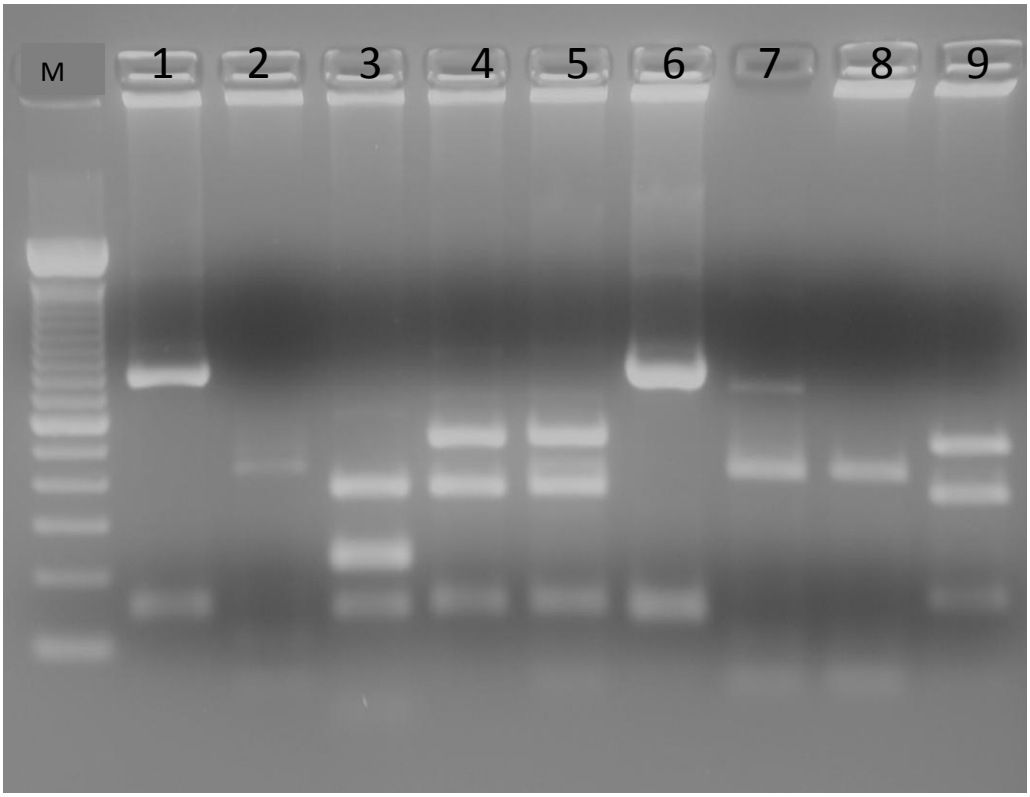


Figure 4.2: Agarose gel (1.5 %) visualization of *Cryptosporidium* secondary PCR products. The gel indicates that samples 1-9 ran against a 100bp Molecular marker (M) are positive for *Cryptosporidium* but cannot be used to identify specific species.

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.

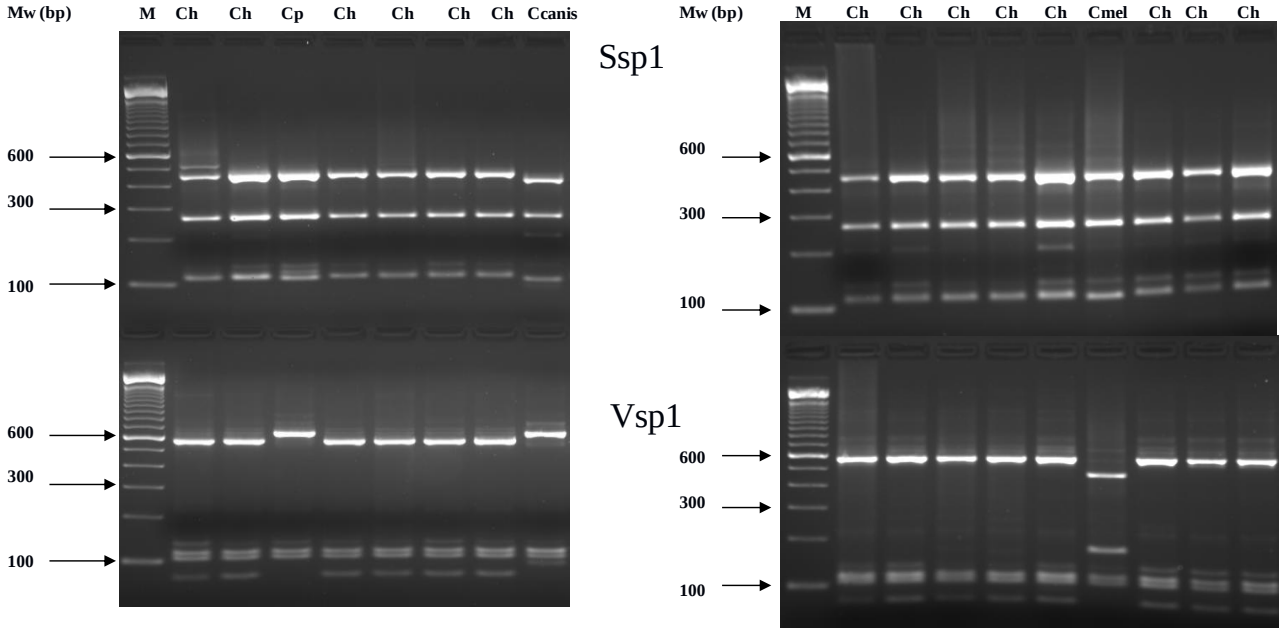


Figure 4.3: Gel Images indicating species of *Cryptosporidium* digested using Ssp 1 and Vsp 1 restriction enzymes. The species are C. *hominis* (Ch) *C. parvum* (Cp), *C. canis*, and *C. meleagridis* (Cmel). Molecular marker (M) is indicated on each gel and the key base pair sizes labeled (100, 300, 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

Species	Asymptomatic n=20 (100%)	Symptomatic n=20(100%)	p-value
C. hominis	9 (45%)	11(55%)	0.701[1]
C. parvum	6 (30%)	4 (20%)	0.656[1]
C. canis	1 (5%)	2 (10%)	0.715[1]
C. meleagridis	3 (5%)	3 (15%)	0.856[1]
C. muris	1 (5%)	0 (0%)	0.513[1]

[1]: Fishers exact test

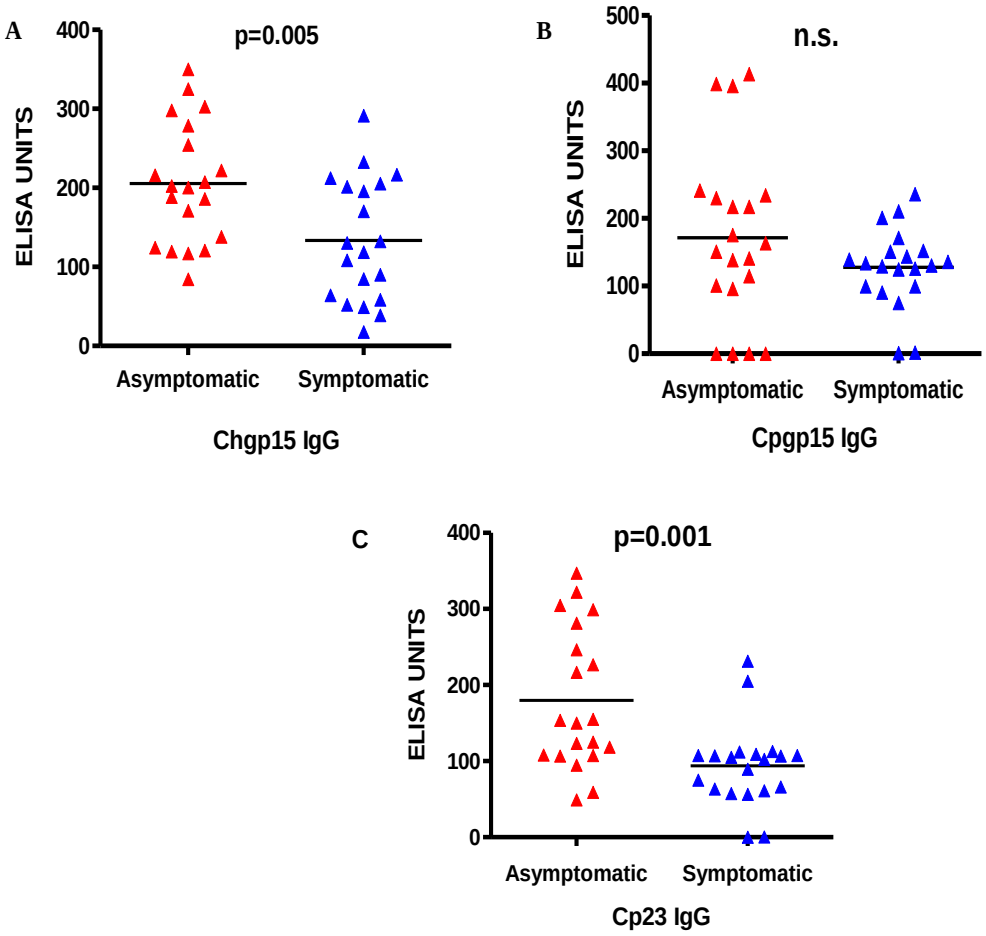
Further, after examination of the stool samples by microscopy for ova and parasites, wide range of parasitic micro-organisms were detected together with *Cryptosporidium.* They included protozoans like *Isosopora belli, Cyclospora spp, Giardia lamblia, Entamoeba histolytica/ dispar, Entamoeba coli, Chilomasttix mensnili, Endolimax nana, Iodomoeba buetschlii*, and *Microsporidia spp*. Helminthes observed included: *Strongyloides stercoralis, Ascaris lumbricoides, Schistosoma mansonii, 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.

