EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPECIES AMONG CHILDREN AND HIV INFECTED INDIVIDUALS IN THE NORTH RIFT REGION OF KENYA

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(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2010

Epidemiology and Molecular Characterization of *Cryptosporidium* Species among Children and HIV Infected Individuals in the North Rift Region of Kenya

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A thesis submitted in partial fulfillment for the degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology.

2010

DECLARATION

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

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DEDICATION

This work is first and foremost dedicated to my loving wife, Mary Chebet for her unreserved support and motivation in all aspects throughout the study period, my dear parents, children, friends and my beloved late uncle Johnson Gwaro Omobe. All this would not have been possible without the boundless grace form the Almighty God.

ACKNOWLEDGEMENTS

This study was conducted at the Kenya Medical Research Institute (KEMRI) in the Centre for Microbiology Research (CMR) and Centre for Biotechnology Research and Development (CBRD), Nairobi under the Institute of Tropical Medicine and Infectious Diseases (ITROMID) Graduate Program of Jomo Kenyatta University of Agriculture and Technology (JKUAT), and at The Moi Teaching and Referral Hospital (MTRH) and Moi University in Eldoret, Kenya.

My sincere and major acknowledgements go to my supervisors Dr. Wangeci Gatei, Dr. Solomon Mpoke and Dr. Gerald Mkoji for their valued guidance and advice throughout the time of this study.

I also recognize the contributions of Prof. H.S. Nyandieka, Prof. F. Esamai, Dr. S. Mining, Dr. J.O. Maina, Dr. A.M. Kwena, Mr. H.N.D. Nyamogoba and the Director of Academic Model of Prevention and Treatment of HIV/AIDS (AMPATH), and the staff at MTRH, Turbo and Burnt Forest AMPATH centres.

Special thanks to Dr. Njeri Wamae, Director, CMR, KEMRI and her staff especially, Dr. Willie Sang, Erastus Mulinge, Ms. Cecilia Mbae, Tabitha Irungu, Jonathan Bett, Bonventure Juma, Njeri Kariuki, Joyce Nyambura and Josephine Maingi for their assistance towards the completion of this work. Others that deserve mention are Lelo E. Agola of CBRD, KEMRI, the Coordinator and the staff of ITROMID at KEMRI.

Finally, thanks without measure go to my wife Mary Chebet and our children Donnah Nyaboke, Brittany Nyaboe and Borter Gwaro Nyamwange for bearing with my absence from home and, to my loving parents, siblings and the entire Omobe family for their support.

TABLE OF CONTENTS

CHAPTER 2:

LITERATURE REVIEW

CHAPTER 3:

MATERIALS AND METHODS

CHAPTER 4:

RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AE	Acetate EDTA buffer
AIDS	Acquired immunodeficiency syndrome
AMPATH	Academic Model for Prevention and Treatment of HIV/AIDS
ASL	Stool Lysis Buffer
BSA	Bovine Serum Albumin
CBRD	Centre for Biotechnology Research and Development
CDC	Centre for Disease Control
CMR	Centre for Microbiology Research
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dNTPs	deoxy nucleoside triphosphates
dsRNA	double stranded Ribonucleic acid
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic acid
EHNRI	Ethiopian Health and Nutrition Research Institute
EMBL	European Molecular Biology Laboratory
HIV	Human immunodeficiency virus
HSP	Heat Shock Protein
ICZN	International Code of Zoological Nomenclature
ILRI	International Livestock Research Institute
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology

KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
MTRH	Moi teaching and referral hospital
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PE	Wash Buffer
PPM	Parts per million
QG	Solubilization and Binding Buffer
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal Ribonucleic acid
SSU rRNA	Small Subunit ribosomal Ribonucleic acid
TBE	Tris borate EDTA buffer
UV	Ultraviolet
ZN	Ziehl-Neelsen

ABSTRACT

This prospective study on C*ryptosporidium* and cryptosporidiosis was carried out in the North Rift region of Kenya. *Cryptosporidium* parasites are leading causes of enteric disease, more so in children and HIV infected individuals. A total of 317 fecal samples from children less than five years of age, and 1794 fecal samples from HIV infected persons seen at Moi referral and Teaching Hospital, Eldoret and at two health centres in the North Rift region of Kenya were collected over a period of one year covering January to December 2005. This study area was chosen because there are no reports on the burden of cryptosporidiosis in this region and therefore the results this study highlight this burden and contribute towards the general picture of cryptosporidiosis in Kenya. Overall prevalence of cryptosporidiosis in children was 9.8% and among HIV infected individuals it was 3.2%. The highest prevalence was observed between March and April, and a smaller peak between June and July. Both periods corresponded to the relatively dry seasons in the North Rift region of Kenya.

The rate of diarrhea in the HIV positive individuals was 35.1% with a cryptosporidiosis prevalence of 6.5%. Cryptosporidiosis was significantly associated with diarrhea (OR= 4.7087, $P \leq 0.002$), skin rash among HIV positive individuals (OR= 2.2145, P=0.0033) and headache (OR= 1.8087, P= 0.0279). However, other symptoms such as abdominal pains, cough, fever and vomiting were not significantly associated with cryptosporidiosis, in both the pediatric and HIV positive study groups. In the pediatric group all subjects were diarrheic and duration of diarrhea of more than two weeks was more likely to be associated with the presence of

cryptosporidiosis (OR= 1.8301) when compared to those with diarrhea for less than one week.

There were no significant sex related differences observed in the cryptosporidiosis prevalence in the children (P= 0.9752) or in the HIV positive persons (P= 0.5029). Similarly, environmental factors such as location of residence, waste disposal, water sources and treatment, presence of animals in homesteads or household size were not significantly associated with cryptosporidiosis.

Genotype analysis based on polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of the 18S rRNA gene fragment revealed that among the children about 82% of the isolates were *C. hominis* and nearly 18% were *C. parvum*. Among the HIV infected persons, on the other hand, about 68% of the isolates were *C. hominis*, about 14% *C. meleagridis*, and 18% were *C. parvum*. Based on the trinucleotide sequence analysis there were 12 subtypes of *C. hominis* and 7 subtypes of *C. parvum* in circulation in the North Rift region of Kenya.

The results suggest that cryptosporidiosis prevalence is comparable to other regions of the world with *C. hominis* being the most common species circulating study area followed by *C. parvum* and *C. meleagridis* in that order. Mixed *C. hominis* and *C. parvum* infections also occurred, and constituted about 1% of the infections. These results suggest that human-to-human transmission is the main mode of spread of cryptosporidiosis in the region.

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. *Cryptosporidium* and Cryptosporidiosis

Cryptosporidium parvum is an apicomplexan protozoan parasite that causes gastrointestinal illness known as cryptosporidiosis in a wide variety of mammals, including humans, cattle, sheep, goats, pigs, and horses. It is also known to infect a wide range of birds, reptiles and fish (Xiao *et al*., 2004, Fayer e*t al.*, 2000). The disease is characterized by watery diarrhea, and while it can resolve without intervention in immunocompetent individuals, it has increasingly become a major public health problem as an opportunistic infection in immunocompromised individuals especially in HIV/AIDs (Hunter and Nichols, 2002). Its transmission and outbreaks have been associated with both drinking and recreational water, farm animals, interpersonal interaction and environmental transmission.

1.1.1 The Taxonomy of *Cryptosporidium*

The taxonomy of *Cryptosporidium*, like the taxonomy of most organisms, remains controversial (Xiao *et al*., 2004). However, the "current" taxonomic position of the genus *Cryptosporidium* is as shown in Table 1.1 (Fayer 2007).

Table 1.1: The current taxonomic classification of *Cryptosporidium*.

Phylum	Apicomplexa
Class	Conoidasida
Subclass	Coccidiasina
Order	Eucoccidiorida
Suborder	Eimeriorina
Family	Cryptosporidiidae
Genus	*Cryptosporidium*

The genome of *C. parvum* consists of 8 chromosomes with few introns, 2 double stranded ribonucleic acid (dsRNA) strands of viral origin, and a total of about 10.4 mega base pairs of DNA (Blunt, *et al*., 1997). Based on morphometric studies of oocysts, genetic characterizations, demonstration of natural and/or some experimental host specificity, and in compliance with International Code of Zoological Nomenclature (ICZN), the currently considered valid species of *Cryptosporidium* species as reviewed by Xiao *et al.* (2004) include *C. andersoni* (cattle), *C. baileyi* (chicken and some other birds), *C. canis* (dogs), *C. felis* (cats), *C. galli* (birds), *C. hominis* (humans), *C. meleagridis* (birds and humans), *C. molnari* (fish), *C. muris* (rodents and some other mammals), *C. parvum* (ruminants and humans), *C. wrairi* (guinea pigs), *C. saurophilum* and *C. serpentis* (snakes and lizards).

Worth noting is that what Tyzzer (1907) described as *C. parvum* in mice is in fact a distinct species whose genotype is biologically and genetically distinct. The mouse genotype has since been named *C. muris*. According to Fayer *et al.*, (2000) and Xiao *et al.* (2004), there are many other cryptic *Cryptosporidium* species that have been

isolated from mammals, all of which were previously assumed to be *C. parvum.* However, their genetic distances are greater or comparable to those among established intestinal *Cryptosporidium* species. Most of the species have co-evolved with the host and therefore have been adapting to the host with time to the extent that genetically related hosts often harbor related species of *Cryptosporidium* (Xiao *et al*. 2004). There are probably many *Cryptosporidium* species that are yet to be named as evidenced by the existence of a wide range of oocysts in surface water of which a few are pathogenic to humans (Xiao *et al*., 2004).

Lack of genetic information on *Cryptosporidium* led to confusion in the taxonomy and accurate identification of *Cryptosporidium* species. However, the development of molecular tools for the identification and characterization of *Cryptosporidium* species, has led to a better understanding of the biology and epidemiology of this important organism (Xiao *et al*., 1998). Indeed phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene has shown that the genus *Cryptosporidium* comprises several distinct species (Xiao *et al*., 1999**)**, and that within each species studied, DNA sequence variations of up to six regions of the SSU rRNA gene have been observed. It is not surprising that the various genotypes of *C. parvum* exhibit considerable genetic polymorphism.

1.1.2. **Life cycle of *Cryptosporidium***

The life cycle of *Cryptosporidium* species begins with ingestion of the sporulated oocysts, the resistant stage found in the environment. The oocyst(s) excyst within the

lumen of the host's small intestines to release four sporozoites. These penetrate the microvilli border and develop into trophozoites, and subsequently into schizonts (meront stage), which reproduce asexually with three nuclear divisions to release type I merozoites. The type I merozoites then invade nearby cells and develop into schizonts or trophozoites to complete the reproductive cycle. Type I merozoites are capable of recycling indefinitely and, thus, the potential exists for new Type I meronts to arise continuously within the host.

However, Type I merozoites can sometimes be triggered into forming a second type of meront (Type II meront), which contains only 4 merozoites. Once liberated, the type II merozoites constitute the initial stages of the sexual reproductive cycle. The Type II merozoites enter cells, where some enlarge and differentiate into macrogametes (macrogametocyte), while others undergo multiple fission inside the cells to form microgametocytes that contain 16 non-flagellated microgametes. The microgametocytes rupture to release microgametes, which then penetrate macrogametes, thus forming a zygote on which a resistant oocyst wall is formed. Meiosis then occurs in the zygote within the oocyst to form four sporozoites. Some of these oocysts are thin walled and can rapture and autoinfect the host while the thick walled ones are passed out in the feces into the environment. Figure 1.1 bellow illustrates the life cycle of *Cryptosporidium* species.

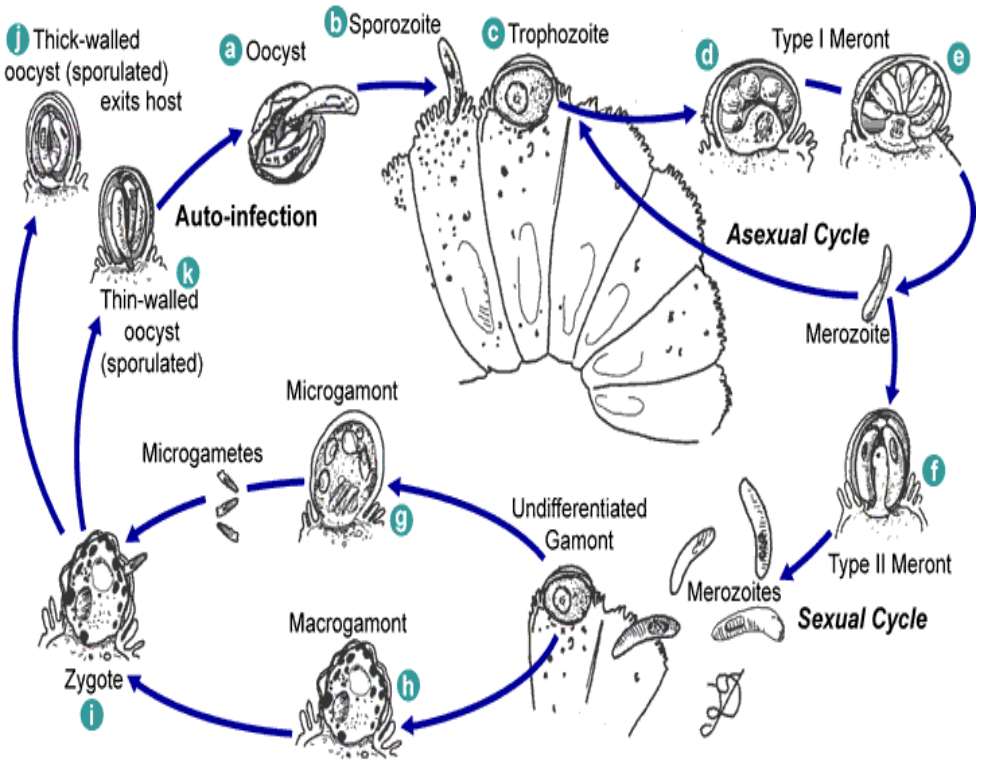

Figure 1.1: The life cycle of *Cryptosporidium* courtesy htttp://www.dpd.cdc.gov/ DPDX/HTML/Frames/A-/Cryptosporidiosis/body_Cryptosporidiosis_life_cycle_lrg.html

1.1.3 **Historical background of cryptosporidiosis**

Cryptosporidium was first identified early in the 20th century as the major cause of diarrhea in mice **(**Tyzer, 1907). The first case of human cryptosporidiosis was discovered in 1976 (Nime *et al*., 1976), and by 1980, *Cryptosporidium* was recognized as a common and debilitating pathogen in patients with HIV/AIDS. The parasite is also a leading cause of childhood diarrhea in developing countries (Bern *et al.*, 2000, Bhattacharya *et al*., 1997, Miron *et al*., 2000, Newman *et al*., 1999); and also, one of the causes of traveler's diarrhea in visitors to developing countries

(Jelinek *et al.*, 1997). *Cryptosporidium* has been linked with water-borne diarrhea outbreaks in developed countries (Glaberman *et al.*, 2002, MacKenzie *et al.*, 1994).

Cryptosporidium parvum occur widely in both source and drinking water and is responsible for water-borne outbreaks of gastroenteritis (Simmons *et al.*, 2001). In developing countries, it is associated with poor sanitation and crowded living conditions and proximity to animals (Miron *et al.*, 1991). Transmission may be through person-to-person, animal-to-person or could be environmental, primarily through water. Caputo *et al.* (1999) reported that sexual activities are significant risk factors for *Cryptosporidium* infection.

Cryptosporidium species possess a unique set of characteristics, which make the organisms such a common and problematic threat to public health. For example, *Cryptosporidium* species are highly infective such that ingestion of as few as nine oocysts may lead to an active infection (Okhuysen *et al.*, 1999). The infection can rapidly spread among children in day care centers (Heijbel *et al.*, 1987, Tangermann *et al.*, 1991), in the elderly, and in hospital staff and patients (Koch *et al.*, 1985). In addition, *Cryptosporidium* oocysts are highly resistant to water treatment protocols by virtue of their size, nature and composition of the oocyst wall (Jenkins *et al.*, 1997). Because of their small size (4–6 microns), the oocysts can easily pass through most conventional filters, including reverse osmosis filters which are considered absolute filters (Frost *et al.*, 2000). Furthermore, the oocysts are highly resistant to chlorination even after exposure to 80 ppm of chlorine for 30 minutes (100 times the

accepted dose for the elimination of coliform bacteria). There is no known effective therapeutic cure for cryptosporidiosis (Stockdale *et al*., 2007).

In acquired immune deficiency syndrome (AIDS) individuals, chronic profuse watery diarrhea, weight loss, and abdominal cramping characterize the illness attributed to *Cryptosporidium* (CDC, 1982). The diarrhea can last up to four months, or until the patient's death. The chronicity of diarrhea is attributed to the ability of the organism to autoinfect hosts especially in the absence of a competent immune system. In these patients, the parasites may be present in the stomach, biliary and pancreatic ducts (Nimish *et al.*, 1996), and respiratory tract (Palmieri *et al*., 2005). *Cryptosporidium* has been found on biopsy or autopsy of immunocompromised patients from the alimentary canal, the hepatobiliary system, and the respiratory tree, whereas in healthy individuals, it is usually limited to the intestines (Ungar, 1990). The prepatent period, which is the interval between exposure to the parasite and the first appearance of oocysts in feces, is generally 3-4 days (Current and Haynes, 1984). Patency, which is the length of time oocysts are shed in the feces, generally lasts 6-18 days in immunocompetent individuals, but may be prolonged in immunosuppressed patients (Dupont *et al*., 1995).

1.2 **Statement of the Problem**

Cryptosporidiosis is a worldwide problem but very little is known about cryptosporidiosis in the North Rift region of Kenya. Therefore, vulnerable populations such as children aged five years and below, and HIV/AIDS infected individuals, their living conditions, access to sanitation facilities, need to be

documented for this region. Occurrence of *Cryptosporidium* is not commonly investigated in routine examination of stool samples in cases of diarrhea and yet it is one of the major opportunistic infections in HIV/AIDS individuals. There is therefore need to document epidemiological situation of cryptosporidiosis in the region.

1.3 **Justification of the Study**

Cryptosporidium has been demonstrated to be an endemic infection in childhood diarrhea in studies in some parts of Kenya and other parts of the world. The endemicity and prevalence of this disease in the North Rift needs to be established, as there is no documentation of the same in this region. Furthermore, the disease is known to be zoonotic and inhabitants of this region extensively practice a wide range of animal husbandry, which could be associated with transmission, and spread of cryptosporidiosis.

HIV/AIDS is now a major health problem in Kenya as evidenced by the fact that up to 50% of the beds in the medical wards in major public hospitals are occupied by HIV/AIDS patients, largely due to opportunistic infections. Among these could be *Cryptosporidium* species which to date have no effective therapeutic cure. HIV/AIDS individuals are most likely to be infected by one or more *Cryptosporidium* species due to their immunocompromised status and hence the possibility of obtaining more isolates for the purposes of this study. *Cryptosporidium* infections are also most likely to occur in children under five years of age, with a

peak occurrence in those under two years of age because their immune system is not fully developed.

It is therefore necessary to establish the epidemiology and magnitude of cryptosporidiosis in HIV/AIDS infected individuals and children under five years of age in order to inform future management and control this disease not only in HIV/AIDS individuals and children under five years of age but in the general population too. It is also important to document the genetic variants of *Cryptosporidium* species circulating in the North Rift region of Kenya and establish how they compare with those from other regions.

1.4 **OBJECTIVES**

1.4.1 **Overall Objective**

To establish the magnitude of cryptosporidiosis as a public health problem in the North Rift region of Kenya and characterize *Cryptosporidium* isolates from humans and domestic animals in the regions.

1.4.2 **Specific Objectives**

1. To determine the prevalence of *Cryptosporidium* infections and associated factors among HIV/AIDS patients, and children under five years of age with diarrhea seen at Moi Teaching and Referral Hospital in Eldoret.

2. To determine the prevalence of *Cryptosporidium* infections in domestic animals from homes of selected *Cryptosporidium* positive patients and establish the relevance of these animals as sources of human infections.

3. To identify and characterize *Cryptosporidium* isolates from humans and domestic animals using molecular approaches, and establish phylogenetic relationships among the parasite isolates.

CHAPTER 2:

LITERATURE REVIEW

2.1 Public Health Significance and Epidemiology of Cryptosporidiosis

Cryptosporidium infections cause gastrointestinal illness characterized by diarrhea as the most common clinical feature in both immunocompetent and immunocompromised persons (Current *et al*., 1991). Apart from occurring in humans, cryptosporidiosis illness is also common in a wide variety of domesticated animals, including, cattle, sheep, goats, pigs, horses, dogs, cats and poultry (Xiao *et al.*, 2004). Cryptosporidiosis also occurs in various wildlife species such as deer, raccoons, opossums, rabbits, rats, mice, and squirrels (Fayer *et al.*, 2000, Xiao *et al*., 2004). In cattle, clinical disease and shedding of the parasite oocysts is usually limited to calves under a few months of age (Atwill, 1995). In humans, clinical disease and shedding of oocysts appears to occur in all ages, but is most common among children and immunocompromised individuals (Atwill, 1995). Shedding of oocysts can last for 3-12 days in calves and range from 9-50 days in humans, and most likely end up, and remain infective in surface water, treatment plants and water distribution systems from where they can be a source of infection (Atwill, 1995).

Once shed the oocysts become non-infectious to animals and humans if the fecal material dries thoroughly before reaching a water body. However, if fecal material is directly deposited in a water body, by the 33rd day, an estimated 60-66% of the oocysts still survive and are capable of infecting and causing disease but after 176 days, 89-99% of the oocysts are incapable of causing disease (Robertson *et al.*, 1992).

Cryptosporidiosis is a major public health problem in both developing and developed countries. It is recognized as an emerging water- and food-borne disease that exerts considerable impact on pediatric health (Tumwine *et al.*, 2005, Gatei *et al.*, 2006, Ayalew *et al*., 2008) and as an opportunistic infection in HIV infected individuals (Ramakrishnan *et al*., 2007, Ribeiro *et al*., 2004, Lim *et al*., 2005). A study in Lima, Peru found that children with cryptosporidiosis experienced growth faltering, both in weight and in height for several months after the onset of infection, suggesting that cryptosporidiosis may have adverse effects on child growth, especially if the infection is acquired during infancy (Checkley *et al*., 1998). Cryptosporidiosis has also been associated with impaired physical fitness and cognitive function in children (Guerrant *et al*., 1999). Similarly, in developed countries water-borne outbreaks have had a significant economic impact. For example, the Milwaukee outbreak in 1993 in USA cost 96.2 million US dollars in medical costs and productivity loses (Corso *et al.*, 2003) and, it is estimated that the United Kingdom spends 23 million sterling pounds per year to meet legal requirements for removal of *Cryptosporidium* from drinking water (Pretty *et al*., 2000).

Recreational water facilities, which attract millions of person events annually, have been reported as sources of outbreaks that affect tens of thousands of individuals (Fayer *et al*., 2000). The diarrhea sickness among children and staff due to cryptosporidiosis in day care centers has been reported in some states of the United States of America (CDC, 1984) and in South Africa (Walters *et al*., 1988). Since these centers can act as focal points for further transmission of *Cryptosporidium*,

public health workers, parents, and day-care providers need to be aware of the potential of *Cryptosporidium* as cause of diarrhea outbreaks.

In acquired immune deficiency syndrome (AIDS) individuals, the illness attributed to *Cryptosporidium* is characterized by chronic profuse watery diarrhea that may last for at least 4 months or continue until the patient's death, weight loss, and abdominal cramping (CDC, 1982).

In 2002, the Center for Disease Control and Prevention (CDC) in a recommendation to the U.S. Public Health Service and the Infectious Diseases Society of America, advised HIV-infected persons to avoid contact with human and animal feces, not to drink water directly from lakes or rivers, that the stool of all pets and animals should be examined for *Cryptosporidium* before they have contact with them, and that they should avoid exposure to calves and lambs, and premises where these animals are raised (CDC, 2002). This was in recognition that *Cryptosporidium* is one of the major opportunistic infections in HIV/AIDS, and that HIV infected individuals are susceptible to a wide range of *Cryptosporidium* species (Gatei *et al*., 2003 & 2002).

A study by Xiao *et al*. (2001) in Lima, Peru showed that cryptosporidiosis was a common endemic childhood infection, and that novel species and genotypes of *C. parvum* were found to infect HIV negative children. In addition to infection with *C. hominis* and *C. parvum*, some children in the Peru study were also infected with *C. meleagridis, C. felis*, and the *C. parvum* dog genotype. They also found that for the zoonotic *Cryptosporidium* parasites, 28-33% of infections were associated with

diarrhea. This is a pointer to the fact that infections hitherto thought to be confined to specific non-human hosts, can now be transmitted to humans.

Genotypes analysis studies have shown that of *C. parvum* genotype 1 (*C. hominis*) is the strain responsible for most human *Cryptosporidium* infections globally, though type II (*C. parvum*) is the most common in humans in the United Kingdom (Ryan-Morgan *et al*., 2002). In contrast, genotype 2 is probably one of the major causes of environmental contamination, and has been found in most oysters examined from Chesapeake Bay, USA, that serve as biologic monitors of estuarine waters (Xiao *et al.*, 1998).

It is therefore evident from the foregoing that diarrhea is one of the major symptoms associated with cryptosporidiosis in both immunocompetent and immunocompromised individuals and children aged five years and below.

2.2 **Pathological Manifestations of Cryptosporidiosis**

The histopathological features in the intestines due to *Cryptosporidium* infection may include atrophy, blunting, fusion or loss of villi, crypt hyperplasia and lengthening that may be accompanied by inflammatory changes such as infiltration of lamina propria with lymphocytes, neutrophils, plasma cells and macrophages (Ungar, 1990). Though these damages can occur throughout the intestine, they are usually most severe in the distal jejunum and ileum (Laurent *et al*., 1999). The pathogenesis of *Cryptosporidium* induced diarrhea is not well understood, but it appears the accompanying diarrhea is reminiscent of cholera-like toxin-mediated process that

leads to hyper secretion of fluids and electrolytes (Guarino *et al*., 1994), increased mucosal prostaglandin production (Argenzio *et al.*, 1994), alterations in intestinal permeability due to increased interferon gamma production (Colgan *et al.*, 1994) and damage to the villi which may cause malabsorption diarrhea with impaired digestion and change in osmotic flux (Current *et al*., 1991). Therefore, the diarrhea in cryptosporidiosis may be caused by a combination of any or all of the conditions discussed above.

Extra intestinal cryptosporidiosis has been demonstrated as evidenced by the presence of *Cryptosporidium* oocysts in bile and sputum of HIV infected individuals in a study by López-Vélez *et al*. (1995) in Spain. In another study by Meamar *et al*. (2006), oocysts of zoonotic *Cryptosporidium parvum* were detected in the sputum and stool samples of an AIDS patient with a 3-month history of intestinal cryptosporidiosis. Palmieri *et al*. (2005) reported pulmonary cryptosporidiosis, which apparently did not have intestinal involvement in an HIV infected heterosexual man. These cases illustrate the potential and ability of this parasite to establish itself in sites that are not its primary point of infestation.

2.3 **Cryptosporidiosis as a Zoonotic Disease**

Cryptosporidium species occur in both humans and animals, and therefore the zoonotic nature of cryptosporidiosis is of great significance from the public health viewpoint. At least 16 *Cryptosporidium* species are currently considered valid species (Fayer, 2007), and of these, 6 are known to infect both humans and animals (Xiao *et al.*, 2004). For example, *C. parvum*, the most common species infecting

humans also infects cattle, sheep, goats, mice and pigs; *C. muris* infects rodents, humans, camels, mountain goats and rock hyrax; *C. hominis* infects both humans and cattle; *C. felis* occurs in cats, cattle and humans; *C. canis* is a parasite of dogs and humans; and *C. meleagridis* parasitizes turkeys, humans and parrots (Xiao *et al*., 2004). All these species have the potential to infect humans through contaminated food, recreational water, drinking water or direct ingestion of infective oocysts (Fayer *et al*., 2000), and more importantly, in immunocompromised individuals and children under five years of age who are especially more vulnerable (Newman *et al*., 1999, Bhatacharya et *al*., 1997, Ribeiro *et al*., 2004).

Pieniazek *et al*. (1999) using DNA sequencing and phylogenetic analysis identified four distinct *Cryptosporidium* genotypes in HIV-infected patients: *C. parvum* genotype 1 (human), *C. parvum* genotype 2 (bovine), *C. parvum*, genotype identical to *C. felis*, and a genotype identical to a *Cryptosporidium sp.* isolate from a dog. This was the first identification of a concurrent human infection with a *C. parvum*, genotype identical to *C. felis*, and the *Cryptosporidium* species isolated from a dog. Similarly, analyses of the 18S ribosomal DNA, heat shock protein-70 (HSP-70), and acetyl coenzyme A synthetase genes of *Cryptosporidium* isolates from HIV-infected individuals from Kenya, Switzerland, and the United States by Morgan *et al*. (2000) led to the identification of four distinct *Cryptosporidium* genotypes designated as *C. parvum* "human" genotype, *C. parvum* "cattle" genotype, *C. felis*, and *C. meleagridis* from the investigated individuals. More interesting, for the first time, *C. meleagridis* was reported from a human host. In addition, *C. canis* and *C. felis* have been found in

HIV positive children in Peru (Xiao *et al*., 2001), and *C. muris* was found in an HIV positive patient in Kenya (Gatei *et al.*, 2002, 2003).

A study by Pedraza-Diaz *et al*. (2001) in the United Kingdom to characterize *Cryptosporidium* associated with human infections using the the18S rDNA gene sequence analysis showed that four out of the five patients investigated were infected with *C. felis*, and that one of these patients was in addition, infected with a yet to be identified *Cryptosporidium* species currently designated the 'dog type'. This was the first report of a dual infection with *Cryptosporidium* species in an immunocompetent individual in the United Kingdom.

It has been established that contrary to earlier beliefs that only bovine and human genotypes of *C. parvum* are potential pathogens of humans, it is now emerging that symptomatic infections with *C. felis, C. meleagridis*, and *C. parvum*-like dog genotype may occur in both immunocompetent and immunosuppressed individuals (Chacin-Bonilla, 2001). In fact, it is now believed that in some regions *C. meleagridis*, a parasite of poultry is responsible for more human infections than was previously thought (Gatei *et al.*, 2002). The expansion of *C. parvum* into humans in some parts of the world may be due, in part, to the intensive husbandry practiced for ruminants and the associated concentration of young animals especially at feeding operations (Xiao *et al.*, 2004). Oocysts shed from humans have been shown to be infectious to calves, lambs, goats, cats and dogs while oocysts from cats, calves and pigs appear to be infectious to humans (Atwill, 1995).

Overall, the observations summarized above suggest that immunocompromised individuals may be susceptible to infection with a wide range of *Cryptosporidium* species and subtypes. There is therefore a need to fully understand the public health implications of *Cryptosporidium* infections of animal origin, and especially, their significance in immunocompromised individuals. Furthermore, animals may serve as a major source of environmental and water contamination through fecal wastes. Other factors that may be associated with cryptosporidiosis include water sources and treatment, sanitary facilities, location of areas of residence and socioeconomic factors.

2.4 **Cryptosporidiosis in Kenya**

There are limited studies on the epidemiology of cryptosporidiosis in Kenya, and virtually nothing is known about the occurrence of the disease in the North Rift region of Kenya. Simwa *et al.* (1989) observed that 3.8% of the 1420 fecal samples examined from children of five years of age and below presenting with diarrhea in Kiambu district in central Kenya, were positive for *Cryptosporidium* oocysts. In a separate study, Estambale *et al.* (1989) observed that 3.8% of 133 stool samples sent for routine microscopic examination at the Kenyatta National Hospital (KNH), Nairobi, Kenya had *Cryptosporidium* oocysts, and that both immunocompromised and immunocompetent individuals were infected. More interesting, Mirza *et al.* (1994) observed that 42% (23/45) of the HIV/AIDS patients seen at the Kenyatta National Hospital, Nairobi had *Cryptosporidium* infections whereas, only 8.6% (2/23) of the HIV negative individuals examined during the same period were positive for the parasite. It appears *Cryptosporidium* is a common infection in

humans in Kenya, and may be an important opportunistic infection in HIV-infected individuals.

Although it is believed that *C. parvum* is commonly present in Kenya, other species such as *C. felis, C. meleagridis* and *C. muris* (Morgan *et al.*, 2000, Gatei *et al.*, 2003) are known to occur as well. In a study in which stool samples were sent for routine laboratory microscopic examination from Narok District Hospital, Thika District Hospital, Machakos Provincial General Hospital, Gertrude's Gardens Children Hospital, Aga Khan Hospital, and Kenyatta National Referral Hospital, the overall prevalence of *Cryptosporidium* infection was established to be 4% (183/4899) (Gatei *et al*., 2006). The prevalence for individual laboratories studied was 7% (7/99) in Narok District Hospital, 4% (45/1,118) in Thika District Hospital, 4% (69/648) in Machakos Provincial General Hospital, 4% (30/690) in Kenyatta National Hospital, 3.5% (17/480) in Aga Khan Hospital, and 2% (15/863) in Gertrude's Gardens Children Hospital. This study further reported that 46% of those children with cryptosporidiosis were living in peri-urban areas, 14.2% in urban centres, and 40% in rural areas.

Majority of the *Cryptosporidium* isolates identified in this study using restriction endonuclease digestion of the PCR product were *C. hominis* (87%), C. *parvum* (9%) and *C. canis* (4%). Others were *C. felis* (2 isolates), *C. muris* (1 isolate), and *C. meleagridis* (1 isolate). It is worth noting that the highest prevalence was reported from Narok district hospital where clients come from the vast Narok district known for its livestock farming and the Maasai Mara game reserve.

From the foregoing discussion it is evident that most of the information available on cryptosporidiosis in Kenya is based on a few field-based or hospital-based studies undertaken in or around Nairobi. The present study was undertaken to establish the burden of cryptosporidiosis in the North Rift region of Kenya among children aged 5 years and less, and in HIV infected patients attending selected health facilities in the region. This region also has livestock rearing as a major activity.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study Design

This study was a prospective cross-sectional descriptive survey that was health facility and laboratory based.

3.2 Study Setting

The study was carried out in Moi Teaching and Referral Hospital (MTRH), Eldoret, and at the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. Parasitological investigations were done in the Microbiology laboratory in the School of Medicine, Moi University while the molecular analysis was carried out at the Centre for Microbiology Research (CMR) and Centre for Biotechnology Research and Development (CBRD), KEMRI, Nairobi and International Livestock Research Institute (ILRI), Nairobi, Kenya.

3.3. Study Area

The study was carried out in the North Rift region of Kenya (Figure 3.1). The communities that live in this part of Kenya are known for rearing cattle, sheep, goats, dogs, cats and poultry. All these animals are potential reservoirs of *Cryptosporidium* infections that can be transmitted to humans. The water sources in this region include wells, springs, rivers, bore holes, rain and piped water, which may easily be contaminated by fecal matter from both humans and domestic animals. Patient recruitment was done at Moi Teaching and Referral Hospital (MTRH), Turbo and Burnt Forest health centers. Samples were also collected from domestic animals in

homesteads of *Cryptosporidium* positive patients living within 10 Km radius from the health centers and 30 Km radius from MTRH.

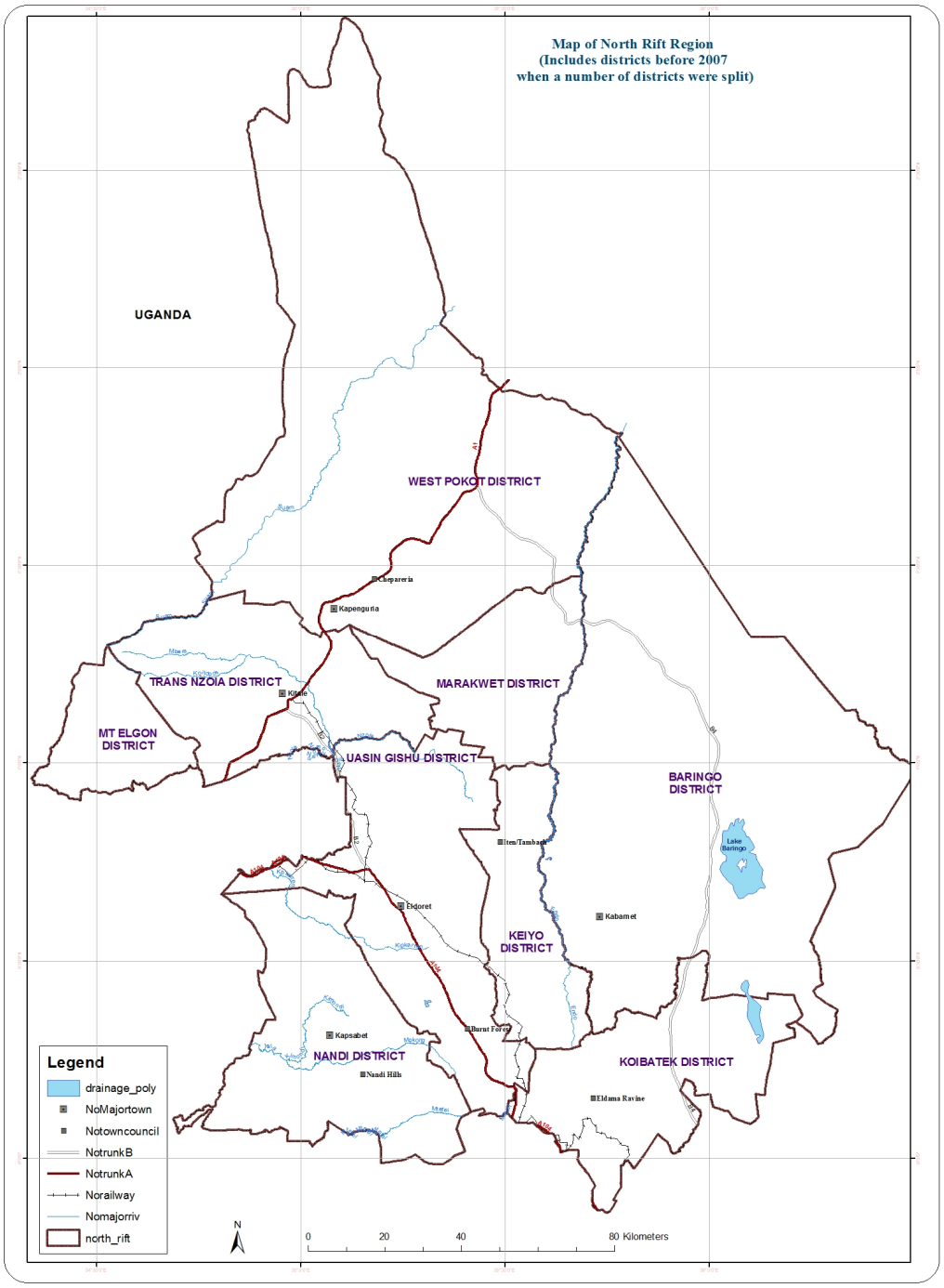

Figure 3.1: A map showing the North Rift Region of Kenya**.**

3.4. **Study Population, Inclusion and Exclusion Criteria**

The study population included inpatient and outpatient HIV/AIDS positive individuals at MTRH, Turbo and Burnt Forest health centers, and children less than five years of age with diarrhea at MTRH. Diarrhea was defined as the passage of three or more loose or watery stools in twenty-four hours. Patients that were critically ill, those that had been resident for less than one month in the North Rift and those not willing to participate in the study were excluded from the study.

3.5. **Sampling and Sample Size Determination**

After informed consent (Appendix 3), convenient sampling method was used in recruiting subjects who met the inclusion criteria into the study. The sample size was determined using the formula provided bellow and based on Kenya's National HIV prevalence of 7.2% in 2002 and the 11% (1618/14603) diarrhea cases seen at the Maternal Child Health (MCH) clinic in MTRH during the same year.

$$n = z^2(pq)/d^2$$

Where: n is the sample size, z is the significant level of the collected data (1.96), p is the expected proportion of the factor of interest in the population, q= 1 - 0.11 and d is the error willing to accept (0.05).

Based on the above information, the minimum samples size for children was 150 and 102 for the HIV positive population.

3.6. D**ata Collection**

Data was collected by use of a structured questionnaire and stool samples were collected for parasitological and molecular data.

3.6.1. **Structured Questionnaire**

The questionnaire (Appendix 4) was used to collect demographic data, individual's information, residence, housing and occupancy, sanitation, and the animals kept at the study subject's homesteads. It also captured information on the geographical location of the respondent's homes for ease of follow up and to facilitate collection of fecal samples from domestic animals in the homesteads of *Cryptosporidium* infected individuals.

3.6.2. **Collection of Human Stool Samples for Isolation of *Cryptosporidium* Oocysts**

Fresh stool samples were collected in unused polypots from known HIV/AIDS cases seen at the AMPATH clinics at MTRH, Turbo and Burnt Forest health centers. Stool samples were also obtained from children under five years of age with diarrhea after assent had been obtained from the parent(s) or guardian. The stool samples were screened for *Cryptosporidium* oocysts immediately or within eight hours after passage. *Cryptosporidium* positive samples were aliquoted into three portions: a portion in 75% ethanol in a screw capped test tube (three volumes of absolute ethanol were added to the stool to obtain 75% ethanol in the final mixture) and kept at room temperature, a portion was preserved in 2.5% potassium dichromate and kept at 4^{o}C and, a portion was kept frozen at -80^{o}C without preservative. The samples

were then transported from the field to Nairobi in cool boxes and were stored at the Centre for Microbiology Research in KEMRI, Nairobi, Kenya until used for DNA isolation and molecular analysis.

3.6.3. **Collection of Stool Samples from Domestic Animals for Isolation of *Cryptosporidium* Oocysts**

The homesteads of *Cryptosporidium* positive patients living within a 10 Km radius from Burnt forest or Turbo health centers, and within a 30 Km radius from MTRH were visited after obtaining permission and consent from the concerned patient or the head of the homestead. Fecal matter from fowls, cattle, goats and sheep in the homesteads were collected in unused polypots with the help of a field veterinary assistant and transported to the parasitology laboratory at School of Medicine, Moi University within eight hours of collection for screening and preservation.

3.6.4 **Parasitological Screening of Stool Samples**

Fresh stool samples collected from the study sites and faecal samples from animals were stained on the same day using the modified Ziehl-Neelsen staining method for *Cryptosporidium* as described by Casemore (1991). Briefly, smears were fixed in 3% acid-alcohol for 5 minutes and then rinsed with distilled water. The smear was stained with carbol fuschin for 5 minutes, decolorized briefly with 1% acid-alcohol and then stained with 0.4% malachite green for 30 seconds. Slides were rinsed with distilled water, dried and observed at 1000X magnification under standard light microscopy (Appendix 5).

3.7. **Molecular Identification of *Cryptosporidium* Strains**

3.7.1. **DNA Isolation from *Cryptosporidium* Oocysts**

Total genomic DNA was isolated from *Cryptosporidium* oocysts in stool samples that had been preserved in 2.5% potassium dichromate or kept frozen at -80^{o}C. The DNA isolation was achieved by use of the instructions in the manufacturer's manual supplied with the QIAamp® DNA Stool Mini Kit (Qiagen Ltd Crawley, West Sussex, United Kingdom). Briefly, a pea-size amount of formed stool or 200 µL of diarrheic stool was aliquoted into a 1.5 ml eppendroff tube, washed 4 times using 800 µL of distilled water and centrifuged at 13000 rpm (16.1g) for 5 minutes. The pellet was then suspended in 1.4 ml of ASL extraction buffer supplied in a QIAamp kit vortexed and the oocysts were ruptured by subjecting them to a freeze-thaw cycle of -80^{o}C for 30 minutes followed by thawing at 75^{o}C for 15 minutes. DNA was then extracted from the suspension using a QIAamp® DNA extraction kit for stool according to the manufacturer's instructions (Appendix 7).

3.7.2. **Identification of *Cryptosporidium* Genotypes**

This was done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 18s rRNA technique described by Morgan *et al*. (2000) and Xiao *et al*. (1999b). The 18S rRNA gene has a conserved segment that is good for phylogenetic analysis and, for species and strain differentiation. This technique involves nested PCR of the small subunit of the 18S gene as described by Xiao *et al*. (1999b) followed by restriction digestion of the nested PCR product using *Ssp*1 and *Vsp*1 DNA restriction enzymes. The primary PCR used forward primer 5'-TTC TAG AGC TAA TAC ATG CG-3' and reverse primer 5'-CCC TAA TCC TTC

GAA ACA GGA-3' (Xiao *et al*., 1999a). The PCR reaction mixture contained 5 µl of Perkin-Elmer 10X PCR buffer (Norwalk, Conn, UK), 3 mM $MgCl_2$, 240 mM (each) deoxynucleoside triphosphate (Promega), 250 nM (each) primer, 1.25 Units of *Taq* polymerase (Boehringer Mannheim, Livingston, United Kingdom), 4µg/µL of non-acetylated BSA (Sigma), and 0.5 µL of DNA template in a total 50 µL reaction mixture. A total of 35 cycles were carried out, each consisting of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, with an initial hot start at 94°C for 3 minutes and a final extension at 72°C for 7 minutes. The PCR products were stored at 4^oC waiting to be used for the secondary PCR (Appendix 8).

The secondary PCR was performed in a total of 50 µL reaction mixture without bovine serum albumin (BSA) using 1 µL of the primary PCR product as template, forward primers 5´-GGA AGG GTT GTA TTT ATT AGA TAA AG-3´ and the reverse primer 5´-AAG GAG TAA GGA ACA ACC TCC A-3´ (Xiao *et al*., 1999) in a final concentration of 500 nM to amplify an 826 to 864 bp internal fragment. The concentrations of other reaction mix and amplification conditions for secondary PCR were the same as the primary reaction (Appendix 8). Amplicons were analyzed by electrophoresis in a 2% agarose gel and visualized after ethidium bromide staining, and genotypes identified by RFLP analysis. Table 3.1 shows the summary of a 50 µL reaction mix of the primary and secondary PCR**.**

Table 3.1: Summary of the reaction mix of primary and secondary PCR.