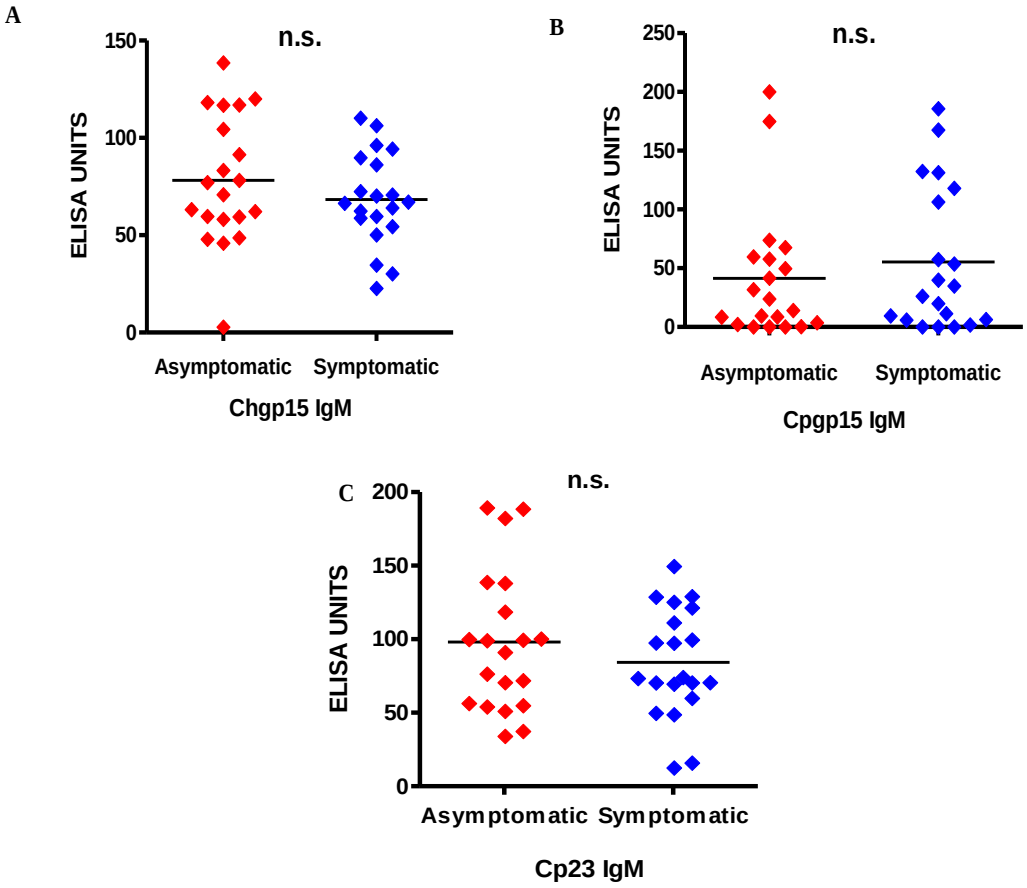


A: Chgp15 IgG, B: Cpgp15 IgG, C: Cp23 IgG

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).



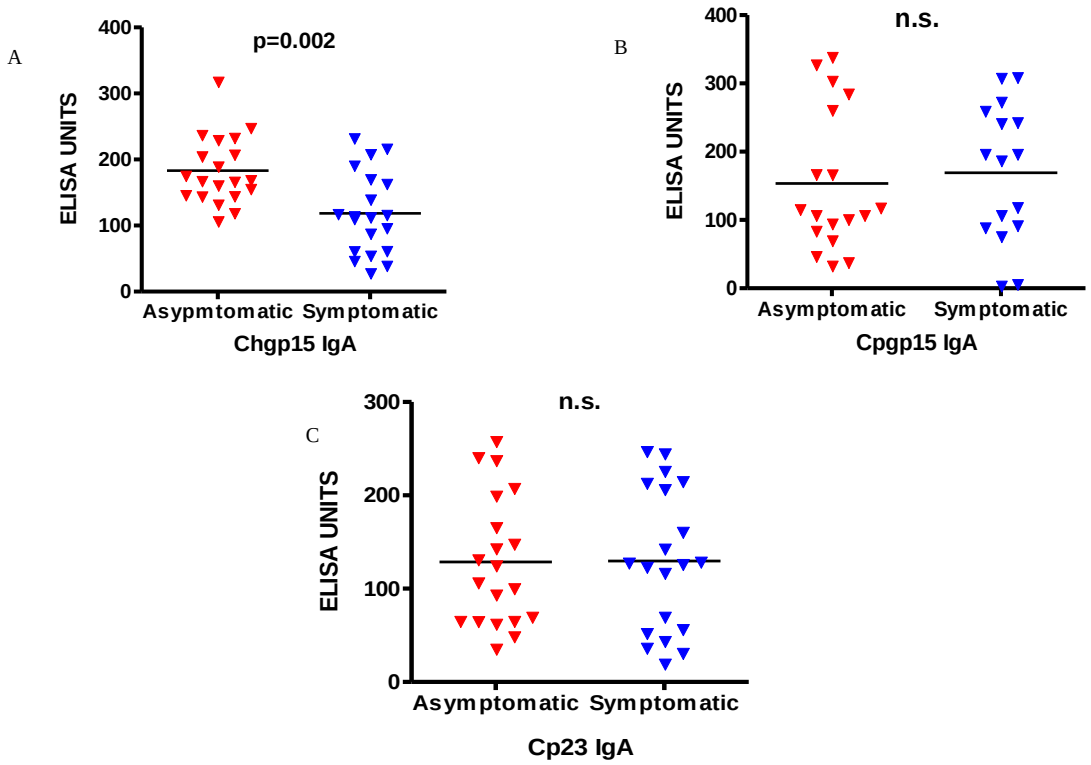
A: Chgp15 IgG, B: Cpgp15 IgG, C: Cp23 IgG

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).