Primary PCR				**Secondary PCR**	
Reagent	Vol.(µL)	Final concentration		Vol.(µL)	Final concentration
10X PCR buffer	5.0	1X		5.0	1X
dNTPs (1.5mM each)	8.0	240 µM each		8.0	240 µM each
Forward primer (10µM)	1.25	250 nM		2.5	500 nM
Reverse primer (10µM)	1.25	250 nM.		2.5	500 nM
$MgCl_2$ (25mM)	6.0	3 mM		6.0	3 mM
BSA (10mg/ml)	2.0	4 µg/µL		-	-
Water	25.75			24.75	
AmpliTaq polymerase	0.25	1.25units		0.25	1.25 units
Template DNA	0.5			1.0	
Total	50.0			50.0	

Restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA secondary PCR products was done by digesting 15 µL of the secondary PCR product in a total reaction volume of 40 µL consisting of 2µL (20 units) of *Ssp*1 (Promega) and 4 µL of 1X restriction buffer for species identification (Appendix 10). The second set of restriction using *Vsp*1 (Promega), was also performed in 40 µL volume except that 1 µL (10-12 units) of the enzyme was used. The restriction reactions were carried out in a water bath at 37°C for a minimum of 1 hour or overnight. The

digestion products were separated on 2% agarose gel and visualized by ethidium bromide staining. Table 3.2 shows the constitution of the RFLP reaction mix.

Table 3.2: Ingredients of RFLP restriction mixture in a final volume of 40 µL using *Ssp*1 and *Vsp*1 restriction enzymes

***Ssp*1 reaction mixture**			***Vsp*1 reaction mixture**	
Ingredient	Vol. (µl)		Ingredient	Vol. (µl)
PCR product	15		PCR product	15
Buffer	4		Buffer	4
Enzyme	2 (20 units)		Enzyme	1 (10 units)
Water	19		Water	20
Total	**40**		**Total**	**40**

3.7.3 **Sub-typing of *Cryptosporidium* Genotypes**

3.7.3.1 **Nested PCR of CP47 Gene**

The CP47 gene was used to subtype the *Cryptosporidium* species. This is a microsatellite gene located in chromosome 6 of *Cryptosporidium* species, which can be used for subtyping the organism. Microsatellite analyses gives improved resolution due to its extensive length polymorphism found in simple repeats located outside coding regions and are more likely to mutate than protein-coding sequences (Feng *et al*., 2000). The following primers were used for a nested PCR amplification of a 380-500 bp fragment of the gene. For the primary PCR forward primer 5'-GCT TAG ATT CTG ATA TGGATC TAT-3' and a reverse primer 5'-AGC TTA CTG GTC CTG TAT CAG TT-3' (Gatei *et al.*, 2007) were used, and for secondary PCR

forward primer 5'-ACC CCA AGA AGG CGG ACC AAG GTT-3' and reverse primer 5'-GTA TCG TGG CGT TCT GAA TTA TCA A-3' (Gatei *et al.*, 2007) were used. The primary PCR was performed in 50 μL volumes containing 1X PCR buffer, 3 mM $MgCl_2$, 240 μM of each dNTP, 250 nM of each primer, 4 μg/μL of non-acetylated BSA, 1.25 units of *Taq* polymerase and 0.5 μL of DNA template. Then 35 PCR cycles (94^{o}C for 45 seconds, 43^{o}C for 45 seconds and 72^{o}C for 60 seconds) were performed with an initial hot start at 95^{o}C for 3 minutes and a final extension step at 72^{o}C for 10 minutes (Glaberman *et al.*, 2002).

The secondary PCR was performed without BSA in 100 μL volumes using 2 μL of the primary PCR product and increasing the final concentration of primers to 500 nM while the rest of the ingredients remained at the same concentration as in the primary PCR. The PCR conditions remained the same except for the annealing temperature which was adjusted to 55^{o}C. The PCR bands were stained with ethidium, visualized under the UV light and photographed. The bands were then carefully excised and kept in a sterile 1.5 ml microfuge tube at -20^{o}C awaiting purification.

The electrophoresed PCR products were extracted and purified from the agarose gel using QIAquick® gel extraction kit and protocol as per the manufacturer's instructions. Briefly, the gel slice was weighed and three volumes of buffer QG (Qiagen, Proprietary composition), incubated at 50^{o}C for 10 minutes. One gel volume of isopropanol was added, centrifuged at 16.1g for one minute in a QIAquick spin column, washed using buffer QG (Qiagen, Proprietary composition) and

centrifuged at 16.1g for five minutes. The DNA was washed by adding buffer PE (Qiagen, Proprietary composition) and finally eluted from the column using buffer EB ((10 mM Tris-HCl, pH 8.5) and kept at -20^{o}C (Appendix 11). To ascertain the yield 5 µL of the DNA was electrophoresed in 2% agarose gel, visualized under UV after ethidium staining and photographed before sequencing.

3.7.3.2 **Sequencing of the Purified CP47 Gene PCR Products**

The two-directional sequencing of the DNA fragments of the CP47 region (Section 3.7.3.1) was done at the International Livestock Research Institute (ILRI) in Nairobi, Kenya. The PCR products were sequenced in both directions using the CP47 specific forward and reverse primers (Section 3.7.3.1) on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences were read using the Analysis Software (http://www.technelysium.com.au/ChromasPro.html) that uses the four colors to color-code analyzed data from all dye/virtual filter set combinations with adenine (A) as green, cytosine (C) as blue, guanine (G) as black, and thymine (T) as red in the electropherogram view.

3.8. **Data Management and Analysis**

3.8.1 **Analysis of Parasitological Data**

The data collected by use of the structured questionnaire (Appendix 4), microscopy and molecular biology techniques were stored and maintained in excel package. Parasitological and epidemiological data were analyzed by use of Epi Info computer

package for dispersion, central tendency and associations (P value of less than 0.05 were considered statistically significant).

3.8.2 **Sequence Analyses for Phylogenetic Relationships and Genetic Diversity**

The CP47 sequencing products were blasted using NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) to check whether they correspond to the CP47 sequences in the database and subsequently aligned by the ClustalW program (www.ebi.ac.uk/Tools/clustalw2/) with manual adjustment **(**Thompson *et al*., 1994**)**. Two types of phylogenetic analyses were done on the aligned sequences to assess relationships among isolates and the distance-based neighbor-joining analysis. The neighbor-joining tree was constructed using the same program (www.ebi.ac.uk/Tools/clustalw2) to show the evolutionary distances between different isolates. Tree reliability was assessed by the bootstrap method (Felsenstein *et al*., 1985). A value of 95% was considered statistically significant **(**Efron *et al*., 1996).

CHAPTER 4

RESULTS

4.1 The Demographic Characteristics of Study Population

Two populations were covered in this study; 1794 HIV infected individuals and 317 children aged 5 years and below (pediatric population), whose ages and sex are shown in Figure 4.1 and Figure 4.2 respectively.

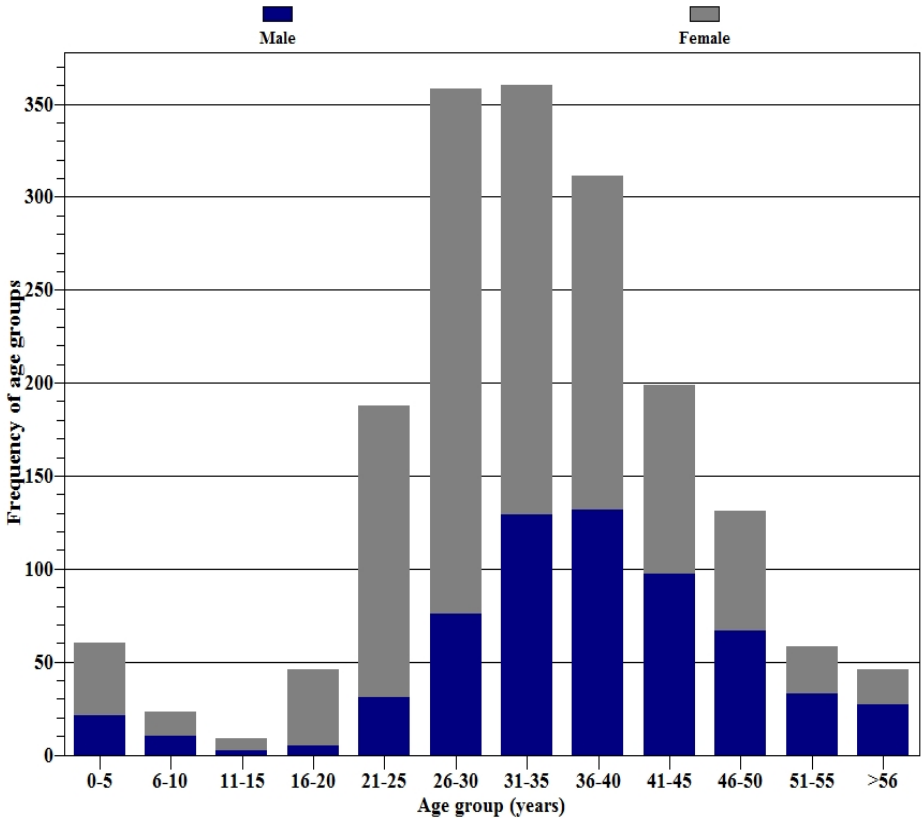

Figure 4.1: Bar graph showing frequency of age groups in years and by sex among HIV positive individuals.

Among the HIV infected population, a proportion of 3.3% (60/1794) comprised of children aged 5 years and below while the remainder 96.7% were above 5 years of age. The average age among those of 5 years of age and below was 3.49 years and

35.04 years among those above 5 years of age. By sex the population was 35.2% (635/1794) male and 64.8% females. As is evident from Figure 4.1 the bulk of the HIV infected population lies between 21-50 years of age and that majority are females.

The pediatric population was 317 comprising of 54.6% males (173/317) and 45.4% female (144/317). The HIV status of this group was not established. The highest proportion of the children seen in this study was under two years of age (Figure 4.2).

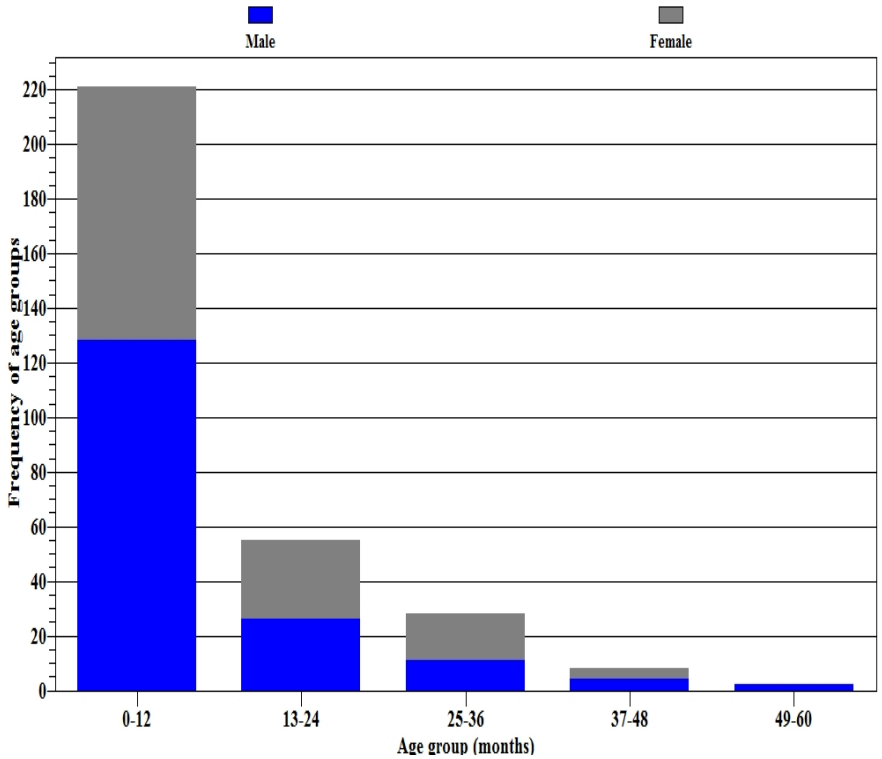

Figure 4.2: Bar graph showing frequency of age groups in months and by sex among the pediatric study population.

4.2 **Microscopic diagnosis of *Cryptosporidium* oocysts.**

The human study population comprised of the HIV positive individuals and children of 5 years and below. *Cryptosporidium* oocysts were diagnosed using modified ZN staining procedure (Section 3.6.4) of the stool samples and demonstrated under a compound microscope at a magnification of 1000X as intense pink-reddish rounded bodies against a green background (Figure 4.3). However, no *Cryptosporidium* oocysts were found in the fecal samples collected from animals in the homesteads of *Cryptosporidium* positive individuals and results to that effect are not discussed here.

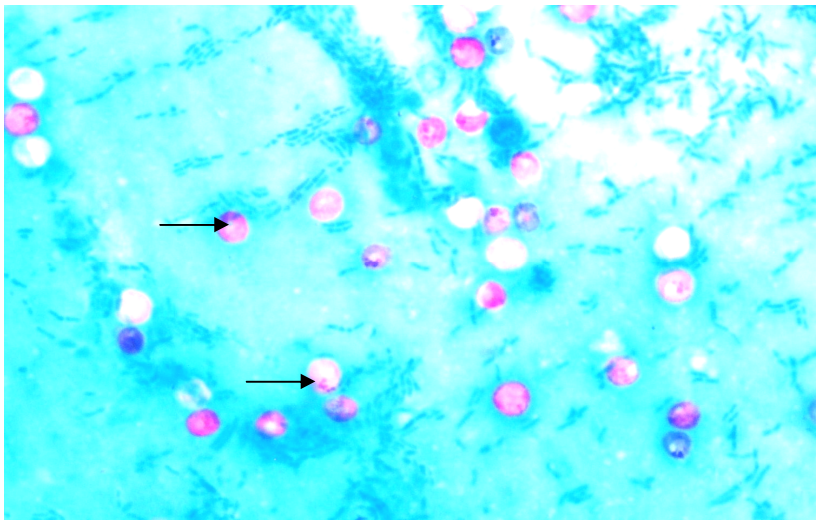

Figure 4.3: A 1000X magnification showing *Cryptosporidium* species stained with the modified ZN stain. The oocysts are seen as pink-reddish rounded bodies

4.3 **Seasonal Distribution of Cryptosporidiosis**:

A bimodal distribution was observed for the combined study populations, with the first peak having a cryptosporidiosis prevalence of 7.2% (31/429) which occurred during February-March period and a second smaller peak observed in June-July with a prevalence of 5.3% (24/457). During the month of April and the period of

September through December low prevalence of cryptosporidiosis was recorded in the overall study population with the lowest prevalence being recorded in the month of December (Figure 4.4).

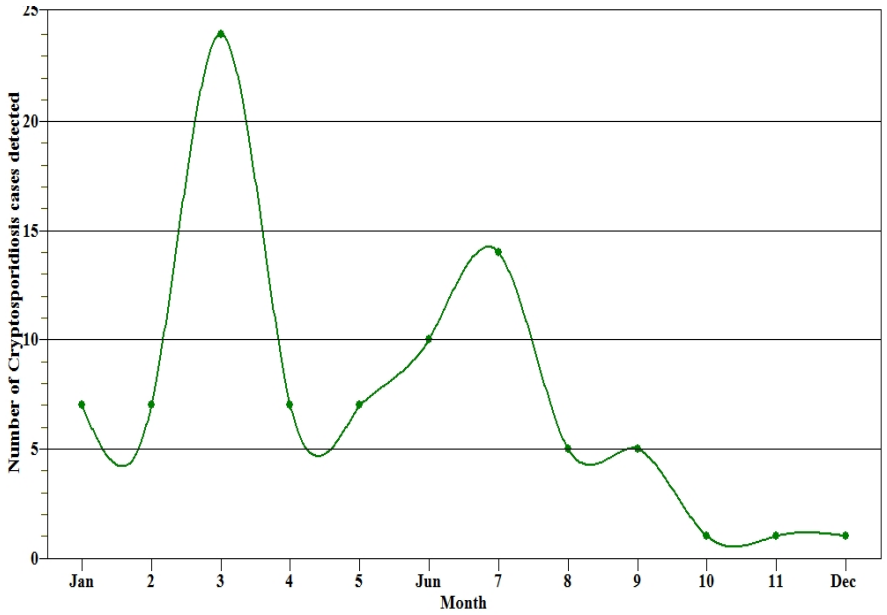

Figure 4.4**:** Graph showing the monthly distribution of cryptosporidiosis from January to December 2005.

4.4 **Prevalence of Cryptosporidiosis in the Study Populations.**

A total of 2111 samples comprising of 1794 from HIV infected individuals and 317 from children age 5 years old and below (hereafter referred to as overall population) were microscopically examined for *Cryptosporidium* oocysts of which 89 (4.2%) turned positive for *Cryptosporidium* oocysts. Males constituted 38.1% of the overall study population while females were 61.9%. The prevalence by sex was 4.4% (35/804) in males and 4.1% (54/1307) in females (Figure 4.5). However, the sex of the subjects did not show any statistically significant difference on the likelihood of *Cryptosporidium* infection (OR= 0.9469, CI=0.6131 - 1.4623, P= 0.8056).

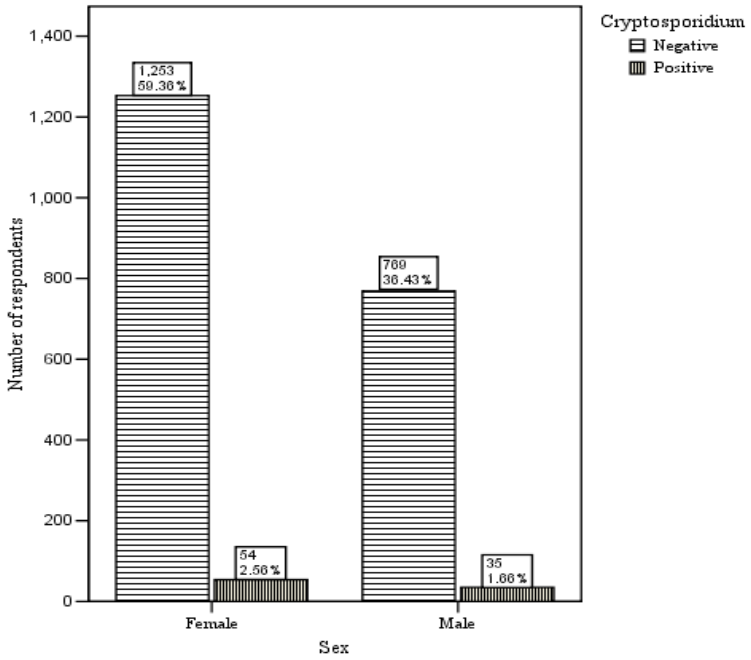

Figure 4.5: Number of *Cryptosporidium* cases by sex in the overall study population (HIV and pediatric patients combined)

A total of 1794 fecal samples obtained from HIV positive patients from the three study sites (MTRH, Turbo Health centre and Burnt Forest Health center) were screened for *Cryptosporidium* infection. The proportion of the sexes is shown in Figure 4.6. The overall prevalence of *Cryptosporidium* in the HIV positive population was 3.2% (Table 4.1).

Table 4.1: The prevalence of *Cryptosporidium* infection by study site and sex among the HIV positive population.

	Children (<5years)				**Adults**			
	Male	Female	**Total**		Male	Female	**Total**	**Grand Total**
MTRH	4	2	**6**		347	566	**913**	919
Burnt Forest	12	25	**37**		110	239	**349**	386
Turbo	5	12	**17**		153	319	**472**	489
Total	**21**	**39**	**60**		**610**	**1124**	**1734**	1794
Prevalence	**9.5%**	**5.1%**	**6.7%**		**2.9%**	**3.4%**	**3.1%)**	3.2%

The prevalence of cryptosporidiosis was 6.7% (4/60) among the HIV positive children. The odds of infection with *Cryptosporidium* were 1.9 times more in male children as compared to female children (OR= 0.5135, CI= 0.0671 - 3.9315, P= 0.5210). The prevalence of cryptosporidiosis was 3.1% (54/1734) in those subjects above five years of age. The prevalence of cryptosporidiosis by sex in individuals 5 years old and above was 3.4% among females and 2.6% among males. There was no significant association between sex of individuals and cryptosporidiosis (OR= 1.3016, CI= 0.7197 - 2.3543, P= 0.3833) though the odds of *Cryptosporidium* infection were slightly higher in females.

The distribution of cryptosporidiosis in the various age groups among the HIV positive population is shown in Table 4.2. The largest proportion of this population lies between ages 21-50 years but the age range with the highest prevalence is 0-10 years.

Table 4.2: Distribution of *Cryptosporidium* isolates by age groups in the HIV positive population.

Age group (years)	Number of subjects	Prevalence of *Cryptosporidium*
0-5	60	6.7% (4/60)
6-10	23	8.7% (2/23)
11-15	9	11.1% (1/9)
16-20	46	2.2% (1/46)
21-25	188	3.7% (7/188)
26-30	358	2.2% (8/358)
31-35	360	1.9% (7/353)
36-40	311	3.5% (11/311)
41-45	199	3.5% (7/199)
46-50	131	5.3% (7/131)
51-55	58	5.2% (3/58)
>56	46	0

The prevalence by sex in the overall HIV population was 2.9% (18/631) in males and 3.4% (40/1163) in females. There was no significant difference between the sexes in terms of the odds of infection with *Cryptosporidium* (OR=1.2130, CI= 0.6895 to 2.1341, P= 0.5023). Figure 4.6 shows the distribution of cryptosporidiosis by sex in the HIV infected population.

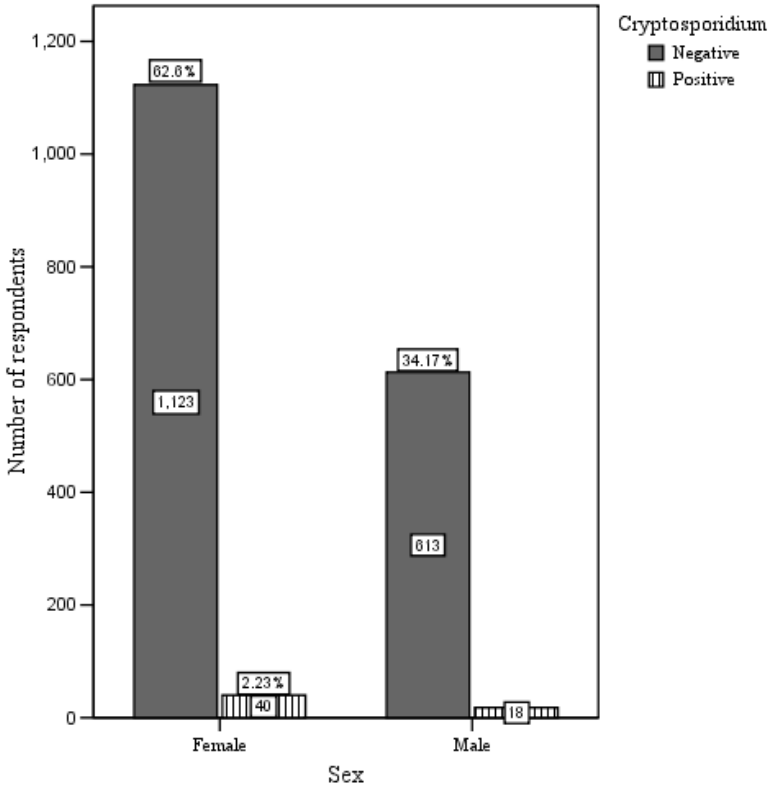

Figure 4.6: *Cryptosporidium* cases by sex in HIV infected patients

When CD4 counts were investigated among the HIV-infected patients, the mean counts in these patients was 231.7 cells per microlitre. However, mean CD4 counts for HIV positive patients with cryptosporidiosis were 3 times lower than among the HIV positive patients without cryptosporidiosis (Figure 4.7). Unconditional logistic analysis of *Cryptosporidium* infection and CD4 count showed that the odds of *Cryptosporidium* infection were higher in patients with lower CD4 counts, and that the difference in CD4 counts between HIV positive patients with and those without cryptosporidiosis was statistically significant, (P= 0.0003).

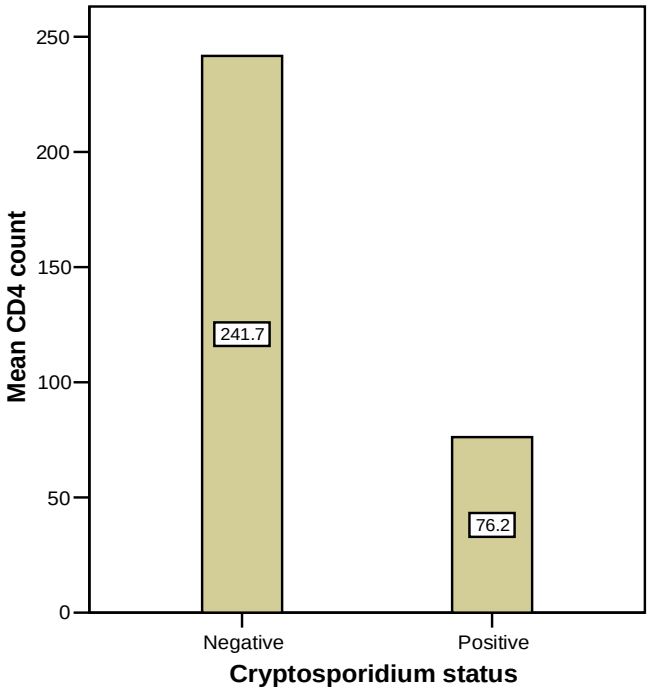

Figure 4.7: Mean CD4 counts in HIV patients with or without cryptosporidiosis.

A total of 317 children aged 5 years and below (the pediatric group) seen at MTRH and whose HIV status was unknown were also investigated for *Cryptosporidium* infection. The prevalence of cryptosporidiosis in these children was 9.8% (31/317) with the cryptosporidiosis prevalence among males being 9.8% and 9.7% among females (Figure 4.8). There was no significant difference in the odds of infection with *Cryptosporidium* between the sexes, (OR= 0.9882, CI= 0.4693 - 2.0810, P= 0.9752).

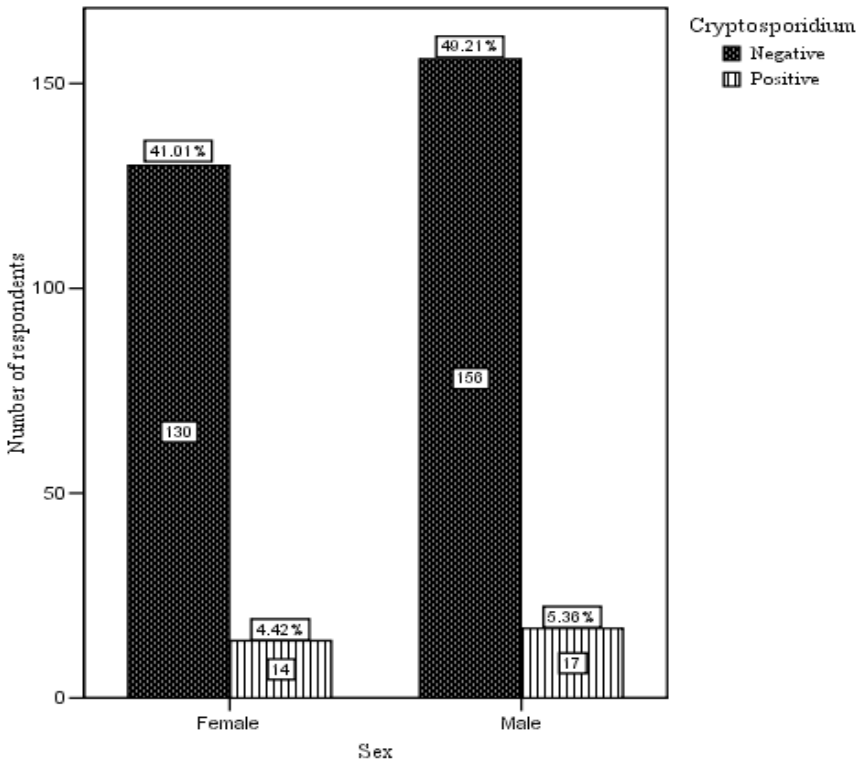

Figure 4.8: *Cryptosporidium* prevalence by sex in the pediatric study group.

When the children were grouped into five age groups and the frequencies of the ages and the prevalence in each age group are shown in the Table 4.3 whereby the infection is seen in children less than 3 year of age.

Table 4.3: Prevalence of cryptosporidiosis by age groups in the pediatric population

Age group (years)	Prevalence	*Cryptosporidium* prevalence
≤ 1	221 (69.7%)	7.7% (7/221)
>1 ≤ 2	56 (17.7%)	14.3% (8/56)
>2 ≤ 3	28 (8.8%)	21.4 (6/28)
>3 ≤ 4	9 (2.8%)	0
>4 ≤ 5	3 (0.9%)	0

There was prevalence of 7.7% among children aged one year and below which was the lowest followed by those between 1-2years old and highest was among children aged between 2-3 years old (Table 4.3). Logistic regression analysis using 1 year old and below as baseline revealed that the odds of infection were two times in children aged 1-2 years (CI= 0.8154-4.9056, P= 0.1300), and are even greater at 3.273 times and statistically significant in children between 2-3 years old (OR= 3.2727, CI= 1.1692 - 9.1611, P= 0.024).

4.5 **Association of Cryptosporidiosis Prevalence with Study Sites**

The prevalence by site was 3.5% (N=919) for the AMPATH centre at MTRH, 3.1% (N=386) for Burnt Forest and 2.9% (N= 489) for Turbo (Table 4.4).

Table 4.4: Prevalence of *Cryptosporidium* infection in the study groups based on study site

Study group	Study site	*Cryptosporidium* prevalence
HIV positive	MTRH	3.5% (32/919)
	Burnt Forest	3.1% (12/386)
	Turbo	2.9% (14/489)
Total		3.2% (59/1794)

The logistic regression output (Table 4.5) for the association of prevalence of cryptosporidiosis and study sites revealed that there was no statistically significant difference in prevalence between the population at MTRH, Burnt Forest health

centre (OR= 0.8894, CI= 0.4531 - 1.7456, P= 0.7333), and Turbo health centre (OR= 0.8170, CI= 0.4317 - 1.5460, P= 0.5345.

Table 4.5: The logistic regression output when prevalence at MTRH is used as baseline

Study site	Odds Ratio	95% C.I.	P-Value
Burnt Forest/MTRH	0.8894	0.4531 - 1.7456	0.7333
Turbo/MTRH	0.8170	0.4317 - 1.5460	0.5345

4.6 **Association of Cryptosporidiosis with Location of Residence**

Prevalence of cryptosporidiosis in those residing in urban areas was 6.3% (4/64), those from the peri-urban areas 4.7% (38/807) and those from rural areas 3.9% (42/1202) as seen in Table 4.5. There was no significant association between location of residence and cryptosporidiosis prevalence (OR=0.7669, CI=0.5314 - 1.1067, P=0.156). The logistic regression output for prevalence of *Cryptosporidium* and location of residence when the urban residence is used as baseline did not show any significant difference in the odds of *Cryptosporidium* infection between those living in the urban areas and, peri-urban (OR= 0.5781, P= 0.2682) and rural residences (OR= 0.4779, P= 0.1312). However, the odds of infection were about 1.8 and 2 times higher in those living in urban locations as compared to the peri-urban and the rural residences respectively.

With respect to the HIV-infected patients, the prevalence of cryptosporidiosis was 7.3% (N=55) for the urban residents, 2.7% (N=635) for peri-urban residents and

3.5% (N= 1066) for rural residents (Table 4.6). There was no significant association between location of residence and prevalence of cryptosporidiosis (OR= 0.9775, P= 0.9245). However, when location of residence were compared, using the urban residence as a baseline, the odds of *Cryptosporidium* infection were found to be 2.9 and 2.2 times higher in the urban residents than for the peri-urban (OR= 0.3507, CI= 0.1138 - 1.0807, P= 0.0680) and rural (OR= 0.4585, CI=0.1575 - 1.3347, P= 0.1526) residents respectively (Table 4.7).

Among the pediatric group (≤5 years old) cryptosporidiosis prevalence was 0% in the urban residents, 12.2% (N=172) in peri-urban residents and 7.4% (N= 136) in rural residents (Table 4.6). However, no statistically significant differences were observed between the peri-urban and rural populations in regard to the prevalence of cryptosporidiosis (OR= 0.5707, CI= 0.2595 - 1.2563, P= 0.1635), (Table 4.7) even then the odds of *Cryptosporidium* infection were about 2 times higher among peri-urban residents than in the rural residents.

Table 4.6: Prevalence of *Cryptosporidium* infection in the study populations according to location of residence.

Residence	Prevalence		
	Overall (N= 2111)	HIV population (N=1794)	Pediatric population (≤5 years old) (N= 317)
Urban	6.3%	7.3%	0
Peri-urban	4.7%	2.7%	12.2%
Rural	3.9 %	3.5%	7.4%

Table 4.7: Logistic regression output comparing cryptosporidiosis prevalence by location of residence using urban area as a baseline

Study population	Baseline: Urban	Odds Ratio	95% C.I.	P-Value
Overall	Periurban/Urban	0.7412	0.2560 - 2.1464	0.5809
	Rural/Urban	0.6104	0.2129 - 1.7500	0.3583
HIV	Periurban/Urban	0.3507	0.1138 - 1.0807	0.0680
	Rural/Urban	0.4585	0.1575 - 1.3347	0.1526
Pediatric	Rural/Urban	0.5707	0.2592 - 1.2563	0.1635

4.7 **Association between Cryptosporidiosis and Water Sources**

About 16% of the 2072 subjects that responded to this item on water sources used river or spring water as the main sources of water for domestic use, 48.6% used wells or boreholes, and 35% used piped tap water (Figure 4.9).

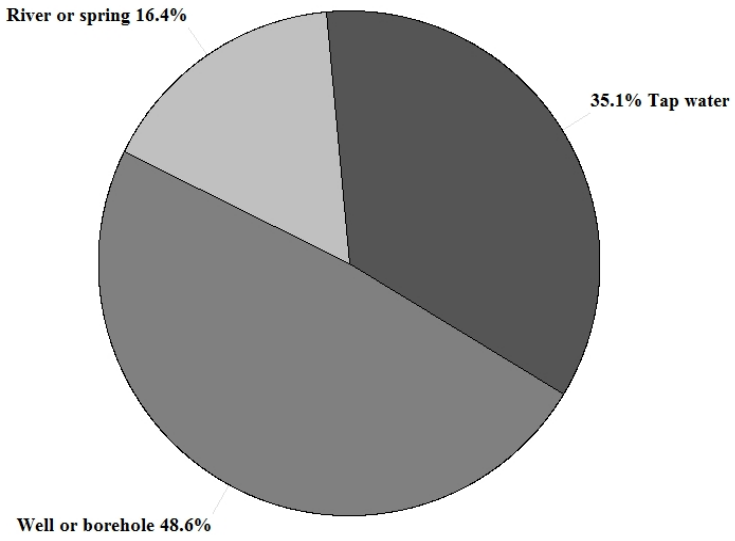

Figure 4.9: Sources of water for the North Rift region study population

The prevalence of cryptosporidiosis was 4.4% (13/339) in those using rivers or springs, 3.3% (33/1006) for well or borehole users and 5.6% (41/727) in those who used piped water. Using logistic regression analysis there was no statistically significant association between *Cryptosporidium* prevalence and water sources (OR= 1.2638, CI= 0.9225 - 1.7314, P= 0.1450). Similarly no statistically significant differences were observed in the odds of *Cryptosporidium* infection between those that used water from rivers/springs and those that used well/borehole water (OR= 0.7326, CI= 0.3929 - 1.3661, P= 3277, or tap water (OR= 1.2910, CI= 0.7043 - 2.3665, P= 0.4088). Even then, the odds of *Cryptosporidium* infection were about 1.3 times higher in those using tap water than in those using river or spring water. Interestingly, it was found that the odds of infection with *Cryptosporidium* were significantly different between those that used well/borehole sources and those that used piped water (OR= 1.7622, CI= 1.1029 - 2.8158, P= 0.0178).

A total of 1755 HIV infected individuals responded to this item on water sources of which 16.6% drew their water from rivers/springs, 50.8% form well/borehole and 32.5% used tap water (Figure 4.10).

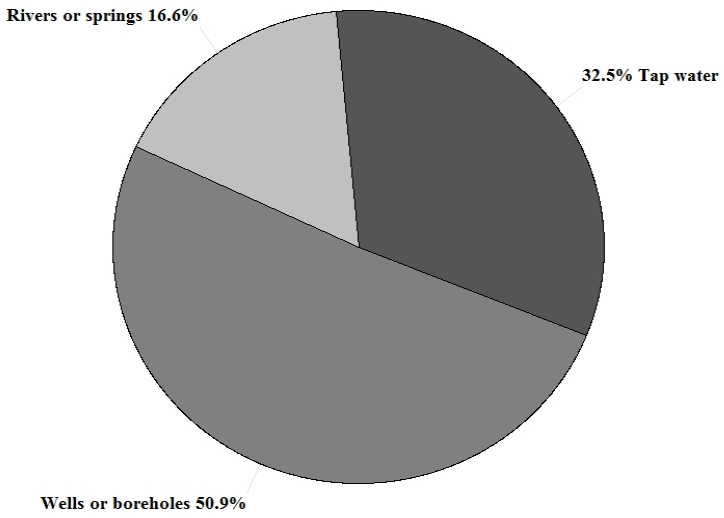

Figure 4.10: Water sources for domestic use by the HIV infected individuals

The prevalence of cryptosporidiosis in the HIV infected individuals, by sources of water was 3.4% (10/292) among river/spring water users, 2.8% (24/893) for well or borehole users and 4.2% (24/570) for piped water users. There was no statistically significant association between cryptosporidiosis prevalence and water sources on logistic regression analysis (OR=1.2055, CI= 0.8165 - 1.7799, P= 0.3472) as shown in Table 4.8. Furthermore, when river/spring water users group was used as baseline, there was no significant difference in odds of *Cryptosporidium* infection between river or spring water users and well or borehole users (OR= 0.7788, P=0.5135), and tap water users (OR= 1.2396, P=0.5754).

Table 4.8: Prevalence and statistical values for cryptosporidiosis according water sources in the study populations.

Source of water	Prevalence		
	Overall (N= 2072)	HIV population (N=1755)	Pediatric population (N= 317)
River/spring	4.4%	3.4%	10.6%
Well/borehole	3.3%	2.7%	8.0%
Tap water	5.6 %	4.2%	10.8%
Odds Ratio	1.2638	1.2055	1.0902
95% CI	0.9225 – 1.7314	0.8165 – 1.7799	0.6474 – 1.8360
P value	0.1450	0.3472	0.7453

The water sources for the pediatric population included river or spring (11.5%), well or borehole (35.6%) and tap water 49.5% (N=317). The prevalence of cryptosporidiosis was 10.6% (5/47) among those using river or spring, 8.0% (9/113) in those using well or borehole water and 10.8% (17/157) for tap water users. The logistic regression analysis showed no significant association between water sources and the prevalence of cryptosporidiosis (OR= 1.0902, CI= 0.6474 - 1.8360, P= 0.7453), as in Table 4.8. The odds of *Cryptosporidium* infection were similar in those using river or spring water, and tapped water users (OR= 1.0200, CI= 0.3551 - 2.9296, P= 0.9707). However, the odds were about 1.3 times higher in those using river or spring sources (OR= 0.7269, CI= 0.2301 - 2.2967, P= 0.5869).

4.8 C**ryptosporidiosis and Water Treatment Methods**

A total of 2071 out of the 2111 enrolled individuals responded to the item on water treatment. The water treatment methods used by the respondents were boiling

(43.6%) sieving (0.8%), chlorination (10.1%). However, 45.5% of the study individuals did not treat their drinking water (Figure 4.11).

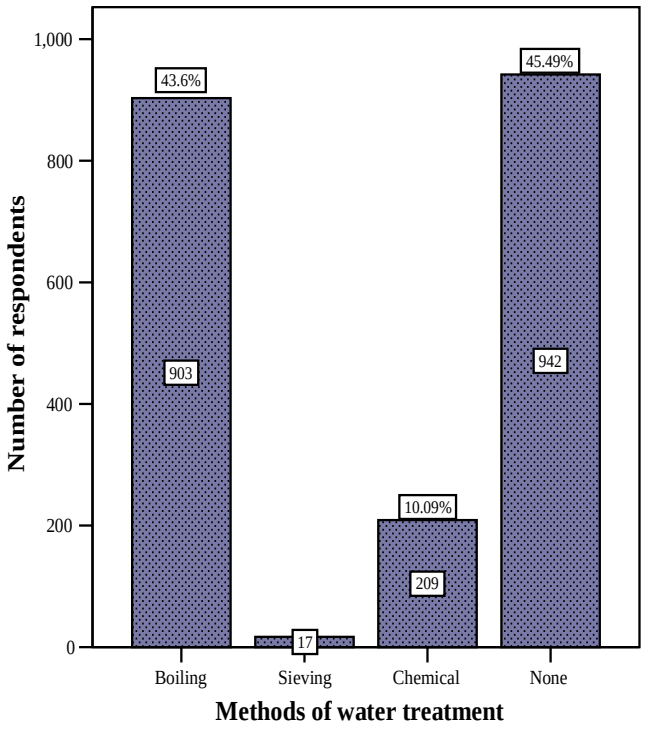

Figure 4.11: Proportion of study individuals using the various water treatment methods.

The prevalence cryptosporidiosis was 4.9% among those who boiled their drinking water, 3.3% among those using water purification chemicals (chlorination) to treat the water and 4.0% among those who never treated their water. There was no statistically significant association between cryptosporidiosis and methods of water treatment (P= 0.3536). When logistic regression analysis was performed using those boiling water as the baseline, there was no significant difference in odds of *Cryptosporidium* infection between the water treatment methods. However, the odds of infection were 2.4 times higher in those who reported using boiled water (OR=0.4225, CI= 0.1494 – 1.1944, P=0.1045) than those using chlorination as a

method of water treatment, whereas the outcomes among those boiling and those who did not treat their water were more or less similar (OR= 0.8289, CI= 0.5221 – 1.3189, P=0.4300).

Table 4.9: Prevalence of cryptosporidiosis and statistical values among individuals using different methods of water treatment.

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Study populations	Methods of water treatment	*Cryptosporidium* prevalence	Odds Ratio	95% CI	P-value
Overall (N= 2072)	Boiling	4.9% (44/903)	0.9323	0.8033 - 1.0820	0.3561
	Sieving	0 (0/17)			
	Chemical	3.3% (7/209)			
	No treatment	4.0% (38/942)			
HIV positive (N=1754)	Boling	4.2% (32/761)	0.8547	0.7104 - 1.0282	0.0960
	Sieving	0 (0/14)			
	Chemical	2.3% (4/172)			
	No treatment	2.7% (22/807)			
Pediatric (N= 317)	Boling	8.5% (12/142)	1.1324	0.8676 - 1.4780	0.3601
	Sieving	0 (0/3)			
	Chemical	8.1% (3/37)			
	No treatment	11.9% (16/135)			

Among the HIV infected individuals, 1754 out of 1794 individuals responded to this item, of which 43.4% said they boiled their drinking water, 0.8% sieved their water, 9.8% used chemical treatment, and 46.0% did not treat their water at all. The prevalence of cryptosporidiosis was 4.2% (N= 762) amongst those that boiled their water, 2.3% (N=172) among those that used chemical (chlorination) water treatment for their drinking water, and 2.7% (N= 807) in those who never treated their drinking

water. The logistic regression output for the association of cryptosporidiosis and water treatment methods showed no significant association (P= 0.0960) as shown in Table 4.9. However, the odds of infection were about 1.8 times higher in those boiling water compared to those using chemical treatment and those who did not treat their water respectively (Table 4.10).

In the pediatric study group (N= 317), 44.8% of the respondents reported boiling drinking water, 0.9% sieved, 11.7% used chemical treatment, and 42.6% did not treat their water at all. The prevalence of cryptosporidiosis in this group stratified by methods of water treatment was 8.5 % among those that boiled their drinking water, 8.1% in those using chemicals and 11.9% among those who did not treat their water at all. However, there was no statistically significant association between cryptosporidiosis and methods of water treatment (P= 0.361), neither were there significant differences between those who boiled their water and, those using chemical treatment, even though the odds of *Cryptosporidium* infection were 1.5 times higher in those who did not treat water (Table 4.10) than in those that boiled their drinking water.

Table 4.10: Odds ratios compared between the different water treatment methods in the total study population, among HIV infected and children.

Study Populations	Boiling as baseline	Odds Ratio	95% - C.I.	P-Value
Overall (combined)	Chemical	0.6765	0.3004 - 1.5238	0.3456
	No treatment	0.8206	0.5264 - 1.2793	0.3829
HIV positive	Chemical	0.5424	0.1894 - 1.5534	0.2545
	No treatment	0.6385	0.3676 - 1.1089	0.1111
Pediatric	Chemical	0.9559	0.2553 - 3.5790	0.9466
	No treatment	1.4566	0.6619 - 3.2052	0.3500

4.9 **Cryptosporidiosis and Human Waste Disposal Methods**

A total of 2065 out of the 2111 requested to participate in this survey responded to this item. The respondents mainly used pit latrines, flash toilets and the bush methods for disposing human waste. Figure 4.12 shows the proportions using the different human waste disposal methods. Majority of the population used pit latrines.

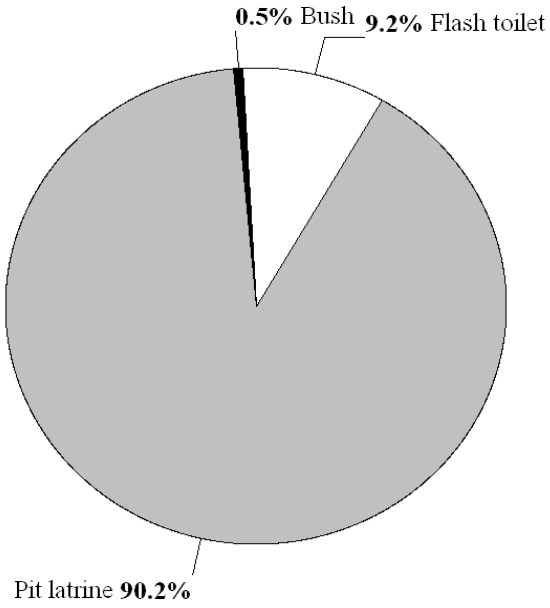

Figure 4.12: Methods of human waste disposal used by the overall population.

The prevalence of cryptosporidiosis was 4.2% (78/1863) among those that disposed human waste in pit latrines, 4.7% (8/191) among those that used flash toilets, and 9.1 % (1/11) among those that used the bush or the environment for human waste disposal. The logistic regression analysis on the overall population showed that there was no statistically significant association between the occurrence of cryptosporidiosis and methods of waste disposal (OR= 1.2237, CI= 0.6665, P= 0.5150) as shown in Table 4.11. However, the odds of infection with *Cryptosporidium* were 2.5 times more in those using the bush method (OR= 2.2885, CI= 0.2896 – 18.0864, P= 0.3325) than in those using the pit latrines (Table 4.12), but the odds for the pit latrine and flash toilet users were almost similar OR=1.1317, CI= 0.5582 – 2.2941, P= 0.7464.).