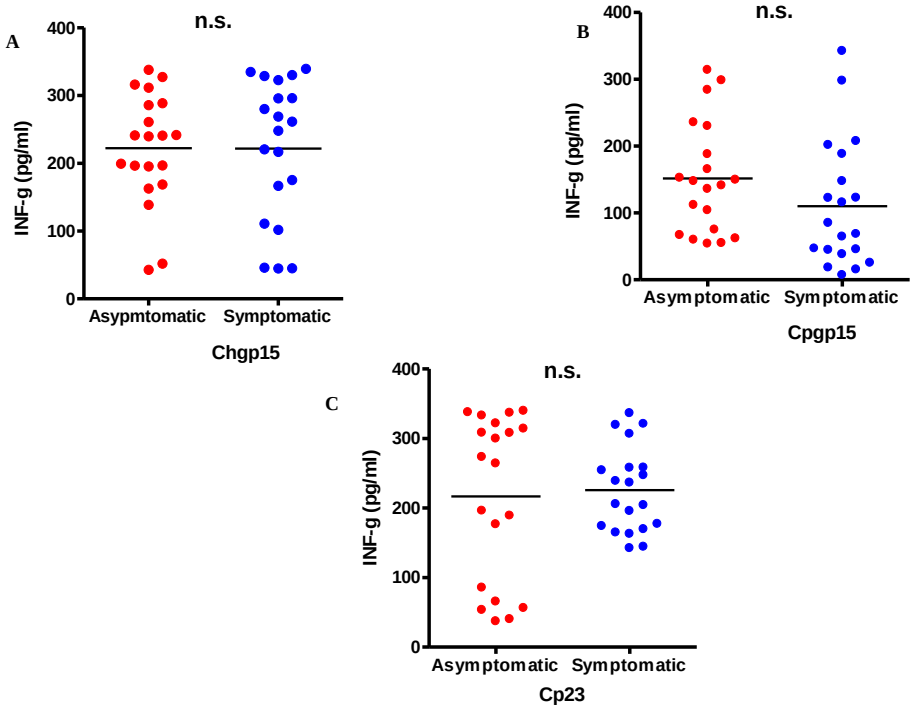
A: Chgp15 IgG, B: Cpgp15 IgG, C: Cp23 IgG

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-γ production to Cp23, was 216.7 ± 26.47 E.U for the asymptomatic patients and 225 ± 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-γ in both symptomatic and asymptomatic patients.



A: Chgp 15 IgG, B: Cpgp 15 IgG, C: Cp23 IgG

Figure 4.7: IFN-γ 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 mm^3. 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 mm^3 with the majority having between 151 and 200 cells per mm^3. Asymptomatic patients $CD4^+$ T cell counts on the other hand had the majority being in the upper categories (>250).

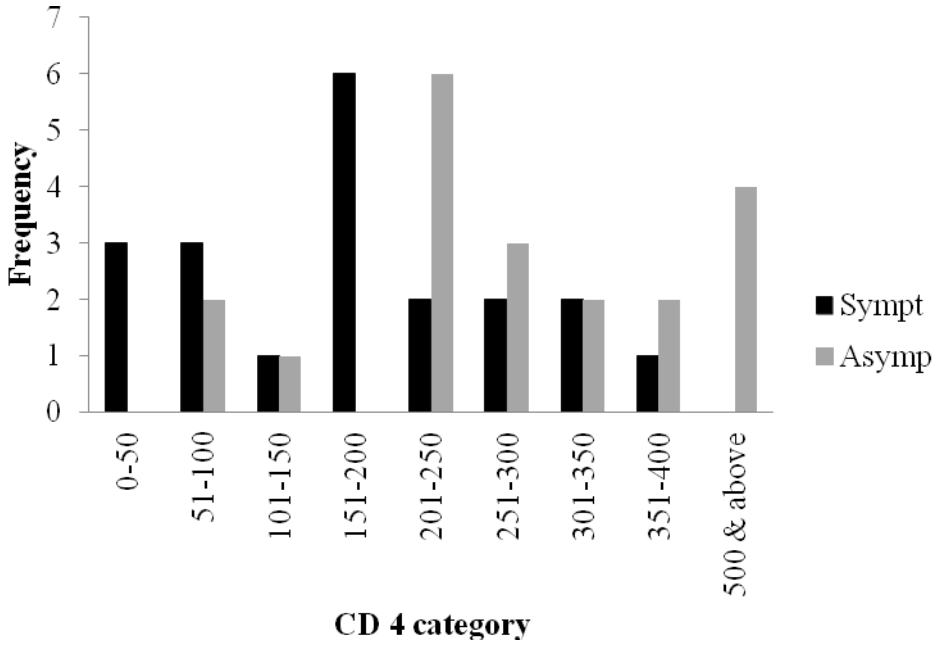


Figure 4.8: Frequency of $CD4^+$ T cell counts in the symptomatic and asymptomatic patients

Table 4.3: Summary of $CD4^+$ T Cells counts and resulting percentage of diarrhea in patients with CD4 count <200 cells/µl

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

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. meleagridis, 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. *meleagridis, 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. *meleagridis, 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* (Ajjampur *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 mansonii,* 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 *Ch*gp15, and Cp23, and fecal IgA levels to *Ch*gp15 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 Cpgp15 (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-*Ch*gp15 IgA ($P < 0.05$) but there was no significant difference in IgA level to either *Cp*gp15 or Cp23 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 sympomatic 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

$CD4^+TCR\alpha\beta^+$ 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 *Cryptosoridium* 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

Abdo, A., Klassen, J., Urbanski, S., Raber, E. and Swain, G. (2003). Reversible sclerosing cholangitis Secondary to Cryptosporidiosis in a renal transplant patient. *Journal of Hepatology* 38: 688–691.

Abubakar, L., Aliyu, H., Arumugam, C., Usaman, N. and Hunter, P. (2007). Treament of cryptosporidiosis in immunocompromised individuals: Systematic review and meta-analysis. *British Journal of Clinical Pharmacology* 63: 387-393.

Aguirre, A., Perryman, E., Davis, C. and McGuire, C. (1998). IL-4 protects adult C57BL/6 mice from prolonged *Cryptosporidium parvum* infection: Analysis of $CD4^{+}\alpha$+IFN-γ^{+} and CD4+α+IL-4+ lymphocytes in gut-associated lymphoid tissue during resolution of infection. *Journal of Immunology* 161: 1891–1900.

Ajjampur, S., Gladstone, P., Selvapandian, D., Muliyil, P., Ward, H. and Kang, G. (2007). Molecular and spatial epidemiology of Cryptosporidiosis in a semi urban community in South India. *Journal of Clinical Microbiology* 45: 915-920.

Ajjampur, S., Rajendran, R. and Banerjee, I. (2008). Closing the diagnostic gap in diarrhoea in Indian children by the application of molecular techniques. *Journal of Medical Microbiology* 57: 239-51.

Ajjampur, S., Sarkar, R., Allison, G., Banda, K., Kane, A., Muliyil, J., Naumova, E., Ward, H. and Kang, G. (2011). Serum IgG Response to Cryptosporidium Immunodominant Antigen gp15 and Polymorphic Antigen gp40 in Children with Cryptosporidiosis in South India. *Clinical Vaccine Immunology* 18: 633-9.

Akiyoshi, E., Dilo, J., Pearson, C., Chapman, S., Tumwine, J. and Tzipori, S. (2003). Characterization of *Cryptosporidium meleagridis* of human origin passaged through different host species. *Infections and immunology* 71: 1828-1832.

Amadi, B., Mwiya, M., Musuku, J., Watuka, A., Sianongo, S., Ayoub, A. and Kelly, P. (2002). Effect of nitazoxanide on morbidity and mortality in Zambian children with Cryptosporidiosis: a randomized controlled trial. *Lancet* 360: 1375-1380.

Batchelor, B., Kimari, J. and Brindle, R. (1996). Microbiology of HIV associated bacteraemia and diarhea in adults from Nairobi, Kenya. *Epidemiology and Infection* 117: 139-144.

Barnes, D., Bonnin, A., Huang, J., Gousset, J., Wu, J., Doyle, J., Dubremetz, J., Ward, H. and Petersen, C. (1998). A novel multi domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. *Molecular Biochemistry in Parasitology* 93-110.

Benhamou, Y., Kapel, N., Huang, H., Matta, D., Meillet, D., Magne, M., Raphael, M., Gentilini, P., Opolon, P. and Gobert, J. (1995). Inefficiacy of intestinal secretory immune responses to *Cryptosporidium* in Acquired Immunodeficiency Syndrome. *Gastroenterology* 108: 627-635.

Bettelli, E., Korn, T. and Oukka, M. (2008). Individuals: Associated risk factors and immune status. *Journal of Infectious Diseases* 60: 76-81.

Blackburn, G., Mazurek, M., Hlavsa, M., Park, J., Tillapaw, M., Parrish, M., Salehi, E., Franks, W., Koch, E., Smith, F., Xiao, L., Arrowood, M., Hill, V., da Silva, A., Johnston, S. and Jones, L. (2006). Cryptosporidiosis associated with ozonated apple cider. *Emerging Infectious Diseases* 12: 684–686.

Blagburn, L. and Current, L. (1983). Accidental infection of a researcher with human *Cryptosporidium*. *Journal of Infectious Diseases* 148: 772-773.

Blanshard, C., Jackson, M., Shanson, C., Francis, N. and Gazzard, G. (1992). Cryptosporidiosis in HIVseropositive patients. *Journal of Medicine* 85: 813–823.

Blewett, D. and Angus, K. (1989). Disinfection and oocysts in Proc. 1st Int. Workshop on *Cryptosporidiosis*, September 7–8, 1988, Edinburgh, 107–115.

Boaventura, S., Santos, S., Cardoso, R., de Andrade, J., Dos Santos, L., Clarêncio, J., Silva, S., Borges, M., Barral-Netto, M., Brodskyn, I. and Barral, A. (2010). Human mucosal leishmaniasis: neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. *European Journal on Immunology* 40 (10): 2830-6.

Borad A. and Ward H. (2011). Human immune responses in Cryptosporidiosis. *Future Microbiology*; 5(3): 507-19.

Bonafonte, M., Smith, L. and Mead, J. (2000). A 23 KDa recombinant antigen of *Cryptosporidium parvum* induces a cellular immune responses on in vitro stimulated spleen and mesentric lymph node cells from infected mice. *Experimental Parasitology* 96: 32-41.

Bull, M., Lee, D., Stucky, J., Chiu, L., Rubin, A. and McElrath, J. (2007). Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *Journal of Immunology Methods* 322**:** 57-69.

Caccio, S., Pinter, E., Fantini, R., Mezzaroma, I. and Pozio, E. (2002). Human infection with *Cryptosporidium felis*, case report and literature review. *Emerging Infectious Diseases* 8: 85 - 86.

Cama, A., Bern, C., Sulaiman, M., Gilman, H., Ticona, E., Vivar, A., Kawai, V., Vargas, D., Zhou, L. and Xiao, L. (2003). *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. *Journal of Eukaryotic Microbiology* 50: Suppl 531–533.

Cama, A., Ross, M., Crawford, S., Kawai, V., Chavez-Valdez, R., Vargas, D., Vivar, A., Ticona, E., Navincopa, M., Williamson, J., Ortega, Y., Gilman, H., Bern, C. and Xiao, L. (2007). Differences in Clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. *Journal of Infectious Diseases* 196: 684-691.

Campbell, D., Stewart, N. and Mead, R. (2002). Susceptibility to *Cryptosporidium parvum* infections in cytokine and chemokine-receptor knockout mice. *Journal of Parasitology* 88: 1014–1016.

Campos, M., Jouzdani, E., Sempoux, C., Buts, P., Reding, R., Otte, B. and Sokal, M. (2000). Sclerosing-cholangitis associated to Cryptosporidiosis in liver-transplanted children. *Europe Journal of Pediatrics* 159: 113–115.

Carcamo, C., Hooton, T. and Weener, H. (2005). Etiologies and manifestations of persistent diarhea in adults with HIV-1 Infection: A case -control study in Lima, Peru. *Journal of Infectious Diseases* 191: 11-19.

Cello, P. and Day, L. (2009). Idiopathic AIDS enteropathy and treatment of gastrointestinal opportunistic pathogens. *Gastroenterology* 136: 1952-65.

Cevallos, A., Zhang, X., Waldor, M., Jaison, S., Zhou, X., Tzipori, S., Neutra, M. and Ward , H. (2000). Molecular Cloning and Expression of a gene encoding *Cryptosporidium parvum* glycoprotein's gp 40 and gp 15. *Infection and Immunity* 68: 4108-4116.

Chalmers, M., Elwin, K., Thomas, L. and Joynson, H. (2002). Infection with unusual types of *Cryptosporidium* is not restricted to immunocompromised patients. *Journal of Infectious Diseases* 185: 270-271.

Chappell, L., Okhuysen, P., Sterling, C., Wang, R., Jabuwoski, W. and Dupont, H., (1999). Infectivity of *Cryptosporidium parvum* in healthy adults with pre existing anti-*C parvum* serum immunoglobulin G. *The American Journal of Tropical Medicine and Hygiene* 60: 157-164.

Chappell, C., Okhuysen, P., Langer-Curry, R., Widmer, G., Akiyoshi, D., Tanriverdi, S. and Tzipori, S. (2006). *Cryptosporidium hominis*, experimental challenge of healthy adults. *American Journal of Tropical Medicine and Hygiene* 75: 851–857.

Chatfield, N., Roberts, M., Dougan, G., Hormaeche, C. and Khan, M. (1995). The development of Oral vaccines against parasites diseases utilizing live attenuated salmonella. *Journal of Parasitology* 110 (Suppl): S17-S24.

Chen, W., Harp, A. and Harmsen, G. (1993). Requirements for CD4+ cells and gamma interferon in resolution of established *Cryptosporidium parvum* infection in mice. *Infections and Immunology* 61: 3928–3932.

Chen, M. and La Russo, F. (2002). Cryptosporidiosis and the pathogenesis of AIDS-cholangiopathy. *Seminar Liver Diseases* 22: 277–289.

Chen, M., O'Hara, P., Nelson, B., Splinter, L., Small, J., Tietz, S. and Limper, H. (2005). Multiple TLRs are expressed in human cholangiocytes and mediate host epithelial defense responses to *Cryptosporidium parvum* via activation of NF-κ B. *Journal of Immunology* 175: 7447–7456.

Chin, S., Harwell, J., Bell, J., Rozycki, G., Ellman, T., Barnett, J., Ward, H., Reinert, S. and Pugatch, D. (2006). Etiology of chronic diarrhea in antiretroviral-naive patients with HIV infection admitted to Norodom Sihanouk Hospital, Phnom Penh, Cambodia. *Clinical and Infectious Diseases* 43: 925-32.

Chou, C., Gudeman, S., O'Rourke, V., Isacescu, R., Detels, G., Williams, R. and Giorgi, V. (1994). Phenotypically defined memory CD4+ cells are not selectively decreased in chronic HIV disease. *Journal Acquired Immune Deficiency Syndrome* 7: 665-75.

Clayton, F., Heller, T. and Kotler, P. (1994). Variation in the enteric distribution of *Cryptosporidium* in Acquired Immunodeficiency Syndrome. *American Journal of Clinical Pathology* 102: 420–425.

Current, L. and Haynes, B. (1984). Complete development of *Cryptosporidium* in cell culture. *Science* 224: 603–605.

Current, L. and Garcia, S. (1991). Cryptosporidiosis. *Clinical Microbiology Review* 4: 325–58

Cozon, G., Biron, F., Jeannin, M., Cannella, D. and Revillard, P. (1994). Secretory IgA antibodies to *Cryptosporidium parvum* in AIDS patients with chronic cryptosporidiosis. *Journal of Infectious Diseases* 169: 696–699.

Dann, M., Okhuysen, C., Salameh, M., Dupont, L. and Chappell, L. (2000). Fecal antibodies to *Cryptosporidium parvum* in healthy volunteers. *Infection and Immunity* 68: 5068-5074.

Davami, H., Bancroft, J. and McDonald, V. (1997). *Cryptosporidium* infection in major histocompatibility complex congeneic stranbins of mice: Variation in susceptibility and the role of T-cell cytokine responses. *Parasitology* 83: 257–263.

De Boer, M., Reijneke, R., Van de Griend, J., Loos, A. and Roos. D. (1981). Large-scale purification and cryopreservation of human monocytes. *Journal of Immunological Methods* 43:225-39.

Deng, M., Lancto, C. and Abrahamsen, S. (2004). *Cryptosporidium parvum* regulation of human epithelial cell gene expression. *International Journal of Parasitology* 34: 73–82.

Dillingham, A., lima, A. and Guerrant, L. (2002). *Cryptosporidium*: Epidemiology and Impact. *Microbes and Infection* 4: 1059-1066.