Table 4.11: Prevalence of cryptosporidiosis in the overall study population, the HIV infected individuals and children in relation to methods of human waste disposal.

Study populations	Method of waste disposal	Cryptosporidium prevalence	Odds Ratio	95% CI	P value
Overall (N= 2065)	Pit latrine	4.2% (78/1863)	1.2237	0.6665 - 2.2468	0.5150
	Flash toilet	4.7% (9/191)			
	Bush	9.1 (1/11)			
HIV population (N=1750)	Pit latrine	3.3% (52/1579)	1.1724	0.5519 - 2.4905	0.6790
	Flash toilet	3.1% (5/161)			
	Bush	10.0% (1/10)			
Pediatric population (N= 317)	Pit latrine	9.1% (26/285)	1.3769	0.4695 - 4.0380	0.5602
	Flash toilet	16.3% (5/31)			
	Bush	0%			

Among the HIV infected, 1750/1794 individuals responded to this item, of which 90.2% (1579/1750) used pit latrines, 9.2% (161/1750) used water closet toilet, and 0.6% (10/1750) disposed their human waste in the open environment (bush). The prevalence of cryptosporidiosis against the various human waste disposal methods is shown in Table 4.11. There was no significant difference between those using pit latrines and those that used flash toilets (OR= 0.8986, CI= 0.3705 – 2.3910, P= 0.4919). However, the odds of infection were 3.3 times higher in the group using the bush method than the using the pit latrine (OR= 3.2628, CI= 0.4058 – 26.2320, P= 0.2661) as shown in Table 4.12.

Among the children (pediatric group) enrolled in the study, 89.9% (285/317) of the responding parent/guardians said they used pit latrines for human waste disposal, 9.8% (31/317) used flash toilets, and 0.3% (1/317) disposed human waste in the environment. The prevalence of cryptosporidiosis was 9.1% (26/285) among pit latrine users and 16.1% (5/31) among flash toilet users. There was no significant

association between cryptosporidiosis and methods of waste disposal though the odds of infection were about 1.5 times higher in those using flash toilets than in those that used pit latrines (OR= 1.5266, CI= 0.4947 - 0.4618, P= 0.4618), as in Table 4.12.

Table 4.12: Odds ratios compared between the total population, HIV infected and children for the different human waste disposal methods.

Study Populations	Pit latrines as baseline	Odds Ratio	95% C.I.	P-Value
Overall (combined)	Flash toilets	1.1317	0.5582 - 2.2941	0.7316
	Bush	2.2885	0.2896 - 8.0864	0.4325
HIV positive	Flash toilets	0.9412	0.3705 - 2.3910	0.8986
	Bush	3.2628	0.4058 - 6.2320	0.2661
Pediatric	Flash toilets	1.5266	0.4947 - 0.4618	0.4618

4.10 **Cryptosporidiosis and Health complaints**

4.10.1 **Cryptosporidiosis and Diarrhea**

In the total study population, 44.8% (946/2111) of the respondents had diarrhea. Of these, 32.0% (303/946) reported having had diarrhea for less than one week, 7.5% for between 1-2 weeks, 11.8% for more than 2 weeks, while 48.6% reported that they did not have diarrhea, even though a casual examination of the stool samples they provided actually showed they were diarrheic. The prevalence of cryptosporidiosis was 7.6% (72/946) among patients with diarrhea, while in patients without diarrhea it was 1.5% (17/1165). There was a statistically significant association of diarrhea with cryptosporidiosis (OR= 5.5631, CI= 3.2556 - 9.5061, P≤ 0.001). There was also a

significant association between the duration of diarrhea and cryptosporidiosis (OR= 0.7302, CI= 0.6247 - 0.8534, P= 0.0001)

The logistic regression analysis for comparing the association of cryptosporidiosis and the various durations of diarrhea and using the group with diarrhea for less than one week as baseline revealed that there was no significant difference between those that had diarrhea for less than 1 week and those that had it for 1-2 weeks (Table 4.13). However, there was a statically significant difference between the group of patients that had diarrhea for less than 1 week and the group that had diarrhea for more than 2 weeks(OR= 2.5734, CI= 1.4154 - 4.6789, P= 0.0019) as shown in Table 16, with the odds infection being 2.6 times higher in the latter. Further, the odds of association with cryptosporidiosis were 2.6 times higher in patients with diarrhea for less than 1 week compared to those who reported no diarrhea, even though their stools were diarrheic (OR= 0.3692, P= 0.003), as in Table 4.13.

Table 4.13: Unconditional Logistic regression output of duration of diarrhea and *Cryptosporidium* infection in the overall population

Diarrhea <1 week as baseline	Odds Ratio	95% C.I.	P-Value
Diarrhea duration Between 1 & 2 weeks	1.0842	0.4539 - 2.5896	0.8556
Diarrhea duration more than 2 weeks	2.5734	1.4154 - 4.6789	0.0019
No diarrhea	0.3692	0.2162 - 0.6304	0.0003

When the association between cryptosporidiosis and the various durations of diarrhea in diarrheic patients were subjected to logistic analysis with the group that had diarrhea for less than 1 week being the baseline, it was revealed that the odds of

having cryptosporidiosis were 2.5 times higher in those who had diarrhea for more than 2 weeks compared to those that had diarrhea for less than 1 week. However, the odds were not significantly different between those who had diarrhea for less than 1week and those who reported no diarrhea but their stool samples were actually diarrheic (Table 4.14).

Table 4.14: Logistic regression output of *Cryptosporidium* infection by the duration of diarrhea and among patients with diarrhea when the group with diarrhea for <1 week is used as baseline

Baseline: Diarrhea <1 week	Odds Ratio	95% C.I.	P-Value
> 1≤-2 weeks	0.7268	0.2432 - 2.1720	0.5678
>2 weeks	2.4871	1.2968 - 4.7701	0.0061
No diarrhea	0.7293	0.4080 - 1.3036	0.2868

Among the HIV individuals (N=1794), 35.1% of the respondents had diarrhea (Figure 4.13).

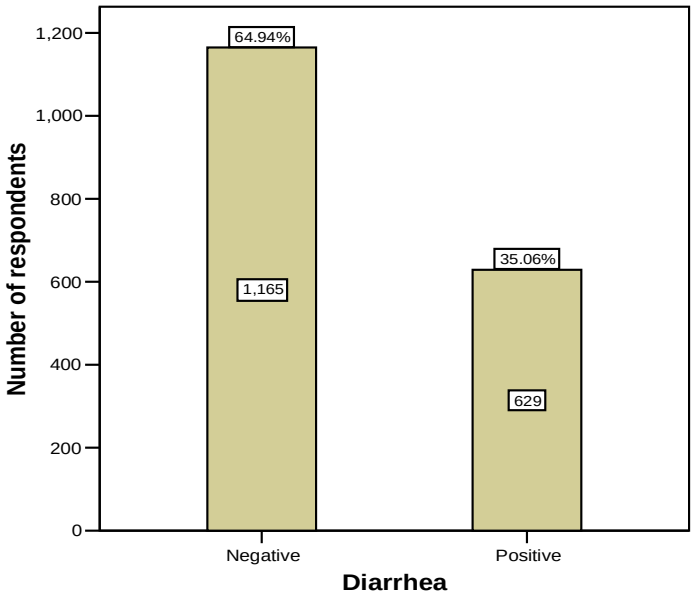

Figure 4.13: Prevalence of Diarrhea among the HIV positive patients

The cryptosporidiosis prevalence among those who were HIV positive and with diarrhea was 6.5% whereas in those without diarrhea it was 1.5%. There was a strong association between diarrhea and the presence of cryptosporidiosis (P≤0.0001) as in Table 4.14. The odds of having cryptosporidiosis were 4.7 times higher in those having diarrhea than in those who did not have diarrhea. Among the HIV infected patients aged five years and below, the prevalence of diarrhea was 43.3% and prevalence of cryptosporidiosis was 11.5% while among the HIV positive individuals above 5 years of age, diarrhea was reported in 34.8% of the patients with a cryptosporidiosis prevalence of 6.3%. The prevalence among the non-diarrheic HIV positive patients is shown in Table 4.15. The odds of having cryptosporidiosis were 4.3 and 4.6 times higher in those with diarrhea among those aged 5 years and below and, those aged above 5 years respectively (Table 4.15).

Table 4.15: Prevalence of diarrhea and cryptosporidiosis among HIV and pediatric study populations.

			Prevalence			Statistical analysis		
Study groups	Age	N	Diarrhea	*Cryptosporidium*		Odds ratio	95% CI	P-value
				Diarrheic	Non Diarrheic			
HIV	Whole group	1794	35.1%	6.5%	1.5%	4.7087	2.6524-8.3591	0.000
	>5 years	1734	34.8%	6.3%	1.4%	4.6869	2.5911-8.4780	0.000
	≤5 years	60	43.3%	11.5%	2.9%	4.3043	0.4211-44.0027	0.2185
Pediatric	≤5 years	317	100%	9.8%				

Among the HIV positive patients with diarrhea, 35.1% (N=629) reported having diarrhea for less than one week, 8.6% for between 1-2 weeks, 16.1% for more than two weeks, and 52.1% reported no diarrhea even though their stools were actually

diarrheic on examination (Figure 4.14). The duration of diarrhea in the latter group was therefore unknown.

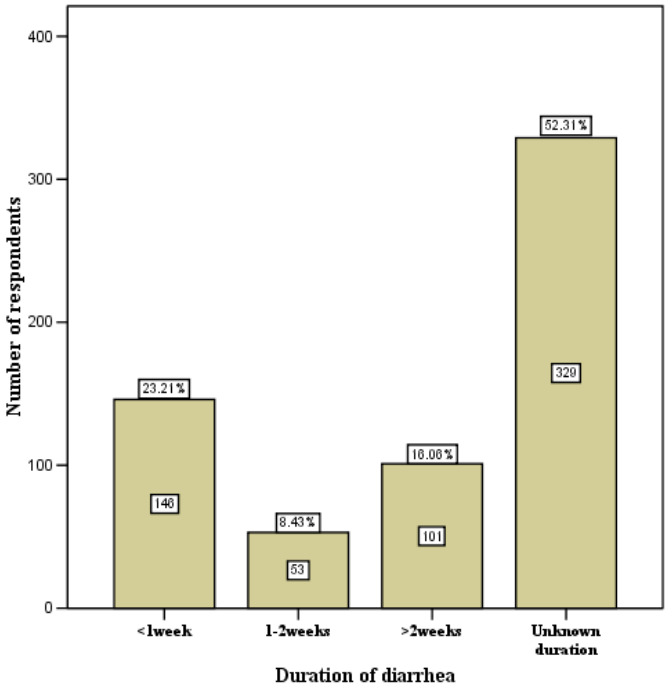

Figure 4.14: Duration of diarrhea in diarrheic HIV positive patients

The prevalence of cryptosporidiosis on the basis of the duration of diarrhea was 4.1% (6/146) among those with diarrhea <1 week, 5.7% (3/53) those with diarrhea for between 1-2 weeks, 16.8% (17/101) in those who had diarrhea for >2 weeks, and 4.6% (15/329) in those patients who reported not to having diarrhea, but whose stools appeared diarrheic. When comparing between the groups while using the group of patients who reported diarrhea for less than one week as baseline, the logistic regression analysis for the association of *Cryptosporidium* infection with the duration of diarrhea showed that the odds of cryptosporidiosis were 1.4 times higher in patients with diarrhea for between one and two weeks and, 4.7 times in patients

with diarrhea for more than two weeks (OR= 4.7222, CI= 1.7915 - 12.4472, P= 0.0017), as in Table 4.16.

Table 4.16: Prevalence of cryptosporidiosis among HIV positive patients with diarrhea and the logistic regression output for the association of cryptosporidiosis with the duration of diarrhea.

Duration of diarrhea	Number	Prevalence	Between group comparison with the ≤1 week group used as baseline		
≤1 week	146	4.1%			
			OR	95% CI	P value
>1≤2 weeks	53	5.7%	1.3725	0.3309 - 5.6926	0.6626
>2 weeks	101	16.8%	4.7222	1.7915 - 2.4472	0.0017
Unknown Duration	328	4.6%	1.1182	0.4250 - 2.9424	0.8209

In the pediatric group, all the respondents were diarrheic with a cryptosporidiosis prevalence of 9.8% (31/317). The proportions of the duration of diarrhea in the pediatric population is shown in Figure 4.15.

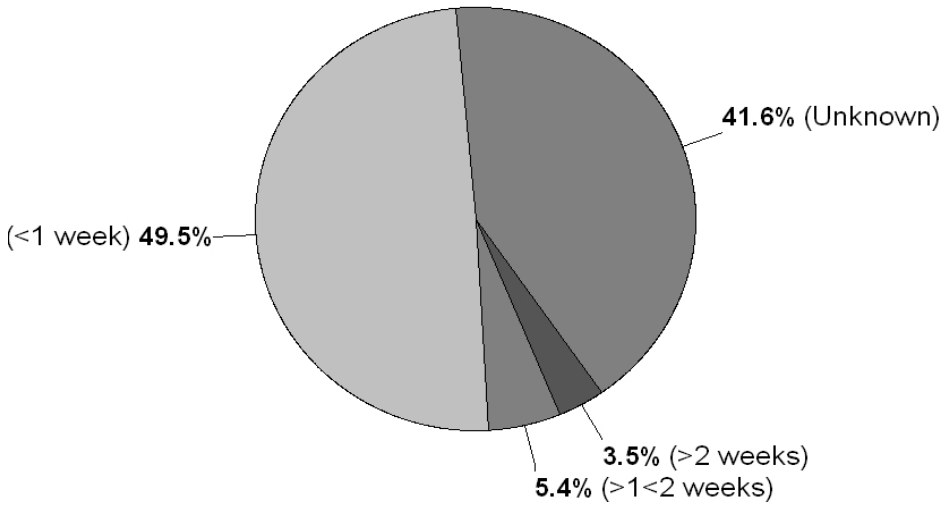

Figure 4.15: Diarrhea duration in children ≤5 years old.

When the prevalence of cryptosporidiosis against the duration of diarrhea was considered, 10.8% (17/157) of the patients had diarrhea for ≤1 week, 5.9% (1/17) for between 1-2weeks, 18.2% (2/11) for >2 weeks, and 8.3% (11/132) in those patients who reported no diarrhea but their stools were diarrheic on examination and therefore duration of diarrhea is unknown (Table 4.17).

Table 4.17**:** Prevalence of cryptosporidiosis by the duration of diarrhea in the pediatric group and the logistic regression output for the in-between group comparisons**.**

Duration of diarrhea	*Cryptosporidium* prevalence	Statistical values for between group comparisons		
≤1 week	10.8%	Odds Ratio	95% CI.	P-Value
>1≤2 weeks	5.9%	0.5147	0.0642 - 4.1284	0.5318
>2 weeks	18.2%	1.8301	0.3648 - 9.1804	0.4627
Unknown duration	8.3%	0.7487	0.3376 - 1.6603	0.4763

When comparing the prevalence of cryptosporidiosis between the groups and using those who had diarrhea for one week and less as the baseline, the logistic regression showed that the odds having cryptosporidiosis were 1.9 times more in those with diarrhea for a duration of less than one week compared to those with diarrhea of between one and two weeks, 1.8 times in those with diarrhea for a duration of more than two weeks when compared to the group with diarrhea for less than one week (Table 4.17).

4.10.2 **Association of Cryptosporidiosis with Abdominal Pain**

In the overall population, the proportion of patients who had complaints of abdominal pain was 28.3% (598/2111). The prevalence of cryptosporidiosis was 4.2% (25/598) among those with abdominal complaints and 4.2% (64/1513) among those patients without complaints of abdominal pains. There was no significant difference in the odds of having cryptosporidiosis between patients with and without abdominal pains (OR= 0.9878, CI= 0.6160 - 1.5841, P= 0.9594). The statistical parameters abdominal pain and other health complaints are shown in Table 4.18.

In the HIV group, 32.8% (588/1794) of the respondents complained of abdominal pains. The prevalence of cryptosporidiosis was 4.3% (25/588) among the patients with abdominal pain complaints and 2.7% (33/1206) in those without abdominal complaints. On logistic regression there was no significant difference in the odds of the occurrence of cryptosporidiosis between having and not having abdominal pains (OR= 1.5784, CI= 0.9298 – 2.6794, P= 0.0910), though the odds of the occurrence of *Cryptosporidium* infection were 1.6 times higher in patients with abdominal pain complaints (Table 4.18).

In the pediatric group, 3.1% (10/319) children had abdominal pains according to the parent or guardian. The rate of cryptosporidiosis among the children with abdominal pains was 0% (0/10) and 10.1% (31/307) in children without abdominal complaints.

Table 4.18: The statistical parameters and association of health complaints with cryptosporidiosis

Study group	Health complaints		Prevalence		Odds ratio	95% CI	P-value
	Symptom	Status	Symptom	*Crypto-sporidium*			
HIV group	Fever	Yes	35.1%	3.0%	0.8993	0.5152-1.5698	0.7088
		No	64.9%	3.3%			
	Cough	Yes	47.7%	3.4%	1.0992	0.6513-1.8548	0.7232
		No	52.3%	3.1%			
	Skin rash	Yes	26.0%	5.4%	2.2179	1.3044-3.7709	0.0033
		No	74%	2.5%			
	Abdominal pain	Yes	32.8%	4.3%	1.5784	0.9298-2.6794	0.0910
		No	67.2%	2.7%			
	Vomiting	Yes	17.3%	4.5%	1.5479	0.8375-2.8610	0.1633
		No	82.7%	3.0%			
	Headache	Yes	42.7%	4.3%	1.8087	1.0664-3.0677	0.0279
		No	57.3	2.4%			
Pediatric group	Fever	Yes	63.3%	9.0%	0.7793	0.3670-1.6650	0.5164
		No	36.7%	11.2%			
	Cough	Yes	57.4%	8.8%	0.7711	0.3670-1.6202	0.4926
		No	42.6%	11.1%			
	Vomiting	Yes	54.9%	7.5%	0.5607	0.2647-1.1877	0.1309
		No	45.1%	12.6%			

4.10.3 **Association of Cryptosporidiosis with Vomiting**

In the overall population, 23.0% (484/2111) of the patients presented with vomiting. The prevalence of cryptosporidiosis among the patients who presented with vomiting was 5.6% (27/484) while it was 3.8% (62/1627) among those patients who did not present with vomiting. On logistic regression there was no significant association of cryptosporidiosis with vomiting (OR= 1.4913, CI= 0.9380 - 2.3710, P= 0.0911), though the odds of the occurrence of the infection were about 1.5 times higher in patients presenting with vomiting (Table 4.18).

In the HIV group, the proportion of patients who presented with vomiting was 17.3% (310/1794). The prevalence of cryptosporidiosis among the patients presenting with vomiting was 4.5% (14/310) and 3.0% (44/1484) in those who did not present with vomiting. There was no significant association between cryptosporidiosis and vomiting (OR= 1.5479, CI= 0.8375 - 2.8610, P= 0.1633), though the odds of having the infection were about 1.5 times higher in patients presenting with vomiting (Table 4.18).

In the pediatric group**,** the proportion of children who presented with vomiting was 54.9% (174/317). The prevalence of cryptosporidiosis in children presenting with vomiting was 7.5% (13/174) while it was 12.6% (18/143) among the children without vomiting. There was no significant difference in the odds of *Cryptosporidium* infection between those presenting with and without vomiting (OR= 0.5607, CI= 0.2647 - 1.1877, P= 0.1309) though the odds were 1.5 times higher in patients without vomiting, (Table 4.18).

4.10.4 **Association of Cryptosporidiosis with fever**

In the total study population, the proportion that had fever complaints was 39.3% (830/2111) and 60.7% (1281/2111) did not have fever. The prevalence of cryptosporidiosis was 4.5 % (37/830) among those who complained of having fever complaints and 4.1% (52/1281) in those who did not complain of fever. On logistic regression analysis there was no significant association between cryptosporidiosis and fever (OR= 1.1028, CI= 0.7167 - 1.6967, P= 0.6564).

Among the HIV positive individuals, 35.1% (629/1794) of the respondents had fever, while 64.9% did not complain of fever. The prevalence of cryptosporidiosis was 3.0% (19/629) among those with fever and 3.3% (39/1165) in those without fever. The logistic regression analysis did not reveal a significant difference in the odds of occurrence of cryptosporidiosis between those with and without fever complaints (OR= 0.8993, CI= 0.5152 - 1.5698, P= 0.7088).

In the pediatric group who were ≤5 years old, 63.3% (201/317) had fever while, the rest did not complaints of fever. The prevalence of cryptosporidiosis was 9.0% (18/201) among the children with fever, and 11.2% (13/116) among those who did not have fever. There was no significant association between *Cryptosporidium* infection and fever (OR= 0.7793, CI=0.3670 - 1.6650, P= 0.5164).

4.10.5 **Association of cryptosporidiosis with cough**

In the overall population, cough was reported in 49.2% (1038/2111) of the respondents. The prevalence of cryptosporidiosis among those with cough was 4.3% (45/1038) and 4.1% (44/1073) in those without cough complaints. On logistic regression analysis there was no significant association between cough and the occurrence of cryptosporidiosis (OR= 1.0598, CI= 0.6932 - 1.6203, P= 0.7886).

In the HIV group, 47.7% (856/1794) of the respondents had **c**ough complaints. The prevalence of cryptosporidiosis among the patients with cough was 3.4% (29/856) and 3.1% (29/938) among the patients without cough complaints. The logistic regression analysis did not show any significant association of cough with the

occurrence of cryptosporidiosis (OR= 1.0992, CI= 0.6513 - 1.8548, P= 0.7232) as in Table 4.18.

In the pediatric group, 57.4% (183/319) of the children were reported having cough by their parents/guardians. The prevalence of cryptosporidiosis was 8.8% (16/182) among the children having cough and 11.1% (15/135) in those who did not have cough. The logistic regression analysis did not show significant association of coughing with the occurrence of cryptosporidiosis (OR= 0.7711, CI= 0.3670 - 1.6202, P= 0.4926) as in Table 4.18.

4.10.6 **Association of Cryptosporidiosis with Skin Rash**

In the overall population, 32.5% (475/2111) of the respondents had skin rash. The prevalence of cryptosporidiosis was 5.3% (25/475) among the patients with skin rash and 3.9% (64/1636) among patients without skin rash. The logistic regression analysis did not show any significant association between occurrence of cryptosporidiosis and skin rash (OR=1.3646, CI= 0.8495 - 2.1919, P= 0.1986), though the odds of infection were about 1.4 times higher in those with skin rash as compared to those who did not have skin rash.

In the HIV group, 26.0% (467/1794) of the respondents had skin rash while 74.0% did not. The prevalence of cryptosporidiosis was 5.4% (25/467) among the patients with skin rash and 2.5% (33/1327) in patients without skin rash. The logistic regression analysis showed a statistically significant association of skin rash with the

occurrence of cryptosporidiosis (OR= 2.2179, CI= 1.3044 - 3.7709, P= 0.0033). The patients with skin rash were about 2.2 times more likely to have cryptosporidiosis compared to the one without skin rash (Table 4.18). The pediatric group did not record skin rash as a health complaint.

4.10.7 **Association of Headache with Cryptosporidiosis**

In the overall population**,** a proportion of 36.7% (775/2111) respondents complained of headache. The prevalence of cryptosporidiosis was 4.3% (33/775) in patients with complaints of headache and 4.2% (56/1335) in patients without headache. There was no significant association of cryptosporidiosis with headache (OR= 1.0158, CI= 0.6545 - 1.5764, P= 0.9444).