Dimicoli, S., Bensoussan, D., Latger-Cannard, V., Straczek, J., Antunes, L., Mainard, L., Dao, A., Barbe, F., Araujo, C., Clement, L., Feugier, P., Lecompte, T., Stoltz, J. and Bordigoni, P. (2003). Complete recovery from *Cryptosporidium parvum* infection with gastroenteritis and sclerosing cholangitis after successful bone marrow transplantation in two brothers with X-linked hyper-IgM syndrome. *Bone Marrow Transplantation* 32: 733–737.

Ehigiator, N., McNair, N. and Mead, R. (2007). *Cryptosporidium parvum*: The contribution of Th 1-inducing pathways to the resolution of infection in mice. *Experimental Parasitology* 115: 107-113.

Enriquez, J. and Sterling, R. (1993). Role of $CD4^{+}$ Th1- and Th2-cell-secreted cytokines in Cryptosporidiosis. *Folia Parasitology* 40; 307–311.

Enriquez, J. and Riggs, M. (1998). Role of immunoglobulin A Monoclonal antibodies against P23 in controlling murine *Cryptosporidium parvum* infection. *Infections and Immunology* 66: 4469–4473.

Feltus, C., Giddings, W., Schneck, L., Monson, T., Warshauer, D. and McEvoy, M. (2006). Evidence supporting zoonotic transmission of *Cryptosporidium* in Wisconsin. *Journal of Clinical Microbiology* 44: 4303–4308.

Flanigan, T., Whalen, C., Turner, J., Soave, R., Toerner, J., Havlir, D. and Kotler D. (1992). *Cryptosporidium* infection and CD4 counts. *Annals of International Medicine* 116: 840–842.

Fleta, J., Sánchez-Acedo, C., Clavel, A. and Quilez, J. (1995). Detection of *Cryptosporidium* oocysts in extraintestinal tissues of sheep and pigs. *Veterinary Parasitology* 59: 201–205.

Fort, M., Lesley, R., Davidson, N., Menon, S., Brombacher, F., Leach, M. and Rennick, D. (2001). IL-4 exacerbates disease in Th1 cell transfer model colitis. *Journal of Immunology* 166: 2793–2800.

Frost, J., Gilli, G., Biorci, F., Muller, M., Craun, F. and Calderon, I. (2000). Serological evidence of *Cryptosporidium* infections in Southern Europe. *Journal of Epidemiology* 16: 385-390.

Frost, J., Kunde, R., Muller, B., Craun, F., Katz, M., Hibbard, J. and Calderon, I. (2003). Serologoical responses to *Cryptosporidium* antigens among users of surface and ground water sources. *Epidemiology of Infections* 131: 1131-1138.

Frost, J., Tolestrup, K., Craun, F., Fairley, K., Sinclair, I. and Kunde, R. (2005). Protective immunity associated with a strong serological response to a *Cryptosporidium*-specific antigen group in HIV-infected individuals. *The Journal of Infectious Diseases* 192: 618-621.

Gassama, A, Papa, S., Fatou, F., Camara, P., Hovette, P., Aissatou, G. and Seng, R. (2001). Ordinary and opportunistic enteropathogens associated with diarrhea in Senegalese adults in relation to Human Immunodeficiency Virus serostatus. *Journal of Infectious Diseases* 5: 192-198.

Gatei, W., Ashford, W., Beechings, J., Kamwati, K., Greensill, J. and Hart, A. (2002a). *Cryptosporidium muris* infection in an HIV -infected adult, Kenya. *Emerging Infectious Diseases* 8: 204-206.

Gatei, W., Suputtamongkol, Y., Waywa, D., Ashford, W., Bailey, W., Greensill, J., Beeching, J. and Hart, A. (2002b). Zoonotic species of *Cryptosporidium* are as prevalent as the anthroponotic in HIV infected patients in Thailand. *Annals of Tropical Medicine Parasitology* 96: 797–802.

Gatei, W., Greensill, W., Ashford, E., Cuevas, M., Parry, A., Clunliffe, N., Beeching, J. and Hart, A. (2003). Molecular analysis of the 18S rRNA gene of *Cryptosporidium* parasites from patients with and without Human Immunodeficiency Virus infections living in Kenya, Malawi, Brazil, the United kingdom and Vietnam. *Journal of Clinical Microbiology* 41: 1458-1462.

Gatei, W., Hart, A., Gilman, H., Das, P., V, Cama. and Xiao, L. (2006a). Development of a multilocus sequence typing tool for *Cryptosporidium hominis*. *Journal of Eukaryotic Microbiology 53 supplement* 1: S43-S48.

Gatei, W., Wamae, N., Mbae, C., Waruru, A., Mulinge, E., Waithera, T., Gatika, M., Kamwati, K., Revathi, G. and Hart, A. (2006b). Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. *American Journal of Tropical Medicine and Hygiene* 75: 78–82.

Germani, Y., Minssart, P. and Vohito, M. (1998). Etiologies of acute, persistent, and dysentric diarrheas in adults in Bangui, Central Africa Republic in relation to Human Immunodeficiency Virus serostatus. *American Journal of Tropical Medicine* 59: 1008-1014.

Gomez Morales, A., Ausiello, M., Urban, F. and Pozio, E. (1995). Crude extract and recombinant protein of *Cryptosporidium parvum* oocysts induced proliferation of human peripheral blood mononuclear cells in vitro. *Journal of Infectious Diseases* 172: 211-216.

Gomez Morales, A., Ausiello, M., Guarino, A., Urbani, F., Spagnuolo, I., Pignata, C. and Pozio, E. (1996). Severe, protracted intestinal Cryptosporidiosis associated with interferon gamma deficiency: Pediatric case report. *Clinical and Infectious Diseases* 22: 848–850.

Gomez-Morales, A., La Rosa, G., Ludovisi, A., Onori, M. and Pozio, E. (1999). Cytokine profile induced by *Cryptosporidium* antigen blood mononuclear cells from immunocompetent and immunosuppressed persons with cryptosporidiosis. 179: 967-973.

Gomez-Morales, A., Mele, R., Ludovisi, A., Bruschi, F., Tosini, F., Rigano, R. and Pozio., E. (2004). *Cryptosporidium parvum*-specific CD4 Th1 cells from sensitized donors responding to both fractionated and recombinant antigenic proteins. *Infectious Immunology* 72:1306-10.

Gooi, C. (1994). Gastrointestinal and liver involvement in primary immunodeficiency. *Gastrointestinal and Hepatic Immunology* pp.19: 169–177.

Grewal, S. and Flavell, R. (1998). CD40 and CD 154 in cell-mediated immunity. *Annual Review of Immunology* 16: 111-135.

Guiton, R., Vasseur, V., Charron, S., Arias, T., Van Langendonck, N., Buzoni-Gatel, D., Ryffel, B. and Dimier-Poisson, I. (2011). Interleukin 17 receptor signaling is deleterious during *Toxoplasma gondii* infection in susceptible BL6 mice. *Journal of Infectious Diseases* 202 (3):427-35.

Guyot, K., Follet-Dumoulin, A., Lelievre, E., Sarfati, C., Rabodonirina, M., Nevez, G., Cailliez, C., Camus, D. and Dei-Cas, E. (2001). Molecular characterization of *Cryptosporidium* isolates obtained from humans in France. *Journal Clinical Microbiology* 39: 3472–3480.

Harp, A., Whitmire, M. and Sacco, R. (1994). *In vitro* proliferation and production of gamma interferon by murine CD4+ T cells in response to *Cryptosporidium parvum* antigen. *Journal of Parasitology* 80: 67–72.

Hashmey, R., Smith, H., Cron, S., Graviss, A., Chappell, L. and White, C. Jr. (1997). Cryptosporidiosis in Houston, Texas. A report of 95 cases. *Medicine (Baltimore)* 76: 118–139.

Hayward, R., Chimura, K. and Cosyns, M. (2000). Interferon-gamma is required for innate immunity to *Cryptosporidium parvum* in mice. *Journal Infectious Diseases* 182: 1001–1004.

Haque, R., Mondal, D, and Karim, A. (2009). Prospective case-control study of the association bettween common enteric protozoal parasites and diarrheal in Bangladesh. *Clinical and Infectious Diseases* 48: 1191-7.

Heine, J., Moon, W. and Woodmansee, B. (1984). Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. *Infections and Immunology* 43: 856–859.

Hill, D., Blewett, A., Dawson, M. and Wright, S. (1990). Analysis of the kinetics, isotype and specificity of serum and coproantibody in lambs infected with *Cryptosporidium parvum*. *Research Veterinary Science* 48: 76–81.