In the HIV group, 42.7% (765/1794) of the patients complained of headaches. The prevalence of *Cryptosporidium* infection among those having headache was 4.3% (33/775) while it was 2.4% (25/1355) in those reporting who reported no headaches. There was a significant association of cryptosporidiosis with complaints of headache, (OR= 1.8087, CI= 1.0664 - 3.0677, P= 0.0279) as in Table 4.18. The odds of the occurrence of cryptosporidiosis were about 1.8 times higher in patients with complaints of headaches compared to those without the complaints. Headache was no recorded as a health complaint among the pediatric group.

4.10.8 **Patients without Complaints**

In the overall population, the proportion of respondents who did not have any kind of health complaints constituted 16.1% (340/2111). Among those who reported no complaints the prevalence of cryptosporidiosis was 2.1% (7/340) while it was 4.6% (82/1771) in those who had one type of health complaint or the other. There was a statistically significant association of occurrence of cryptosporidiosis with the presence health complaints (OR= 0.4330, CI= 0.1984 - 0.9451, P= 0.0356). The odds of the occurrence of cryptosporidiosis were about 2.3 times more in those with at least a health complaint compared to those without health complaints.

In the HIV group, 17.8% (320/1794) of the respondents did not have any kind of health complaints. The prevalence of cryptosporidiosis was 1.6% (5/320) in those without health complaints while it was 3.6% (53/1474) among those who had one complaint or the other. There was no significant association of cryptosporidiosis with health complaint (OR= 0.4256, CI= 0.1688 - 1.0732, P= 0.0703) though the odds were about 2.3 times higher in patients with health complaints compared to those without any heath complaint.

In the pediatric group**,** 6.3% (20/317) of the children did not have any of the health complaints. The prevalence of cryptosporidiosis was 10% (2/20) among children without complaints and 9.8% (29/297) in children who had one health complaint or the other. There was no significant difference in the odds of the occurrence of

cryptosporidiosis between those having health complaints and those who did not have any complaints (OR= 1.0268, CI= 0.2268 - 4.6488, P= 0.9726).

4.11 **Cryptosporidiosis and Domesticated Animals**

4.11.1 **Association of domestic animals with Cryptosporidiosis**

The association between domestic animals and cryptosporidiosis was examined for cats, dogs, chicken, sheep, goats and cattle among the HIV positive and pediatric populations. In the HIV study group, a proportion of 10.4 % (187/1794) reported that they had cats in their homesteads 21.5% (387/1794) had dogs, 50.4%, had chicken 27.9% (501/1794) had sheep, 17.2% (309/1794) had goats and 35.7% (640/1794) had cattle. The prevalence of cryptosporidiosis among those with or without the various domestic animals is indicated in Table 4.19. There was no significant association of cryptosporidiosis with the presence of any of the animals in the homesteads (Table 4.19).

Table 4.19: Domestic animals and the statistical values associated with cryptosporidiosis in the study groups

Study group	Domestic animals		Prevalence		Odds ratio	95% CI	P-value
	Animal	Status	Animals	*Crypto-sporidium*			
HIV group	Cats	Yes	10.4 %	4.3%	1.3917	0.6496 - 2.9817	0.3952
		No	89.6%	3.1%			
	Cattle	Yes	35.7%	3.3%	1.0242	0.5942 - 1.7653	0.9314
		No	64.3%	3.2%			
	Chicken	Yes	50.4%	2.8%	0.7393	0.4359 - 1.2537	0.2623
		No	49.6%	3.7%			
	Dogs	Yes	21.5%	1.8%	0.4898	0.2205 - 1.0880	0.0796
		No	78.5%	3.6%			
	Goats	Yes	17.2%	3.2%	1.0013	0.5008 - 2.0020	0.9972
		No	82.8%	3.2%			
	Sheep	Yes	27.9%	3.2 %	0.9826	0.5473 - 1.7643	0.9532
		No	72.1%	3.2 %			
Pediatric group	Cats	Yes	12.9%	5.0%	0.4501	0.1032 - 1.9634	0.2881
		No	87.1%	10.5%			
	Cattle	Yes	22.4%	7.0%	0.6410	0.2369 - 1.7347	0.3813
		No	77.6%	10.6%			
	Chicken	Yes	67.5%	8.7%	0.8225	0.3644 - 1.8565	0.6381
		No	32.5	10.3%			
	Dogs	Yes	16.7%	9.4%	0.9535	0.3487 - 2.6072	0.9261
		No	84.3%	9.8%			
	goats	Yes	14.8%	6.4%	0.5893	0.1719 - 2.0201	0.4002
		No	85.2%	10.4%			
	Sheep	Yes	19.9%	9.5%	0.9642	0.3778 - 2.4608	0.9392
		No	80.1%	9.8%			

In the pediatric group, 12.9% (40/317) had cats, 16.7% (53/317) had dogs, 67.5% (214/317) had chicken, 19.9% (63/317) had sheep, 14.8% (47/317) had goats and 22.4% (71/317) had cattle in their homesteads. The prevalence of cryptosporidiosis among those with and without the various domestic animals together with other statistical findings is shown in Table 4.19. It is worth noting that there was no statistically significant association of cryptosporidiosis with the domestic animals in homesteads.

4.11.2 **Cryptosporidiosis and Patients without Animals**.

In the overall population, the homesteads, which did not have any animals, constituted 38.1% (804/2111) while 61.9% had one or more types of animals in their homesteads. The cryptosporidiosis prevalence was 4.2% (34/804) among those without animals and 4.2% (55/1307) among those who had at least one type of animal. On logistic analysis, there was no significant difference in the odds of *Cryptosporidium* infection between having and not having animals in the homesteads (OR= 1.0051, CI= 0.6494 - 1.5559, P=0.9816).

In the HIV group, 37.3% (669/1794) of respondents did not have any animals in their homesteads while 62.6% (1125/1794) had at least one type of animal. The prevalence of cryptosporidiosis among those who did not have animals was 3.6% (24/669) and 3.0% (34/1125) among those who had animals. On logistic regression analysis there was no significant difference in the odds of *Cryptosporidium* infection between those having and those not having animals in their homesteads, (OR= 1.1940, CI= 0.7017 - 2.0316, P= 0.5133).

In the pediatric group, 42.6% (135/317) of the respondents reported not having any animals in their homesteads while 58.0% had at least one type of animal or the other. The prevalence of cryptosporidiosis among those who did not have animals was 7.4% (10/135) and 11.5% (21/182) in those who had at least one type of animal. The logistic regression output did not show any significant difference in the odds of

Cryptosporidium infection between those with and without animals within the homesteads, (OR= 0.6133, CI= 0.2788 - 1.3492, P= 0.2242) though the odds were about 1.6 times higher among those who had at least one type of animal in their homesteads compared to those who did not have any animals in their homesteads.

4.12 **Association of Cryptosporidiosis with Housing and Occupancy**

In the total study population, the average number of rooms in the houses of the respondents was 2.5 in the urban residences with the mean number of 3 occupants per room, 1.8 in peri-urban with an average of 4 persons per room, and 2.3 in rural residences with a mean of 5 persons per room. The mean number of rooms and occupants was significantly different between the residences (P≤ 0.002) and (P= ≤0.001) respectively. There was no significant association of cryptosporidiosis with number of rooms in a house (OR= 0.8976, CI= 0.7231 – 1.1144, P= 0.3278) or the number of occupants (OR= 1.0227, CI= 0.9334 – 1.1204, P= 0.6304) on logistic regression analysis.

The analysis of variance (ANOVA) showed that the mean number of rooms was significantly different between the areas of residence occupied by the respondents who were negative for cryptosporidiosis (P≤ 0.001), but there was no significant difference in the number of rooms in houses occupied by cryptosporidiosis positive patients in the various residences (P= 0.1481).

In the HIV group, the overall mean number of rooms per house was 2 with a mean occupancy of 4 persons. The mean number of rooms in the various residencies was 2.6 in urban, 1.9 peri-urban and 2.4 rooms in rural residences with the mean number of occupants being 3, 5 and 4 respectively. The number of rooms did not show significant association with cryptosporidiosis on logistic analysis among HIV positive patients (OR= 0.8252, CI= 0.6287 - 1.0829, P= 0.1659) neither did the number of occupants per house (OR= 1.0217, CI= 0.9157 – 1.1401, P= 0.7008).

The mean number of rooms was significantly different between areas of residence ($P \leq 0.002$). The mean number of rooms among the cryptosporidiosis negative patients was 2.6 in the urban residence, 1.9 peri-urban and 2.4 rural areas of residence. In the cryptosporidiosis positive patients, the mean number of rooms was 1.3 in urban, 1.6 in peri-urban and 2.2 in the rural residences. The mean number of rooms was significantly different between the residences in both the cryptosporidiosis negative ($P \leq 0.002$) and cryptosporidiosis positive patients (P= 0.0425). Further, there was no significant difference in the odds of *Cryptosporidium* infection between those occupying houses with 2 rooms (OR= 0.6716, CI= 0.3548-1.2711, P= 0.2213), 3 rooms (OR= 0.6439, CI= 0.3102-1.3367, P= 0.2375), and 4 rooms (OR= 0.5915, CI= 0.2377-1.4721, P= 0.2590) when single roomed houses were used as baseline in the logistic regression analysis. However, the odds of infection with *Cryptosporidium* were about 1.5, 1.6, and 1.7 times higher in single roomed houses compared to 2, 3, and 4-roomed houses respectively.

In the pediatric group, the overall mean number of rooms was 2.0. The mean number of rooms in the urban residences was 2.1 with a mean occupancy of 4 persons, peri-urban 1.7 with 4 persons and 2.2 in the rural residences with a mean occupancy of 5 persons. The mean number of rooms and occupancy were significantly different between the residences ($P \leq 0.001$) and ($P = 0.003$) respectively. On stratifying for cryptosporidiosis the mean number of rooms ($P \leq 0.001$) and mean occupancy ($P = 0.003$) were found to be significantly different in the cryptosporidiosis negative households. In the cryptosporidiosis positive homesteads, the means of number of rooms were not significantly different in the cryptosporidiosis positive households ($P= 0.7951$) but the mean occupancy was significantly different ($P = 0.003$) between the areas of residence. On logistic analysis though, there was no significant association between the number of rooms (OR= 1.1864, CI= 0.8342 - 1.6872, P= 0.3415) and the mean occupancy (OR= 1.0271, CI= 0.8563 - 1.2319, P= 0.7731) with the occurrence of cryptosporidiosis.

However, the odds of *Cryptosporidium* infection were 2 times, 1.3 times and 1.8 times higher in single roomed houses than in 2, 3 and 4-roomed houses respectively.

4.13 **MOLECULAR ANALYSIS**

4.13.1 **The Circulating *Cryptosporidium* Genotypes**

Out of the 89 isolates obtained 72 amplified, while 17 failed to amplify. The secondary PCR (Section 3.7.2) band products (826-864 bp) that are diagnostic of the *Cryptosporidium* species are shown in Figure 4.16.

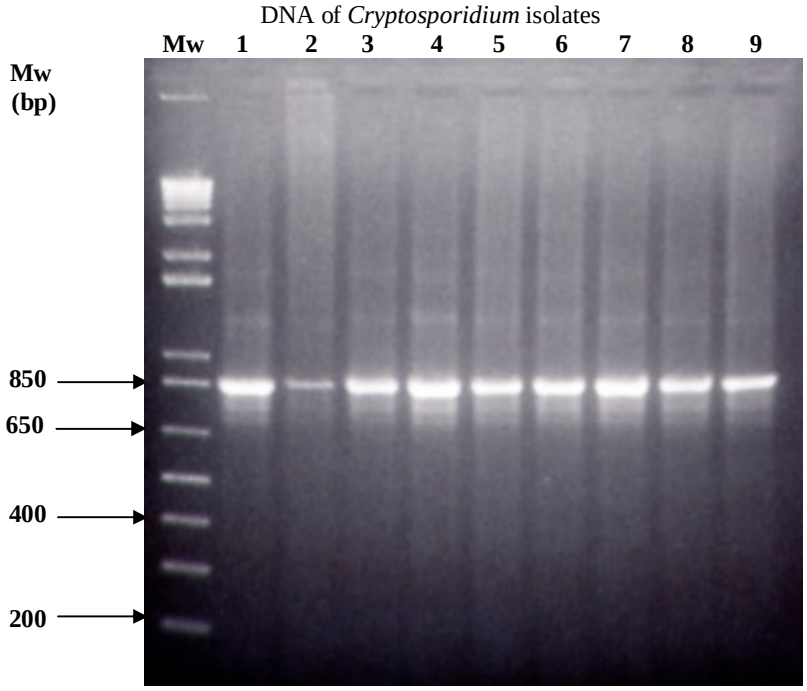

Figure 4.16: Agarose gel electrophoresis showing the nested PCR products of the of 18S rRNA gene (826 - 864 bp), diagnostic of *Cryptosporidium* species separated on 1.5% agarose gel stained with ethidium bromide. Lane 1:Sample A243, lane 2: P125, lane 3:A566, lane4: C504, lane5: P253, lane 6: P252, lane7: A594, lane 8: C537 and lane 9: A659.

The RFLP analysis using *Ssp*1 enzyme, and *Vsp*1 restriction enzymes (section 3.7.2) was used to identify the genotypes whose sample gel photograph is shown in Figure 4.17.

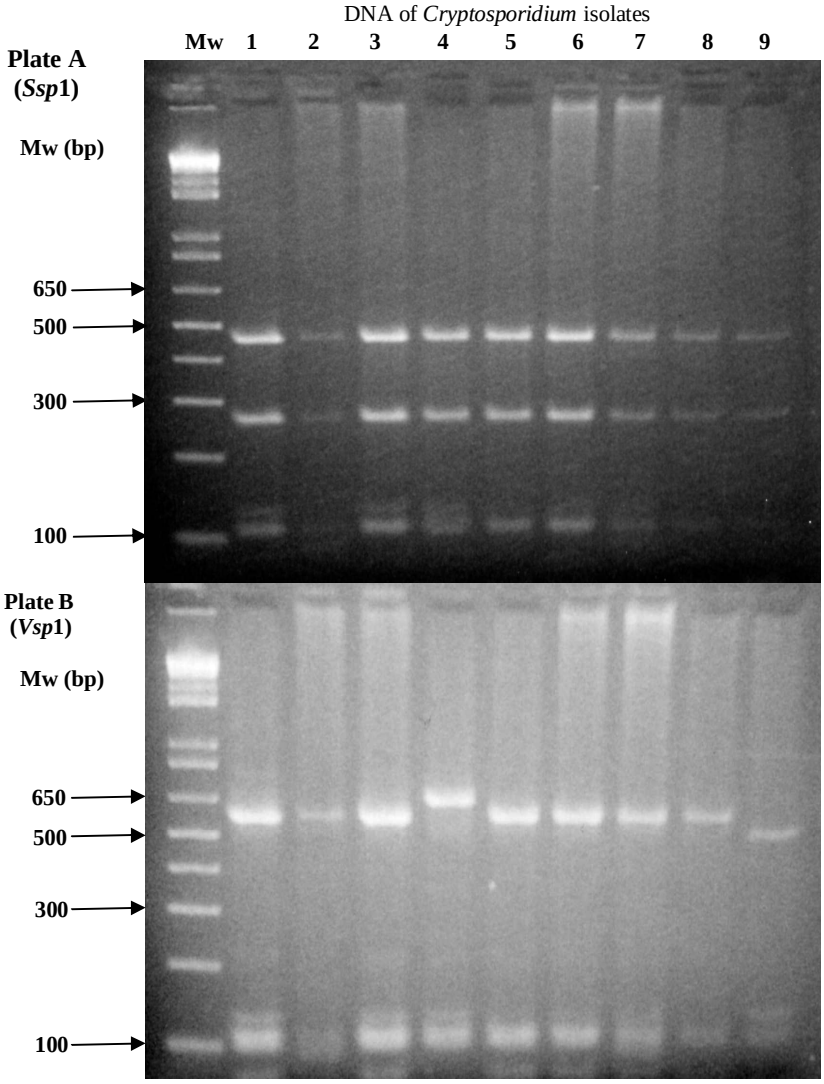

Figure 4.17: Genotyping of the *Cryptosporidium* species by a nested PCR-RFLP procedure based on the 18s rRNA gene sequences. Plate A: *Ssp*1 digestion products and Plate B *Vsp*1 digestion products. The fragments were separated on 2.0% agarose gel stained with ethidium bromide. Lane1 Molecular weight marker, Lanes 2,3,4,6,7 and 8, *C. hominis*, lane 5 *C .parvum* and lane 9 *C. meleagridis*.

The 72 isolates that amplified were genotyped into *C. hominis* 73.6% (53/72), *C. meleagridis* 8.3% (6/72) and *C. parvum* 18.1% (13/72) as shown in Figure 4.18.

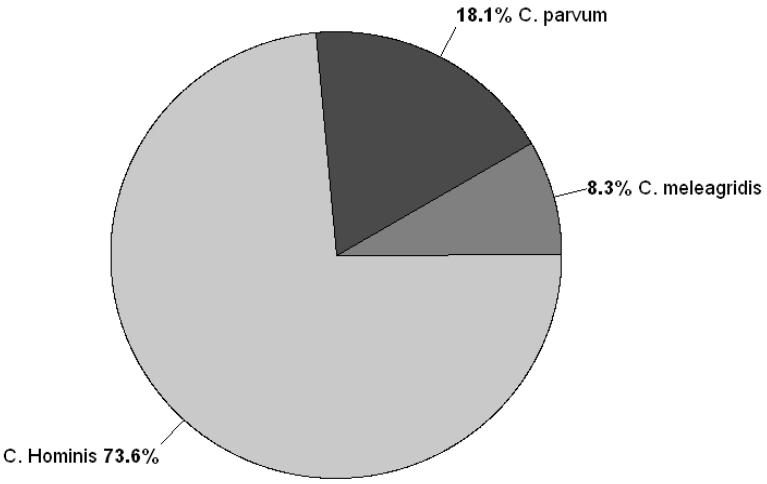

Figure 4.18: The proportion of the three genotypes of *Cryptosporidium* isolates.

4.13.2 **Distribution of the *Cryptosporidium* Species**

In the HIV population, *C. hominis* was found in 68.2% (30/44). Of these, one was from the urban residence, 33.3% (10/30) were form peri-urban residences and 63.3% (19/30) were from rural residences. *C. parvum* constituted 18.2% (8/44) of the total isolates from HIV positive patients, of which was one isolate was from a peri-urban residence and seven isolates were from the rural residences. The proportion of *C. meleagridis* in the total isolates was 13.6% (6/44). The isolates were one from urban residence, two from peri-urban and 3 from the rural residences. The distribution of *Cryptosporidium* genotypes in the study populations by study site and areas of residence is summarized in Tables 4.20 and 4.21 respectively.

Table 4.20: Distribution of *Cryptosporidium* isolates among the study populations by study site

STUDY GROUP	Study site	Number of isolates	*C. hominis*	*C. parvum*	*C. meleagridis*	Failed to amplify
HIV Positive population	Burnt Forest	12	3	1	1	7
	MTRH	32	19	2	5	6
	Turbo	14	8	5	-	1
Pediatric population	MTRH	31	23	5	-	3

In the Pediatric population, only two genotypes were isolated that is *C. hominis*, which constituted 82.1% (23/28) of the isolates. The *C. hominis* isolates were from peri-urban areas 60.9% (14/23) and 39.1% (9/23) were from patients from rural areas of residence. *Cryptosporidium parvum* constituted 17.9% (5/28) of the isolates of which four were from patients staying in the peri-urban areas and one from the rural residence. The youngest patient with *C. hominis* infection was six months old and the oldest was twenty eight months old.

Table 4.21: Distribution of *Cryptosporidium* isolates among the study populations by areas of residence

Study population	Area of residence	Number of isolates	*C. hominis*	*C. parvum*	*C. meleagridis*	Failed to amplify
HIV positive population	Urban	4	1	-	1	2
	Peri-urban	17	10	1	2	4
	Rural	37	19	7	3	8
Pediatric population	Urban	-	-	-	-	-
	Peri-urban	21	14	4	-	3
	Rural	10	9	1	-	0

4.13.3 **Sub-typing of the *Cryptosporidium* Species**

The nested PCR of the CP47 locus (Section 3.7.3.1) gave bands of 300-500 bp as shown in Figure 4.19.

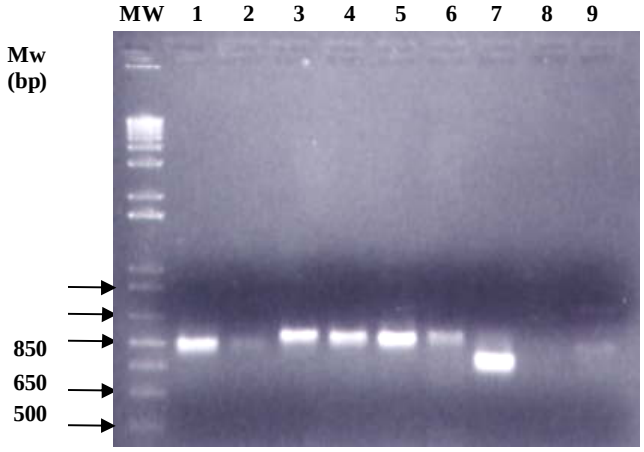

Figure 4.19: Agarose gel electrophoresis picture showing the 300-500 bp band products of the nested PCR of CP47 gene on 1.5% agarose gel stained with ethidium bromide. Lane 1:A659, lane 2: A643, lane 3: P94, lane4: C295, lane 5: C105, lane 6:P190, lane 7:P263 and Lane 9: A347 Sample in lane 8 failed to amplify

After the purification of the DNA from the agarose gel, 5µL of the yield were run on a 2% agarose gel to ascertain the presence of DNA as shown in Figure 4.20.

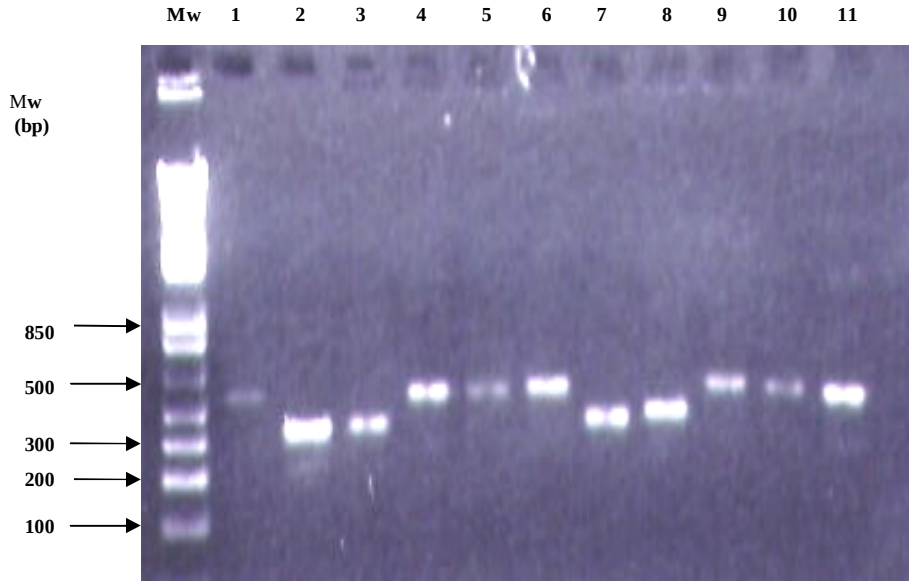

Figure 4.20: Agarose gel electrophoresis picture of the purified CP47 PCR fragments (300 – 500 bp) separated on a 2% agarose gel stained with ethidium bromide. Lane 1: Sample A130, lane 2: A1126, lane 3: A113, Lane 4: P233, lane 5:A242, lane 6:P229, lane 8: P238, Lane 9: P102, Lane 10:P112 and lane 11:R161

4.13.3.1 **Sequencing of the CP47 PCR Products**

Of the 46 samples that were sequenced, 6 failed to amplify during sequencing while 5 amplified very short fragments that could not be blasted and another five were less than 300 base pairs and therefore could not be analyzed. The sequences obtained from the sequencer after manual adjustments are shown in Appendix 1.