Hochrein, H., O'Keeffe, M., Luft, T., Vandenabeele, S., Grumont, J., Maraskovsky, E. and Shortman, K. (2000). Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *Journal of Experimental Medicine* 192: 823–833.

Houpt, R., Bushen, Y., Sam, E., Kohli, A., Asgharrpour, Calfee, P., Guerrant, L., Maro, V., Ole-Nguyaine and Shao, F. (2005). Short report:Asymptomatic *Cryptosporidium hominis* among Human Immunodeficiency Virus-infected patients in Tanzania. *American Journal of Tropical Medicine and Hygiene* 73: 520- 522.

Huang, K., Akiyoshi, E., Feng, X. and Tzipori, S. (2003). Development of patent infection in immunosuppressed C57Bl/6 mice with a single *Cryptosporidium meleagridis* oocyst. *Journal Parasitology* 89: 620–6

Huang, B. and White, C. (2006). An updated review on *Cryptosporidium* and Giardia. *Gastroenterology Clinical Journal of North America* 35: 291-314.

Janoff, N., Mead, S., Mead, R., Echeverria, P., Bodhidatta, L., Bhaibulaya, M., Sterling, R. and Taylor, D. (1990). Endemic *Cryptosporidium* and *Giardia lamblia* infections in a Thai orphanage. *American Journal of Medicine and Hygiene* 43: 248-256.

Kalia, V., Sarkar, S., Gourley, S., Rouse, T. and Ahmed, R. (2006). Differentiation of memory B and T cells. *Current Opinion in Immunology* 18: 255–264.

Kapel, N., Benhamou, Y., Buraud, M., Magne, D., Opolon, P. and Gobert, J. (1996). Kinetics of mucosal ileal gamma-interferon response during cryptosporidiosis in immunocompetent neonatal mice. *Parasitology Resource* 82: 664–667.

Katsumata, T., Hosea, D., Ranuh, G., Uga, S., Yanagi, T. and Kohno, S. (2000). Short report: Possible *Cryptosporidium muris* infection in humans. *American Journal Tropical Medicine and Hygiene* 62: 70–72.

Khader, S., Bell, G., Pearl, J., Fountain, J., Rangel-Moreno, J., Cilley, E., Shen, F., Eaton, M., Gaffen, L., Swain, L., Locksley, M., Haynes, L., Randall, D. and Cooper, M. (2007). IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nature Immunology* 8(4):369-77.

Khan, A., Rogers, A., Karim, M., Ahmed, S., Hibberd, L., Calderwood, B., Ryan, T. and Ward, H. (2004). *Cryptosporidium* among Bangladesh children with diarhea: a prospective, matched, case-control study of clinical features, epidemiology and systemic antibody responses. *American Journal of Tropical Medicine and Hygiene*. 71: 412-419.

Kirkpatrick, B., Daniel, M., Jean, S., Pape, J., Karp, C., Littenberg, B., Fitzgerald, D., Lederman, M., Nataro, J. and Sears, C. (2002). Cryptosporidiosis stimulates an inflammatory intestinal response in malnourished Haitian children. *Journal in Infectious Diseases* **186**: 94-101.

Kirti, K., Khurana, S., Wanchu, A. and Malla, N. (2009). Serum Immunoglobulin A, G and M response to *Cryptosporidium parvum* in Cryptosporidium-HIV co-infected patients. *Journal in Infectious diseases* 9: 179-187.

Kuhls, T., Mosier, D., Abrams, V., Crawford, D. and Greenfield, R. (1994). Inability of interferon gamma and amino guanidine to alter *Cryptosporidium parvum* infection in mice with severe combined immunodeficiency. *Journal of Parasitology* 89: 480–485.

Lean, I., McDonald, S., Bajaj-Elliott, M., Pollok, R., Farthing, J. and McDonald, V. (2003). Interleukin-4 and transforming growth factor beta have opposing regulatory effects on gamma interferon-mediated inhibition of *Cryptosporidium parvum* reproduction. *Infections and Immunology* 71: 4580–4585.

Leav, A., Mackay, M., Anyanwu, A., Cevallos, A., Kindra, G., Rollins, N., Bennish. L., Nelson, R. and Ward, H. (2002). Analysis of sequence diversity at the highly polymorphic Cpgp40/15 locus among *Cryptosporidium* isolates from Human Immunodeficiency Virus infected children in South Africa. *Infectious Diseases* 70: 3881-3890.

Leav, B., Mackay, M. and Ward, H. (2003). Cryptosporidium species: new insights and old challenges. *Clinical Infectious Diseases* 36: 903-908.

Leoni, F., Amar, C., Nichols, G., Pedraza-Diaz, S. and McLauchlin, J. (2006). Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhea in England between 1985 and 2000. *Journal of Medical Microbiology* 55: 703–707.

Lopez-Velez, R., Tarazona, R., Garcia, C., Gomez-Mampaso, E., Guerrero, A., Moreira, V. and Villanueva, R. (1995). Intestinal and extraintestinal cryptosporidiosis in AIDS patients. *Europe Journal of Clinical Microbiology and Infectious Diseases* 14: 677–681.

Lin, Y., Slight, S. and Khader, S. (2010). Th17 cytokines and vaccine-induced immunity. *Seminar on Immunopathology* 32(1): 79-90.

Lumadue, J., Manabe, Y., Moore, R., Belitsos, P., Sears, C. and Clark, D. (1998). A clinicopathologic analysis of AIDS-related cryptosporidiosis. *AIDS* 12: 2459–2466.

Mallon, M., Macleod, A., Wastling, J., Smith, H., Reilly, B. and Tait, A. (2003). Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium parvum*. *Journal Molecular Evolution* 56: 407–417.

McDonald, T. and Spencer, J. (1992a). Cell-mediated immune injury in the intestine. *Clinical Gastroenterology* 21: 367–386.

McDonald, V., Deer, R., Uni, S., Iseki, M. and Bancrofti, G. (1992b). Immune responses to *Cryptopsoridium muris* and *Cryptosporidium parvum* in adult immunocompetent or immunocomprised (nude and SCID) mice. *Infection and Immunology* 60(9): 3325-31.

McDonald, V. and Bancroft, G. (1994). Mechanism of innate and acquired resistance to *Cryptosporidium parvum* infection in SCID mice. *Parasite Immunology* 16: 315-20.

McDonald, A., Mac Kenzie, W., Addiss, D., Gradus, M., Linke, G., Zembrowski, E., Hurd, M., Arrowood, M., Lammie, P. and Priest, J. (2001). *Cryptosporidium parvum*-specific antibody responses among children residing in Milwaukee during the 1993 waterborne outbreak 183: 1373-1379.

McDonald, S., Grady, J., Eliot, M., Notley, J. and Alexander, B. (2004). Protection against the earrly phase of *Cryptosporidium parvum* infection conferred by interleukin-4 induced expression of T-helper 1 cytokines. *Journal of Infectious Diseases* 190: 1019-1025.

McLauchlin, J., Amar, C., Pedraza-Diaz, S. and Nichols, G. (2000). Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom, results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *Journal Clinical Microbiology* 38: 3984–3990.

Mead, J., Arrowood, M., Sidwell, R. and Healey, M. (1991). Chronic *Cryptosporidium parvum* infections in congenitally immunodeficient SCID and nude mice. *Journal Infectious Diseases* 163: 1297–1304.

Meisel, J., Perera, D., Meligro, C. and Rubin, C. (1976). Overwhelming watery diarrhea associated with *Cryptosporidium* in an immunosuppressed patient. *Gastroenterology* 70: 1156–1160.

Mor, S., Tumwine, J., Ndeezi, G., Srinivasan, M., Kaddu-Mulindwa, D., Tzipori, S., Griffiths J. (2010). Respiratory cryptosporidiosis in HIV-seronegative children in Uganda: potential for respiratory transmission. *Clinical Infectious Diseases*. 50(10): 1366-72.

Morgan, U., Xiao, L., Sulaiman, I., Weber, R., Lal, A., Thompson, R. and Deplazes, P. (1999). Which genotypes/species of *Cryptosporidium* are humans susceptible to? *Journal Eukaryotic Microbiology* 46: 42S and 43S.

Morse, T., Nichols, R., Grimason, A., Campbell, B., Tembo., K. and Smith, H. (2007). Incidence of cryptosporidiosis in paediatric patients in Malawi. *Epidemiology and Infections* 135: 1307-1315.

Moss, D., Chappell, C., Okhuysen, P., Dupont, H., Arrowood, M., Hightower, A. and Lammie, P. (1998). The antibody responses to 27-, 17- and 15-kDa Cryptosporidium antigens following experimental infection in humans. *The Journal of Infectious Diseases* 178: 827-833.

Muthusamy, D., Rao, S. and Ramani, S. (2006). Multilocus genotyping of *Cryptosporidium* species isolates from Human Immunodeficiency Virus infected individuals in South Africa. *Journal of Clinical Microbiology* 44(2): 632-634.

Mwachari, C., Batchelor, B., Paul, J., Waiyaki, P. and Gilks, C. (1998). Chronic diarrhoea among HIV-infected adult patients in Nairobi, Kenya. *Journal of Infections* 37(1): 48-53.

Mwachari, C., Meier, S., Muyodi, J., Gatei, W., Waiyaki, P. and Cohen, C. (2003). Chronic diarrhea in HIV-1 infected Adults in Nairobi, Kenya: Evaluation of risk factors and the WHO treatment algorithm. *AIDS* 17: 2124-2126.

Nannini, C., Okhuysen, P., (2002). HIV1 and the gut in the era of highly active antiretroviral therapy. *Current Gastroenterology Report* 4: 392-8.

Navin, T. and Juranek, D. (1984). Cryptosporidiosis: Clinical, epidemiologic and parasitologic review. *Review Infectious Diseases* 6: 313–327.

Nime, F., Burek, J., Page, D., Holscher, M. and Yardley J. (1976). Acute enterocolitis in human being infected with the protozoan *Cryptosporidium*. *Gastroenterology* 70: 592-598.

Noble, A. and Kemeny, D. (1995). Interleukin-4 enhances interferon-gamma synthesis but inhibits development of interferon-gamma-producing cells. *Immunology* 85: 357–363.

O'Connor, M., Shaffie, R., Kang, G. and Ward, H. (2011). Cryptosporidiosis in patients with HIV/AIDS *AIDS*. 25(5): 549-60.

Okhuysen, P., Chappell, C., Crabb, J., Sterling, C. and DuPont, H. (1999). Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. *Journal of Infectious Diseases* 180: 1275–1281.

Ong, C., Eisler, L., Alikhani, A., Fung, V., Tomblin, J., Bowie, R. and Isaac-Renton, L. (2002). Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: First report of human infections with a cervine genotype. *Emerging Infectious Diseases* 8: 263–268.

Ong, C., Priest, J., Copes, R., Khan, M., Fyfe, M., Marion, S., Roberts, J., Lammie, P. and Isaac-Renton, J. (2005). Enzyme immunoassay of *Cryptosporidium* specific immunoglobulin G antibodies to assess longitudinal infection trends in six communities in British columbia, Canada. *American Journal of Tropical Medicine and Hygiene* 73: 288-295.

Panterburg, B., Dann, S.M., Wang, H., Robinson, P., Castellanos-Gonzalez, A., Lewis, D. and White, A. (2008). Immune responses to human *Cryptosporidium* sp infection. *Infection and Immunity* 1: 23-29.

Peeters, J., Villacorta, I., Vanopdenbosch, E., Vandergheynst, D., Naciri, M., Ares Maras, E., and Yvorre, P. (1992). *Cryptosporidium parvum* in calves: Kinetics and immunoblot analysis of specific serum and local antibody responses (Immunoglobulin A [IgA], IgG, and IgM) after natural and experimental infections. *Infections and Immunology* 60: 2309–2316.

Peng, M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I., Glaberman, S., Lal, A. and Xiao, L. (2001). A comparison of *Cryptosporidium* sub genotypes from several geographical regions. *The Journal of Eukaryotic Microbiology Supplement 3*: 28s-31s.

Perryman, L., Kapil, S., Jones, M. and Hunt, E. (1999). Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. *Vaccine* 17: 2142–2149.

Pieniazek, N., Bornay-Llinares, F., Slemenda, S., Da Silva, A., Moura, I., Arrowood, M., Ditrich, O. and Addiss, D. (1999). New *Cryptosporidium* genotype in HIV-infected persons. *Emerging Infectious Diseases* 5: 444-449.

Preidis, G., Wang, H., Lewis, D., Castellanos-Gonzalez, A., Rogers, K., Graviss, E., Ward, H. and White, A. Jr. (2007). Seropositive human subjects produce Interferon Gamma after stimulation with recombinant *Cryptosporidium hominis* gp 15. *American Journal on Tropical Medicine and Hygiene* 77: 583-585.

Priest, J., C. Bern, L. Xiao, J., Roberts, J., Kwon, A., Lescano, W., Checkley, L., Cabrera, D., Moss, M., Arrowood, C., Sterling, R., Gilman and Lammie, P. (2006). Longitudinal analysis of *Cryptosporidium* species-specific immunoglobulin G antibody responses in Peruvian children. *Clinical Vaccine Immunology* 13**:** 123-31.

Putignani, L., Sallicandro, P., Alano, P., Abrahamsen, A., Crisanti, A. and Spano, F. (1999) Chromosome mapping in *Cryptosporidium parvum* and establishmentof a long range restriction map for chromosome VI. *FEMS microbiology letter* 175: 231-238.

Qadri, F., Jonson, G., Begum, Y., Wenneras, C., Albert, M., Salam, **M**. and Svennerholm, A. (1997). Immune response to the mannose-sensitive hemagglutinin in patients with cholera due to Vibrio cholerae O1 and O0139. *Clinical Laboratory Diagnosis and Immunology* 4: 429-34.

Ramanathan, S., de Kozak, Y., Saoudi, A., Goureau, O., Van der Meide, P., Druet, P. and Bellon, B. (1996). Recombinant IL-4 aggravates experimental autoimmune uveoretinitis in rats. *Journal of Immunology* 157: 2209–2215.

Rehg, J. (1996). Effect of Interferon-gamma in experimental *Cryptosporidium parvum* infection. *Journal of Infectious Diseases* 174: 29–232.

Riggs, M., Stone, A., Yount, P., Langer, R., Arrowood, M. and Bentley, D. (1997). Protective Monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. *Journal of Immunology* 158: 1787–1795.

Riggs, M. (2002). Recent advances in Cryptosporidiosis: The immune response. *Microbes and Infection* 4: 1067-1080.

Robinson, P., Okhuysen, P., Chappell, C., Lewis, D., Shahab, I., Lahoti, S. and White, A. Jr. (2001). Expression of IL-15 and IL-4 in IFN-gamma-independent control of experimental human *Cryptosporidium parvum* infection. *Cytokine* 15: 39–46.

Rossi, P., Rivasi, F., Codeluppi, M., Catania, A., Tamburrini, A., Righi, E. and Pozio, E. (1998). Gastric involvement in AIDS associated cryptosporidiosis. *Gut* 43: 476–477.

Samie, A., Bessong, P., Obi, C., Sevilleja, J., Stroup, S., Houpt, E. and Guerrant, R. (2006). *Cryptosporidium* species: Preliminary descriptions of the prevalence and genotype distribution among school children and hospital patients in the venda region, Limpopo province,South Africa. *Experimental Parasitology* 114: 314-322.

Sandhu. S., Priest, W., Lammie, P., Hubbard, A., Colford, J. and Eisenberg, J. (2006). The natural history of antibody responses to *Cryptosporidium* parasites in Men at high risk of HIV Infection. *Journal of Infectious Diseases* 194: 1428-1437.

Sang-Mee .G., Jong-Yi, C., Yung-Oh., shin. and Min S. (2008). Antibody responses to *Cryptosporidium* antigen in HIV positive patients in the Republic of Korea. *Korean Journal of Parasitology* 2: 71-75.

Schmidt, W., Wahnschaffe, U., Schafer, M., Zippel, T., Arvand , M., Meyerhans, A., Riecken, E.O. and Ullrich, R. (2001). Rapid increase of mucosal CD4+T followed by clearance of intestinal Cryptosporidiosis in an AIDS patients receiving highly active antiretroviral therapy. *Gastroenterology* 120: 984-987.