4.13.3.2 **Sequence Alignment**

The 30 samples that yielded clean CP47 sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The aligned sequences (Appendix 1) were used to draw the phylogenetic trees.

4.13.4 **Phylogenetic Tree**

Based on aligned sequences (Appendix 1) of the CP47 gene the phylogenetic tree in Figure 4.21 was obtained which separated the thirty isolates into two distinct groups, *C. parvum,* eleven samples and *C. hominis,* nineteen samples.

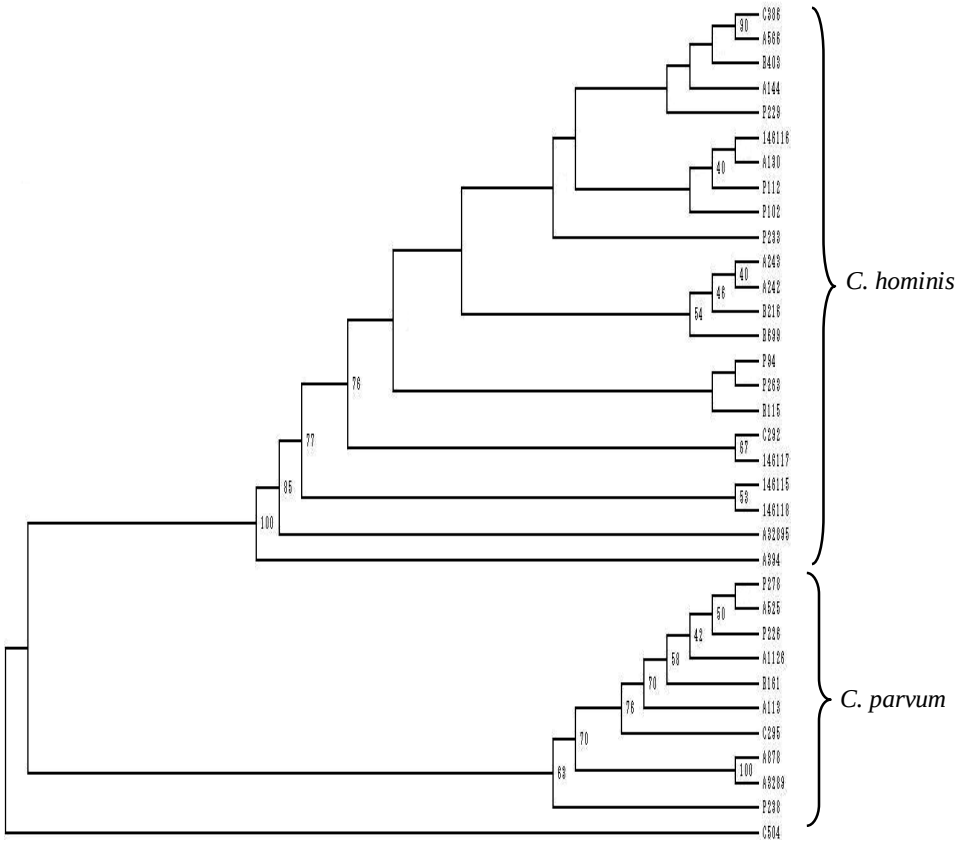

Figure 4.21**:** Phylogenetic tree of *C. hominis* and *C. parvum* isolates. Bootstrap values greater than 40% are shown.

Figure 4.21 shows a clear distinction between the genotypes of *C. hominis* and *C. parvum* isolates from the North Rift region of Kenya.

Based on sequence length polymorphism the *C. hominis* genotype was further divided into 7 sub-types A394, A32895, P94, P263, C292, and R115, R699, B216, A242 and A243, R403, A144, P229, P102, A130, P112, P233 and C386 and A566)

while among the *C. parvum* had four sub-genotypes C504, A878 and A3289, P238 and C295, A113, A1126, R161, A525, P226, and P298 (Figures 4.21).

4.13.6 **Trinucleotide Analysis**

This was done by assigning the CP47 locus of *C. hominis* as type I and *C. parvum* as type II and for the subtype identification, the trinucleotide TAA was coded as A and the TGA/TAG was coded as G with the following digit showing the number of each trinucleotide repeat. Twelve subtypes within the *C. hominis* species were identified as IA43G14 (5.26%), IA47G22 (5.26%), IA48G23 (5.26%), IA52G29 (37.5%), IA52G30 (5.26%), IA53G29 (5.26%), IA53G31 (5.26%), IA54G30 (5.26%), IA54G31 (10.52%), IA55G32 (5.26%), IA56G33 (10.52%), and A57G26 (5.26%) while in the *C. parvum*, eight subtypes were identified as IIA30G12 (9.1%), IIA31G14 (9.1%), IIA34G14 (9.1%), IIA35G15 (18.2%), IIA36G14 (9.1%), IIA36G16 (9.1%), IIA37G15 (27.3%) and IIA38G15 (9.1%).

CHAPTER 5

DISCUSSION

5.1 The Prevalence and Seasonality of Cryptosporidiosis in the North Rift Region

In the present study the overall prevalence of cryptosporidiosis in the North Rift region of Kenya was 4.2% (Section 4.4) with *C hominis* being the most prevalent followed by *C. parvum* and *C. meleagridis* in that order (Section 4.13.1). These findings are similar to those obtained from studies elsewhere in Kenya (Estambale *et al.,* 1989, Simango *et al,*. 2004, Gatei *et al,*. 2006). Prevalence of cryptosporidiosis in the North Rift region revealed a bimodal seasonal pattern of distribution with a higher peak during the dry season (February – March) and a smaller peak (June - July). The transmission during both seasons was more of anthroponotic than zoonotic. This pattern was similar to that observed by Gatei *et al.* (2006) in Central Kenya, Bern *et al*. (2002) in Brazil, and Amin (2002) in the United States. Elsewhere in the world, however, peak transmission is during hot and humid seasons as reviewed by Fayer *et al.* (1986). A peak transmission season during the late summer have been observed in Canada (Laupland and Church, 2005), South Africa (Fripp *et al.*, 1991), and USA (Jonathan and Michael, 2007), whereas in Spain peak transmission of cryptosporidiosis occurred during autumn-winter period (Clavel *et al.*, 1996). Results of the present study and previous studies suggest that seasonal prevalence of cryptosporidiosis varies from place to place probably due to differences in risk factors and determinants that may be unique for different regions, which in turn may influence the pattern of *Cryptosporidium* transmission. Water was

not implicated as the main transmission route of *Cryptosporidium* in the north Rift region but rather the findings incriminate anthroponotic and zoonotic transmission as routes of transmission of cryptosporidiosis. More comprehensive studies need to be undertaken to gain insight into the factors and issues influencing the transmission of *Cryptosporidium* species in the North Rift region of Kenya.

5.2 **HIV and Cryptosporidiosis**

Prevalence of cryptosporidiosis in the North Rift region observed among the HIV infected persons was 3.2% (Section 4.4), which falls within the range of 3-4% observed in Malaysia (Lim *et al.*, 2005) and in Los Angels, USA (Sorvillo *et al.*, 1994). Fayer *et al.* (1986)) observed that cryptosporidiosis is highest in poorly developed countries because of poor hygienic and low socioeconomic standards. Prevalence of cryptosporidiosis was higher among the HIV infected children aged 5 years or less than in those HIV infection persons aged above 5 years old. The HIV infected patients who were five years or less had a higher risk of infection with *Cryptosporidium* than those HIV infected aged 5 years old and above. Though the study demonstrated a higher prevalence of cryptosporidiosis in children with HIV/AIDS, the finding was not statistically significant probably due the small number (60/1794) of HIV positive children that were captured during the study. Even then, these findings are in tandem with reports by Moolasart *et al.* (1995) in Thailand, Skeets *et al.* (1990) in Oregon, and Sorvillo *et al.* (1994) in Los Angeles County, both in the USA which showed that cryptosporidiosis prevalence was higher in HIV infected children than in the HIV positive adults. The higher prevalence and

risk of infection with *Cryptosporidium* in children is probably because children have greater susceptibility to infections attributable to an immature immune system, and the possibility of a fecal-oral transmission of the infections. There is need, therefore, for the health care providers and those involved in the management of HIV/AIDS in children to come up with novel ways of preventing and controlling diarrhea illnesses in HIV positive children, and the inclusion of cryptosporidiosis diagnosis as routine procedure in the examination of stool of diarrheic children.

The mean CD4 count among the cryptosporidiosis infected HIV patients was 76.21 cells per microlitre of blood as compared with the mean of 244.1 cells per microlitre among the cryptosporidiosis negative patients (Section 4.4) indicating that patients are more likely to contract *Cryptosporidium* infection with the lowering of the immune status as evidenced by low CD4 counts. However, the mean CD4 counts were not significantly different between diarrheic and non-diarrheic patients. There was a strong association of cryptosporidiosis and diarrhea ($P \leq 0.002$), and that the odds of infection were significantly higher in those patients who had diarrhea for more than two weeks compared to those who had it for less than two weeks, ($P = 0.0017$). This shows that cryptosporidiosis is strongly associated with chronic diarrhea in HIV infected patients. There is need however for a more comprehensive assessment of all possible opportunistic diarrheic agents in HIV patients to establish the complete picture of the etiology of diarrhea in HIV patients and the impact of the interaction of these agents on the severity of diarrhea and the progression of the HIV/AIDS syndrome.

Cryptosporidiosis prevalence of 3-44% among HIV/AIDS patients have been reported by Mwachari *et al*. (2000) in Nairobi Kenya, Ribeiro *et al*. (2004) in Brazil, Bonilla *et al*. (1992) in Venezuela, Taherkhani *et al* (2007) in Iran, Prakash *et al* (2004) in Nepal and Ramakrishnan *et al*. (2007) in India using the same diagnostic technique used in the current study. These trends of the reported burden of cryptosporidiosis suggest that each country or region should be treated as being unique due to the varied ecological conditions, human activities and socioeconomic factors that may influence the various routes of transmission, and therefore collective condemnation as high prevalence regions or otherwise should be treated with caution. There is need for more elaborate investigations to cover the divergent and convergent factors that influence the transmission of cryptosporidiosis.

About 74% of the isolates were identified as *C. hominis*, 8.3% as *C. meleagridis* and as 18.1% *C. parvum* by PCR-RFLP analysis of the 18s RNA gene. In the HIV infected patients on the other hand, 68.2% of the isolates were *C. hominis*, 18.2% *C. parvum* and 13.6% were *C. meleagridis*. These findings compare very well with those of by Gatei *et al*. (2003) using *Cryptosporidium* isolates from Kenya, Malawi, Brazil, Vietnam, and United Kingdom. They also compare well with those of Coupe *et al.* (2005) in Dijon Hospital in France, Zavvar *et al*. (2008) in Iran and Raccurt *et al.*, (2006) in Haiti in which *C. hominis* was found to be the most prevalent among HIV infected patients followed by *C. parvum* and then *C. meleagridis*. The foregoing suggests that person-to-person and probably environmental transmission is more common than zoonotic transmissions in the North Rift region of Kenya. In contrast, the prevalence of *C. parvum* in parts of some European countries namely France,

England and Northern Ireland is reported to be higher than *C. hominis* (Guyot *et al*., 2001, Leoni *et al*., 2006, Lowery *et al*., 2001). There are no explanations proffered so far, as to why the trends in these regions are at variance with the rest of the world.

5.3 **Cryptosporidiosis in the Pediatric Population**

The HIV status of the children enrolled in this study, who were under five years of age, was not determined. A prevalence as 9.8% was recorded in this study (Section 4.4), which is higher than that of 4% observed by Gatei *et al*. (2006) which also concluded that cryptosporidiosis is one of the major causes of diarrhea in children. Higher prevalence values of between 10% and 32% in Africa have been reported in Uganda (Tumwine *et al*., 2003), Tanzania (Cegielski *et al*., 1999) and Egypt (Abdel-Messih *et al*., 2005). In Switzerland, a prevalence of 5.5% was reported in children (Glaecer *et al*., 2004). It is worth noting that all the studies cited above used the modified ZN-staining to demonstrate the presence of *Cryptosporidium* oocysts in stool samples. In as much as there are variations observed in the prevalence levels between regions, these reports and the results of the current study reinforce the fact that *Cryptosporidium* infections are among the major causes of morbidity among children not only in the North Rift region but in other parts of the world. In addition, each region it appears may have unique factors that determine the epidemiology and transmission of *Cryptosporidium* species. This further mitigates for the routine stool examination for *Cryptosporidium* oocysts, more so in children with diarrhea as a measure of effective management of diarrhea in children.

Molecular analysis of *Cryptosporidium* isolates from children showed that 82.1% of the isolates were *C. hominis*, 17.9% as *C. parvum* (Section 4.13.2). The foregoing suggests that there is more of an anthroponotic and or environmental transmission, than a zoonotic transmission in children in the North Rift region of Kenya. These findings are consistent with those of Tumwine *et al.* (2003) among children in Mulago hospital in Kampala in which 73.7% of the isolates were *C hominis* and 19.2% were *C. parvum.* Raccurt *et al*. (2006) has also reported that 72% of the children regardless of their HIV status in a study conducted in Haiti were infected with *C. hominis*. These studies underpin the roles played by person-to-person and environmental transmission of cryptosporidiosis. Therefore, there is need for concerted efforts towards proper sanitation, personal hygiene and environmental management in a bid to reduce and control the spread of this disease.

5.4 **Cryptosporidiosis and Location of Residence**

There was no significant association between areas of residence and cryptosporidiosis in the HIV positive population though the odds of infection were about 2.8 and 2.2 times higher in urban areas of residence compared to peri-urban and rural areas of residence respectively (Section 4.6). This may be explained, though not investigated in the current study, by the fact that transmission of HIV is higher in the urban and peri-urban settings because the commercial sexual activities involving long distance truck drivers and the locals along the busy Mombasa-Nairobi-Nakuru-Malaba highway that runs through the study area.

However, the trend was opposite in the pediatric group whereby cryptosporidiosis prevalence was higher in children from peri-urban and rural areas of residence compared to those residing in urban areas. Even then, living in the peri-urban areas presented a greater risk of infection (OR= 1.75) with *Cryptosporidium* than living in the rural areas (Section 4.6). This trend compares well with the findings of Gatei *et al.* (2006) elsewhere in Kenya, which showed that most children with cryptosporidiosis lived in peri-urban areas followed by those in rural areas and then urban areas. The peri-urban residential areas present greater risk because they are densely populated mostly with families of low socioeconomic status, have limited or poor sanitary infrastructure and water supply system, a situation that is made worse by having pit latrines located a few meters from water points within small plots. Elsewhere in the Texas-Mexico border, Leach *et al*. (2000) has also demonstrated that areas that are densely populated and with poor sanitary facilities portend greater risk for *Cryptosporidium* infection among children. The rural and peri-urban areas of the North Rift region of Kenya need a comprehensive field based study to investigate fully the risk factors associated with cryptosporidiosis.

5.5 **Cryptosporidiosis, water and Sanitation**

The main method of human waste disposal used by the respondents was pit latrines (Section 4.9). However, those disposing their waste in the bush were at a highest risk of infection with *Cryptosporidium* among the HIV patients while the flash toilet users were at a higher risk of infection among the pediatric population. The association of higher risk with flash toilets is a pointer to the effect that having a

flash toilet without commensurate and proper hygienic conditions and practices may not protect children from infections including cryptosporidiosis. Elsewhere, unsanitary living conditions that include and not limited to paucity of clean drinking water, improper sewage or waste disposal facilities have been associated with *Cryptosporidium* infections (Palit *et al*., 2005).

The study populations mostly used wells or boreholes and piped water as the main sources of domestic water (Section 4.7). However, there was no significant association between sources of water and cryptosporidiosis and indeed the odds of infection were similar in the HIV positive population based on water sources. However, users of piped water amongst the pediatric group were at a greater risk of infection followed by those using wells or boreholes thus incriminating the hygienic conditions, which may be favoring anthroponotic transmission. These findings are similar to those along the Mexican-Texas border (Leach *et al*., 2000) and in Guatemala (Laubach *et al*., 2004) which implicated consumption of municipal water, contaminated water and poor sanitation services as risk factors for *Cryptosporidium* infection. However, without carefully case controlled studies, implication of water sources may be anecdotal. Even then, good environmental management practices and personal hygiene still remain central in reducing chances of infection since most families in the rural areas use either springs or wells or boreholes as sources of water for domestic use which can be easily contaminated by human and animal waste.

The various water treatment methods investigated in this study did not confer protection against acquiring *Cryptosporidium* infections (Section 4.8). In fact, those boiling their drinking water were at a higher risk than those using chemicals to treat their drinking water and yet boiling water is known to be the most effective method of killing the *Cryptosporidium* oocysts (CDC, 1994). This is so probably due to unhygienic handling and storage of drinking water after boiling.

5.6 Cryptosporidiosis and Health Symptoms

Diarrhea was significantly associated with the presence of cryptosporidiosis among the HIV positive patients (OR= 4.7), and that it was more frequent in those living in the urban residences (Section 4.10.1). The rural and urban residences were similar in the association of diarrhea with cryptosporidiosis. However, diarrhea was more frequent among the HIV positive children with a cryptosporidiosis prevalence of 11.7% and a significantly higher statistical chance of having cryptosporidiosis (OR= 4.3) compared to the children without diarrhea. These results are in tandem with those reported by Barboni *et al*. (2008) in Argentina (13.7%), Moolasart *et al*. (1995) in Thailand (19%), among diarrheic HIV positive children. The prevalence of cryptosporidiosis among diarrheic children in the present study is way below that of 33.3% reported by Brandonisio *et al*. (1993) in Italy, 73.6% by Tumwine *et al*. (2005) in Uganda among HIV positive children with persistent diarrhea. The report by Tumwine *et al*. (2005) is one of the highest rates reported, a trend that may be explained by the fact that the study used commercial kits based on direct-fluorescent monoclonal antibody test for the detection of *Cryptosporidium* oocysts that is more sensitive than the ZN staining microscopy that was used in this study. These studies

therefore, give credence to the fact diarrhea illness is worse in HIV infected children because of the lowered immunity and the possibility of the presence of many other diarrheic agents that compound the episodes, hence the need of concerted efforts of preventing and controlling diarrhea in these children.

Except for headache (OR=, 2.218, P= 0.0033) and skin rash (OR= 2.2179, P=0.0279), other health complaints such as fever, cough and abdominal pains did not show any statistically significant association with cryptosporidiosis. Headache and skin rash are common complaints among HIV patients and are therefore an indication of a declining immune status, which in turn is associated with increased chances of acquiring opportunistic infections including cryptosporidiosis as evidenced by the low CD4 counts among those patients infected with *Cryptosporidium* species in the current study (Section 4.4). In the pediatric group, none of the health complaints was significantly associated with cryptosporidiosis. These results are in agreement with those of Adjei *et al*. (2004) in diarrheic children in Korle-Bu Teaching Hospital, Accra, Ghana in which the clinical symptoms of abdominal pain, nausea, vomiting, fever and blood in stool were not significantly associated with cryptosporidiosis. However, this is converse to the findings of a study conducted in Nairobi, Kenya by Gatei *et al.* (2006) in which vomiting was significantly associated with cryptosporidiosis in children and by Al-Hindi *et al*. (2007) in Al-Nasser pediatric hospital in which abdominal pain was significantly associated with cryptosporidiosis in children aged five years and below. These observations therefore indicate that it is difficult to diagnose cryptosporidiosis on basis of clinical symptoms alone.

5.7 **Cryptosporidiosis and Domestic Animals**

In the current study, the association of cryptosporidiosis with the keeping of livestock was investigated because cryptosporidiosis has been associated with both farm and wild animals (Fayer *et al*., 2000). Unexpectedly, the odds of infection were higher among those who did not have animals within their homesteads. For instance, the odds were 1.6 times more in those without cattle, 2.2 times more in those without cats, 1.7 in those without goats, 1.2 in those without chicken, 1.04 in those without dogs and 1.03 in those without sheep (Section 4.11.1). In all instances, it is implied that the odds of infection were higher in those without domesticated animals suggesting that zoonotic transmission is not a major route of the transmission of cryptosporidiosis in the North Rift region. The findings of this study negates the initial assumption that since the North Rift region is famed for animal husbandry, then cryptosporidiosis particularly that due to *C. parvum* could be significantly associated with domesticated animals. However, the findings of this study are in tandem with those of Adjei *et al*. (2004) in Ghana, Pereira *et al*. (2002) in Brazil, which also found no significant association of cryptosporidiosis with the keeping of or proximity to domestic animals. It is, however in contrast with published reports by Mahdi *et al*., (2002), Kiang *et al*., (2006) whereby domestic and farm animals have been directly associated with cryptosporidiosis outbreaks among humans. More elaborate studies need to be carried out to establish the nature of transmission of *Cryptosporidium* species in the North Rift and other areas of Kenya given that this study did not involve persons working in large animal farms neither did it check for the prevalence of cryptosporidiosis in animals in such farms.

5.8 Cryptosporidiosis and Housing

Respondents from peri-urban residential areas had the lowest average number of rooms per a household, an indication of a low socioeconomic status, followed by the rural and finally the urban residential areas of residence (Section 4.12). The mean number of rooms was significantly different between the residential areas among the HIV positive population, the cryptosporidiosis negative and positive households. However, in the pediatric group the mean number of rooms among the cryptosporidiosis negative households was statistically different between the areas of residence but similar among the cryptosporidiosis positive patients and that the mean number of occupants per house was statistically different in both *Cryptosporidium* positive and negative households. In both the HIV and pediatric populations, the odds of infection with *Cryptosporidium* were higher in households with a higher household occupancy per room. The foregoing suggests that regardless of where the respondents resided, it is the socioeconomic status and not the area of residence, which primarily impacts on sanitation, personal hygiene and ones ability to improve the health status of the household members that predisposes one to the possibility of infection with *Cryptosporidium* parasites.

5.9 The Circulating *Cryptosporidium* Genotypes and Sub-types

The leading cause of cryptosporidiosis in the North Rift region of Kenya was found to be *C. hominis* followed by *C. parvum* and *C. meleagridis* in that order (Section 4.13.2). Based on length polymorphism, there were 7 C. *hominis* subtypes, which

were obtained mainly from patients who resided in rural and peri-urban areas. Inasmuch as the CP47 sequence of isolates in the current study had up to 99% similarity with other Kenyan isolates deposited with the European Molecular Biology Laboratory (EMBL) data bank by Gatei *et al*., (2006), they were different based on the trinucleotide repeat analysis as described by Gatei *et al*. (2007). The subtyping based on the trinucleotide repeats yielded 12 subtypes of *C. hominis* from the North Rift region of Kenya (Section 4.13.6), which interestingly were different from those, reported elsewhere in Kenya by Gatei *et al*. (2002), neither did the subtypes show any bias in distribution between the rural or peri-urban areas of residence. These results and the report of Gatei *et al*. (2002), in which a batch of Kenyan samples revealed that there were 9 unique subtypes of *C. hominis*, implies that there are many *Cryptosporidium* subtypes circulating in Kenya some of which could be unique to specific regions of the country.