Scriba, T. (2008). Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *Journal of Immunology* 180: 1962-70

Seder, R. and Ahmed, R. (2003). Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nature Immunology* 4: 835–842.

Siobhan, M. and Tzipori, S. (2008). Cryptosporidiosis in children in sub saharan Africa: A lingering challenge. *Clinical Infectious Diseases* 47(7): 915-921.

Smith, L., Priest, J., Lammie, P. and Mead, J. (2001). Human T and B cells immunoreactivity to a recombinant 23-kDa *Cryptosporidium parvum* antigen. *Journal of parasitology* 87: 704-707.

Soave, R., Danner, R., Honig, C., Ma, P., Hart, C., Nash, T. and Roberts, R. (1984). Cryptosporidiosis in homosexual men. *Annals of International Medicine* 100: 504–511.

Soave, R. (1990). Treatment strategies for Cryptosporidiosis. *Ann. N. Y. Acad. Sci*. 616, 442–451.

Sopwith, W., Osborn, K., Chalmers, R. and Regan, M. (2005). The epidemiology of Cryptosporidiosis in North England. *Epidemiology and Infections* 133: 785-793.

Sturbaum, G., Jost, H. and Sterling, R. (2003). Nucleotide changes within three Cryptosporidium parvum surface protein encoding genes differentiate genotype I from genotype II isolates. *Molecular Biochemistry and Parasitology* 128: 87-90.

Stark, D., Barratt, J., van Hal, S., Marriott, D., Harkness, J. and Ellis, J. (2009). Clinical significance of enteric protozoa in the immunosuppressed human population. *Clinical Microbiology Review* 4: 634-50.

Theodos, C. (1998). Innate and cell-mediated immune responses to *Cryptosporidium parvum*. *Advanced Parasitology* 40: 87-119.

Tilley, M. and Upton, S. (1991). Sporozoites and merozoites of *Cryptosporidium parvum* share a common epitope recognized by a Monoclonal antibody and two-dimensional electrophoresis. *Journal on Protozoology* 38: 48S–49S.

Tilley, M., McDonald, V. and Bancroft, G. (1995). Resolution of cryptosporidial infection in mice correlates with parasite-specific lymphocyte proliferation associated with both Th1 and Th2 cytokine secretion. *Parasite Immunology* 17: 459–464.

Trotz-Williams, L., Martin, D., Gatei, W., Cama, V., Peregrine, A., Martin, S., Nydam, D., Jamieson, F. and Xiao, L. (2006). Genotype and subtype analyses of *Cryptosporidium* isolates from dairy calves and humans in Ontario. *Parasitology* 99: 346–352.

Tumwine, J., Kekitiinwa, A., Bakeera-Kitaka, S., Ndeezi, G., Downing, X., Akiyoshi, D. and Tzipori, S. (2005). Cryptopsoridiosis and microsporidiosis in Uganda children with and without concurrent infection with human immunodeficiency virus. *American Journal of Tropical Medicine and Hygiene* 73: 921-925.

Tzipori, S., Angus, K., Campbell, I. and Gray, E. (1980). *Cryptosporidium* evidence for a single-species genus. *Infections and Immunology* 30: 884–886.

Tzipori, S. and Ward, H. (2002). Cryptopotidiosis: Biology, pathenogenesis and disease. *Microbes in Infection* 4: 1047-1058.

UNAIDS, 2009. AIDS Epidemic Update: November 2009, Geneva :Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) 2009.

Ungar, B., Soave, R., Fayer, R. and Nash, T. (1986). Enzyme immunoassay detection of immunoglobulin M and G antibodies to *Cryptosporidium* in immunocompetent and immunocompromised persons. *Journal of Infectious Diseases* 153: 570-578.

Ungar, B., Burris, J., Quinn, C. and Finkelman, F. (1990). New mouse models for chronic *Cryptosporidium* infection in immunodeficient hosts. *Infections and Immunology* 58: 961–969.

Ungar, B., Kao, T., Burris, J. and Finkelman, F. (1991). *Cryptosporidium* infection in an adult mouse model. Independent roles for IFN-gamma and CD4+ T lymphocytes in protective immunity. *Journal of Immunology* 147: 1014–1022.

Urban, J. Jr., Fayer, R., Chen, S., Gause, W., Gately, M. and Finkelman, F. (1996). IL-12 protects Immunocompetent and immunodeficient neonatal mice against infection with *Cryptosporidium parvum*. *Journal of Immunology* 156: 263–268.

Wanke, C., Cohan, D. and Thummmakul, T. (1999). Diarrheal diseases in patients infected with Human Immunodeficiency Virus in Bangkok, Thailand. *American Journal of Tropical Medicine* 60: 871-4.

Wanyiri, J. and Ward, H. (2006). Molecular basis of *Cryptosporidium*-host cell interactions: Recent advances and future prospects. *Future Microbiology (Review)* 1(2): 201-208.

Wanyiri, J., O'Connor, R., Allison, G., Kim, K., Kane, A., Qiu, J., Plaut, A. and Ward, H. (2007). Proteolytic processing often *Cryptosporidium* glycoprotein gp40/15 by human furin and by a parasite-derived furin-like protese activity. *Infection and Immunity* 75: 184-192.

Weaver, C., Harrington, L., Mangan, P., Gavrieli, M. and Murphy, K. (2006). Th17: An effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24: 677–688.

Weber, R., Ledergerber, B. and Zhinden, R. (1999). Enteric infections and diarrhea in Human Immunodeficiency Virus-infected persons. *Archives of International Medicine* 159: 1473-1480.

White, A., Robinson, P., Okhuysen, P., Lewis, D., Shahab, I., Lahoti, S., DuPont, H. and Chappell, C. (2000). Interferon-gamma expression in jejunal biopsies in experimental human cryptosporidiosis correlates with prior sensitization and control of oocyst excretion. *Journal of Infectious Diseases* 181**:** 701-9.

White, A. Jr. (2005). Cryptosporidiosis (*Cryptosporidium hominis* and *Cryptosporidium parvum*, other species). *Principles and Practice of Infectious Diseases*. Philadelphia: Elsevier Churchill Livingstone 24: 3215-3228.

Wyatt, C., Brackett, E. and Savidge, J. (2001). Evidence for the emergence of a type-1-like immune response in intestinal mucosa of calves recovering from cryptosporidiosis. *Journal of Parasitology* 87: 90–95.

Wynn, T. (2003). IL-13 effector functions. *Annual Review of Immunology* 21: 425–456.

Xiao, L., Morgan, U., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R., Fayer, R. and Lal, A. (1999). Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Applied Environmental Microbiology* 65: 3386–3391.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R. and Lal, A. (2001). Identification of 5 genotypes of Cryptosporidium parasites in children in lima peru. *Journal of Infectious Diseases* 183: 492-497.

Xiao, L., Bern, C., Arrowood, M., Sulaiman, I., Zhou, L., Kawai, V., Vivar, A., Lal, A. and Gilman, R. (2002). Identification of the *Cryptosporidium* pig genotype in a human patient. *Journal of Infectious Diseases* 185: 1846–1848.

Xiao, L. and Ryan, U. (2004a). Cryptosporidiosis: an update in molecular epidemiology. *Current Opinion in Infections Diseases* 17: 483-490.

(Xiao, L. and Ryan, U. (2008). Molecular eipdemiology. In: Ronald Fayer LX, editor. *Cryptosporidium* and cryptosporidiosis. second ed. Boca Raton, FL: CRC Press. p. 119-72.

Xiao, L., Fayer, R., Ryan, U. and Upton, S. (2004b). Cryptosporidium taxonomy :recent advances and implications for public health. *Clinical Microbiology Review* 17: 72-97.

Xiao, L. and Fayer, R. (2008). Molecular characterisation of species and genotypes of Cryptosporidium and Giardia and assessment of zoonotic transmission. *International Journal on Parasitology* 38: 1239–1255.

Yoder, J. and Beach, M. (2007). Cryptosporidiosis surveillance-United States, 2003-2005. *surveillance summary* 56: 1-10.

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 Na_2HPO_4

2.4g of KH_2PO_4

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 Na_2CO_3 i.e. 0.16g

ii. 0.035M $NaHCO_3$ 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 $^{\circ}$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 $MgCl_2$.

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