There were four subtypes identified among the *C. parvum* isolates by use of restriction fragment length polymorphism, but 8 subtypes were identified using the trinucleotide repeat analysis. Most of the *C. parvum* subtypes were isolated from patients residing in rural and peri-urban areas. This is the first report of the *C. parvum* subtypes in Kenya using the CP47 microsatellite gene. Since this genotype of *Cryptosporidium* can be transmitted both through the anthroponotic, zoonotic route and even through environmental contamination (Feng *et al*. 2000), it is difficult to ascertain the extent to which these modes of transmission are involved in the spread of cryptosporidiosis due to it in the North Rift region of Kenya. This therefore calls for a deeper understanding of the epidemiology of cryptosporidiosis in this

region so as to zero in on determinants that govern the distribution of the various genotypes and subtypes. One patient (1.1% of all the cryptosporidiosis positive patients) who was also HIV positive and lived in a rural area had a mixed infection of *C. parvum* and *C. hominis*, a finding that is consistent with that of Leoni *et al*. (2006) in Britain. The presence of mixed infection is an indication of contact with human or animal waste or water that was contaminated with either genotype or both.

This study has revealed that there are more *C. hominis* subtypes that are circulating in the region as compared to *C. parvum* subtypes. There is need, therefore, for a more comprehensive study to look into the *C. hominis* and *C. parvum* populations in terms of the circulating genotypes and subtypes in the North Rift Region of Kenya using more subtyping tools in an endeavor to establish associations of cryptosporidiosis with the various parameters that influence and/or encourage genetic mixing and transmission.

CHAPTER 6

CONCLUSIONS AND RECOMENDATIONS

6.1 Conclusions

This study established the prevalence among HIV positive patients as 3.2% with *C. hominis* being the leading cause of cryptosporidiosis followed by *C. parvum* and then *C. meleagridis* in that order. It has also established indeed as other studies that mixed *C. hominis* and *C. parvum* constitute about 1% of the cryptosporidiosis burden. The average CD4 count in HIV positive persons with cryptosporidiosis was 76.21 cells per µl. In children of five years and below the prevalence was found to be 9.8% whereby *C. hominis* was the leading cause of cryptosporidiosis followed by *C. parvum*.

All the *C. hominis* and *C. parvum* isolates from children were from the peri-urban and rural areas while in the HIV infected patients most of the isolates were from those living in the urban areas. Overall, the frequency of *C. hominis* was higher in the peri-urban population as compared to the rural population, whereas *C. parvum* frequency was higher in the rural residences. Most of the homes of cryptosporidiosis positive subjects did not have domesticated animals thereby suggesting that transmission of the disease is mainly through anthroponotic and/or environmental transmission though zoonotic cannot be ruled out.

Overall, the main species circulating in the North Rift region were *C. hominis* with 12 subtypes, *C. parvum* with 8 subtypes and *C. meleagridis*, which was not subtyped. The subtypes did not show any bias based on the location of areas of residence.

Diarrhea and skin rash were significantly associated with cryptosporidiosis among the HIV positive patient while the other health complaints in both children and the HIV positive patients were not significantly associated with cryptosporidiosis.

The risk of infection with *Cryptosporidium* was higher in households with less number of rooms per house though the number of occupants did not influence the occurrence of the infection. Living in the peri-urban and rural areas exposed children to greater risks of infection with *Cryptosporidium* parasite while living in the urban residential areas presented a greater risk of infection with *Cryptosporidium* among the HIV infected persons.

The people using bush for human waste disposal were at a higher risk of infection but there was no difference in the risk of infection among those using wash closets and pit latrine.

Piped water and water drawn form wells/boreholes was associated with a higher risk of infection because most peri-urban homesteads use tap and/or well/borehole water while people in the rural areas mostly used well/borehole water. Boiling water did not protect or prevent HIV positive individuals from being infected with *Cryptosporidium* parasites though failing to treat water exposed children under the age of five years to a higher risk of infection.

The presence or absence of various types of domestic animals within the homesteads did not show any significant association with cryptosporidiosis. The role of environmental and zoonotic transmission have not been clearly elucidated by this study and therefore more location specific studies need to be undertaken to assess the impacts of these parameters in the transmission of cryptosporidiosis.

6.2 Recommendations

That stool samples be examined for *Cryptosporidium* oocysts as a routine procedure among diarrheic children and HIV patients in health facilities.

That a comprehensive field based study be undertaken to establish the risk factors associated with the transmission of cryptosporidiosis in the North rift region and indeed other areas of Kenya.

The role of environmental and zoonotic transmission have not been clearly elucidated by this study and therefore more location specific studies need to be undertaken to assess the impacts of these parameters in the transmission of cryptosporidiosis.

That, there is need for conducting an MLST study using multiple sites so as to establish the true nature of the population structure and the transmission dynamics of *Cryptosporidium* parasites not only in the North Rift region of Kenya but in Kenya as a whole.

Health education emphasizing on the improvement of sanitation and proper handling of human and animal waste disposal be conducted regularly among mothers and those living with the HIV/AIDS.

6.3 **Study Limitations**

Finances were a major limitation in this study since my PhD programme was self-sponsored. This made it difficult to meet the time limits because I had to save for some time to purchase the needs of the study. The other limitation was that *Cryptosporidium* oocysts were not isolated from the fecal samples obtained from domesticated animals and therefore that objective of the study was not achieved.

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APENDIX 1

ALIGNED CP47 DNA SEQUENCES

CLUSTAL 2.0.10 multiple sequence alignment

```
P94             ----------------------------------------------------CAATCA 6
P263            ----------------------------------------------------CAATCA 6
C386            -------------------------------------------------GATTTATCAATCA 13
A566            ------------------------------------------------TGATTTATCAATCA 14
P233            ----------------------------------------------------------TCA 3
R115            -------------------------------------------------------------CA 2
C292            ------------------------------------------------------------- 
R403            ------------------------------------------------------------- 
A144            ------------------------------------------------------------- 
P229            ------------------------------------------------------------- 
P102            ----------------------------------------------------AAATCA 6
A130            -------------------------------------------------TTATCAAATCA 11
P112            -------------------------------------------------TTATCAAATCA 11
EU146117        GGACCAAGGTTTTTCTAGTCCAGCTTCTAACAAACTGTCATCGAGTGATTTATCAAATCA 60
R699            ----------------------------------------------------ATCAAATCA 9
A243            -------------------------------------------------TTATCAAATCA 11
B216            ------------------------------------------------TTTATCAAATCA 12
A242            ----------------------------AAAAAGTCATCGAGTGCTTTATCAAATCA 29
A32895          -------------------------------------------------TTATCAAATCA 11
EU146116        GGACCAAGGTTTTTCTAGTCCAGCTTCTAACAAACTGTCATCGAGTGATTTATCAAATCA 60
EU146115        GGACCAAGGTTTTTCTAGTCCAGCTTCTAACAAACTGTCATCGAGTGATTTATCAAATCA 60
EU146118        GGACCAAGGTTTTTCTAGTCCAGCTTCTAACAAACTGTCATCGAGTGATTTATCAAATCA 60
A394            -------------------------------------------------TTTATCAAATCA 12
A878            ------------------------------------------------------------- 
A3289           ------------------------------------------------------------- 
P278            ------------------------------------------------------------- 
P226            ------------------------------------------------------------- 
C295            ------------------------------------------------------------- 
A525            ------------------------------------------------------------- 
R161            ------------------------------------------------------------- 
A1126           ------------------------------------------------------------- 
C504            ------------------------------------------------------------- 
A113            ------------------------------------------------------------- 
P238            ------------------------------------------------------------- 


P94             ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 66
P263            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 66
C386            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 73
A566            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 74
P233            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 63
R115            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 62
C292            -TCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 59
R403            --------------------------------AGCACGTGTTAGCACTAATAATAATAA 27
A144            ---------------TTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 44
P229            ---------CTTTACATTTAAGGATAAGTTATTAGCACGTGTCAGCACTAATAATAATAA 51
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A130            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 71
P112            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 71
EU146117        ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 120
R699            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 69
A243            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 71
B216            ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 72
A242            ATCAACTCCCTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 89
A32895          ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 71
EU146116        ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 120
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EU146118        ATCAACTCACTTTACATTTAAGGATAAGTTATTAGCACGTGTTAGCACTAATAATAATAA 120
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A878            --------------------------------GGTTTTTCCAGCCCGACTTCTAACAA 26
A3289           ----------------------GGCGGACCAAGGTTTTTCCAGCCCGACTTCTAACAA 36
P278            ----------------CCCAGAAGGCGGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 43
P226            --------------------AAGGCGGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 38
C295            ------------------------------CAAGGTTTTTCTAGTCCGGCTTCTAACAA 29
A525            -----------------CCAGAAGGCGGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 42
R161            -----------------CCAGAAGGCGGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 42
A1126           ----------------------GGCGGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 36
C504            ------------------------------------------------------ACAA 4
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A113            ---------------------------GGACCAAGGTTTTTCTAGTCCGGCTTCTAACAA 33
P238            ------------------------------------------------------------


P94             TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAAT----- 121
P263            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAAT-- 124
C386            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 133
A566            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 134
P233            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 123
R115            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAAT----- 117
C292            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATAATAA 119
R403            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 87
A144            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 104
P229            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 111
P102            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 126
A130            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 131
P112            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 131
EU146117        TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATAATAA 180
R699            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATG---- 125
A243            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATG---- 127
B216            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATG---- 128
A242            TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATG---- 145
A32895          TAATAATAATAATAATAATAATAATAATAATAATAATAATAATGATAATGAT----- 126
EU146116        TAATAATAATAGTAATAATAGTAATAATAATAATAATAATAATAATAATAATGATAA 180
EU146115        TAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATAATGATAATGA 180
EU146118        TAATAATAATAATAATAATAGTAATAATAATAATAATAATAATAGTAATAATGATAATG- 179
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A3289           --ACTTTCATCAAGTGATTTATCAAATCAATCA-ACTCACTTTACATTTAAGGATAA--- 90
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P226            --ACTGTCATCGAGTGATTTATCAAATCAATCA-ACTCACTTTACATTTAAGGATAA--- 92
C295            --ACTGTCATCGAGTGATTTATCAAATCAATCA-ACTCACTTTACATTTAAGGATAA--- 83
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R161            --ACTGTCATCGAGTGATTTATCAAATCAATCA-ACTCACTTTACATCTAAGGATAA--- 96
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A113            --ACTGTCATCGAGTGATTTATCAAATCAATCA-ACTCACTTTACATTTAAGGATAA--- 87
P238            --ACTGTCATCGAGTGATTTATCAAATCAATCA-ACTCACTTTACATTTAAGGATAA--- 54
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P94             -------------------GATAATGATAGTGATAATGATAATGATAATGATAATGATAA 162
P263            -------------------GATAATGATAGTGATAATGATAATGATAATGATAATGATAA 165
C386            TGATAGTGATA------ATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 187
A566            T------------------GATAGTGATAATGATAATGATAATGATAATGATAATGATAA 176
P233            T------------------GATA---------------------------------- 128
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A144            TGATAGTG------------ATAATGATAATGATAATGATAATGATAATGATAATGATAA 152
P229            TGATAGTG------------ATAATGATAATGATAATGATAATGATAATGATAATGATAA 159
P102            TGATAGTGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 186
A130            TGATAGTGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 191
P112            TGATAGTG------------ATAATGATAATGATAATGATAATGATAATGATAATGATAA 179
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R699            --------------------ATAATGATAGTGATAATGATAATGATAATGATAATGATAA 165
A243            --------------------ATAATGATAGTGATAATGATAATGATAATGATAATGATAA 167
B216            --------------------ATAATGATAGTGATAATGATAATGATAATGATAATGATAA 168
A242            --------------------ATAATGATAGTGATAATGATAATGATAATGATAATGATAA 185
A32895          ------------------------------------------------AATGATAATGATAA 140
EU146116        TGATAGTG------------ATAATGATAATGATAATGATAATGATAATGATAATGATAA 228
EU146115        TAGTG---------------ATAATGATAATGATAATGATAATGATAATGATAATGATAA 225
EU146118        --------------------ATAGTGATAATGATAATGATAATGATAATGATAATGATAA 219
A394            ------------------------------------------------------------
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A3289           -------------------------GTTATTAGCACGAGT--TAGCACTAATAATAACAT 123
P278            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 130
P226            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 125
C295            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 116
A525            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 129
R161            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 129
A1126           -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 123
C504            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 91
A113            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 120
P238            -------------------------GTTATTAGCACGTGT--TAGCACTAATAATAATAA 87


P94             TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 222
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A566            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 236
P233            -----ATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 183
R115            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 218
C292            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 239
R403            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 195
A144            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 212
P229            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 219
P102            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 246
A130            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 251
P112            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 239
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R699            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 225
A243            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 227
B216            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 228
A242            TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 245
A32895          TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 200
EU146116        TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 288
EU146115        TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 285
EU146118        TGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAATGATAA 279
A394            ------------------------------------GATAATGATAATGATAATGATAA 150
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A3289           TCATAATAATCACGATCATAATAATAATAATG---------ATAACGATAATGATAATAA 174
P278            TAATAATAATAATAATAATAATAATAGTAATA---------ATAATGATAATGATGATAA 181
P226            TAATAATAATAATAATAATAATAATAGTAATA---------ATAATGATAATGATGATAA 176
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R161            TAATAATAATAATAATAATAATAATAATAATAGTAATAATAATGATAATGATGATAA 189
A1126           TAATAATAATAATAATAATAATAATAATAGTAATA---ATAATGATAATGATGATAA 180
C504            TAATAATAATAATAATAATAATAATAGTAATA---------ATAATGATAATGATGATAA 142
A113            TAATAATAATAATAATAATAATAATAATAGTAATA---ATAATGATAATGATGATAA 177
P238            TAATAATAATAATAATAATAATAATAATAGTAATA---ATAATGATAATGATGATAA 144
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P263            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 285
C386            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 307
A566            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 296
P233            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 243
R115            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 278
C292            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 299
R403            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 255
A144            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 272
P229            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 279
P102            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 306
A130            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 311
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EU146117        TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 360
R699            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 285
A243            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 287
B216            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 288
A242            TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 305
A32895          CGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 260
EU146116        TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 348
EU146115        TGATAATGATAATGATAATGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 345
EU146118        TGATAATGATAATGATAGTGATAATGATAATGATAGTGGCAATAACATTAATGTTAATAA 339
A394            TGATAATGATAATGATGATAATGATAATAATGATAGTGGTAATAATATTAATGTTAATAA 210
A878            TGACAATGATAATGATAATAATAGTGATAATGATA------ATAATATTAATGTTAATAA 218
A3289           TGACAATGATAATGATAATAATAGTGATAATGATA------ATAATATTAATGTTAATAA 228
P278            CGATAATGATAATGATAATGATAATGATAATGATAGTGGTAATAATATTAATGTTAATAA 241
P226            CGATAATGATAATGATAATGATAATGATAATGATAGTGGTAATAATATTAATGTTAATAA 236
C295            CGATAATGATAATGATAATGATAATGATAATGATAGTGGTAATAATATTAATGTTAATAA 227
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C504            CGATAATGATAATGATAATGATAATGATAATGATAGTGGTAATAATATTAATGTTAATAA 202
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P238            CGATAATGATAATGATAATGATAATGATAATGATAGTGGTAATAATATTAATGTTAATAA 204
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P94             TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 340
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A566            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 354
P233            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 301
R115            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 336
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A144            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 330
P229            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 337
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P112            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 357
EU146117        TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 418
R699            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 343
A243            TATCGTTTCAAATGGGATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 345
B216            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 346
A242            TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-ATTAT 363
A32895          TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 318
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EU146115        TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 403
EU146118        TATCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCACAGAAAAAC-AAAAT 397
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A878            TGTTGCTTCAAATGCAATAAATCAT---TCTAC-AATAAATTCCATAGAAAAAC-AAAAT 273
A3289           TGTTGCTTCAAATGCAATAAATCAT---TCTACTAATAAATTCCATAGAAAAACTAAAAT 285
P278            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 299
P226            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 294
C295            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 285
A525            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 304
R161            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 307
A1126           TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 298
C504            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 260
A113            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 295
P238            TGTCGTTTCAAATGCAATAAATAATCAATCTAC-AATAAATTCCATAGAAAAAC-AAAAT 262
                * * * ********  ****** **   ****  *********** ******** *  **

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P263            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAAA----------- 391
C386            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAAAT---------- 414
A566            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAAAT---------- 403
P233            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAAATC--------- 351
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C292            TCAGAGATTATTAAAAACGTGGCTT--GCAAAAACTAGCGAGATACAAATT--------- 406
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A144            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAA------------ 377
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P112            TCAGAGATTATTAAAAACGTGGCTTT-GCAAAAACTAGCGAGATACAAA----------- 405
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A878            TCCGAGATTATTAAAAACGTGGCTTTTACAAAAGCTAGCGAGACACAAATTTTTAATAAA 333
A3289           TCCGAGATTATTAAAAACGT------------------------------------- 305
P278            TCCGAGATTATTAAAAACGTGGCTTTTACAAAAACTAACGAGATACAA-TTTCTAATAAA 358
P226            TCCGAGATTATTAAAAACGTGGCTTT-ACAAAAACTAACGAGATACAA------------ 341
C295            TCCGAGATTATTAAAAACGTGGCTTTTACAAAAACTAACGAGATACAAAT---------- 335
A525            TCCGAGATTATTAAAAACGTGGCTTTTACAAAAACTAACGAGATA--------------- 349
R161            TCCGAGATTATTAAAAACGTGGCTTT-ACAAAAACTAACGAGATACAAA----------- 355
A1126           TCCGAGATTATTAAAAACGTGGCTTT-ACAAAAACTAACGAGATACAAAT---------- 347
C504            TCCGAGATTATTAAAAACGTGGCTTT-ACAAAAACTAACGAGATACAAA----------- 308
A113            TCCGAGATTATTAAAAACGTGGCTTTTACAAAAACTAACGAGATACAAATCT-------- 347
P238            TCCGAGATTATTAAAAACGTGGCTTT-ACAAAAACTAACGAGATACAA------------ 309
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APPENDIX 2

PATIENT INFORMATION SHEET

TITLE OF THE STUDY: Molecular characterization of *Cryptosporidium* species in the North Rift region of Kenya

PRINCIPAL INVESTIGATOR: Caleb Isaboke Nyamwange, MSc.

INSTITUTION OF AFFLIIATION: ITROMID, KEMRI Nairobi, and Moi University.

PARTICIPATION: You are being asked to voluntarily participate in a research study. Refusal to participate in the study will involve no penalty to which you are otherwise entitled. You (your child) may discontinue your (your child's) participation at any time without penalty or loss of benefits. The principal investigator may withdraw you (your child) from the study if we are unable to obtain a stool sample from you (your child) but you will not lose any benefits if this happens.

INTRODUCTION: This is a cross-sectional study in which we are interested in finding out the prevalence, species and subspecies of *Cryptosporidium* present in humans and animals in North Rift region of Kenya as a way of assessing the seriousness of *Cryptosporidium* as a pathogen.

PROCEDURES TO BE FOLLOWED

Questionnaire: If you agree to participate or act as a legal representative for an adult or child to, participate in the survey, we will ask you (your child) some questions about that age, sex, occupation, village, residence, history of illness, sources and treatment of water, size of house, number of people sleeping in the same house.

Lab tests: A stool sample will be taken from you and examined for *Cryptosporidium* and the rest shall be preserved for speciation and typing of the parasites.

Risks: There is no risk in participating in this study.

Benefits: The direct benefit will be that diagnosis as to whether you are or not infected with this organism will prompt appropriate management of you condition. The results of the whole study will contribute towards the Knowledge of the epidemiology of the disease.

Compensation: There is no compensation to volunteers for their participation.

Duration of participation: This study requires only a single stool sample and administration of a questionnaire. Permission may also be sought for the principal investigator to visit your home to collect samples from your animals and household contacts.

Confidentiality of the records: The records and information gathered from you (your child) will be coded for purposes of confidentiality and that your name will not be identified from these records. No identity of any specific patient including you (your child), in this study will be disclosed in any public reports or publications. You will receive a copy of the signed consent form.

Use of stool samples: The stool samples obtained in this study will not be used for any purpose other than the ones stated in the protocol and consent form. After the

analysis is done, your stool sample shall be discarded and will not be kept in the laboratory.

Persons and places for answers to research related questions: Caleb Isaboke Nyamwange C/o Center for Biotechnology Research and Development, P.O. Box 54810, Tel 020-722541, Nairobi.

OR

Secretary KEMRI/National Ethical Review committee P.O. Box 54840, Nairobi, Tel. 020-722541 ext. 2295

APPENDIX 3

INFORMED CONSENT FORM

Project Title: Molecular characterization of C*ryptosporidium* species in the North Rift region of Kenya

1. Patient's name_____

   If minor,

   Give guardian's name_____ Relationship _____

   Date of birth _____ Age _____ Sex_____

   Address _____

   Telephone _____ fax _____ Email _____

2. **Purpose of the study:** To assess possible sources of the infections, and characterize the genotypes of *Cryptosporidium* from both humans and domesticated animals in the North Rift Region of Kenya. This study has been approved by the *Scientific Steering Committee* of KEMRI after clearance by the *Ethical Review Committee.*

   This study is under the direction of _____ of _____(Centre).

3. **Procedure to be followed**

   In this study to be carried in the north rift region of Kenya, for up to 12 months, you will give two samples of stool for parasitological examination and molecular analysis.

4. **Risks:** The procedure for the collection of stool samples does not pose any risk to you.

5. **Benefits:** You will not have direct benefit(s) from the study but the results of the study will contribute towards the Knowledge of the epidemiology of the disease.

6. **Confidentiality of the Records:** The records and information gathered from you will be coded for purposes of confidentiality and that your name will not be identified from these records. No identity of any specific patient in this study will be disclosed in any public reports or publications.
7. **Basis of participation**: It is important for you to know that you have the freedom to decline to participate in the study
8. **Signature**

I have read the above information and have had an opportunity to ask questions and all of my questions have been answered. I consent to taking part in the study. I fully understand there are no risks associated with the collection of samples. I have been given a copy of this consent form.

Signature_____ Date_____

(Patient)

Signature_____ Date_____

(Parent or Legal Guardian)

I, the undersigned, have fully explained the relevant details of this study to the patient named above and/or the person authorized to consent or assent for the patient. I am qualified to perform this role.

Signature_____ Name_____ Date_____

(Investigator)

Signature_____ Name_____ Date_____

(Witness)

Address of witness _____

APPENDIX 4

QUESTIONNAIRE

1. Patient's code _____
2. Patient's Name _____ADM/OPD No. _____ Age_____
3. If under 5 years give fathers or mothers or guardians name (circle which)

   _____
4. Sex
   - ❖ Male
   - ❖ Female
5. Residence

   Estate/Village_____ house No._____

   Sub-Location_____

   Location_____ Division _____

   District_____ Province _____
6. The number of rooms of the house in which you stay _____

7 Do you share your house with anybody? If yes who and how many?

I) My children _____ How many children _____

ii) Relatives_____ How many _____

iii) Any other_____ How many _____

7 Do you keep any of the following animals? If yes, how many? (Including their young ones)

- ❖ Cats _____ how many _____
- ❖ Dogs _____ how many _____
- ❖ Goats _____ how many _____

- ❖ Sheep _____ how many _____
- ❖ Cattle _____ how many _____
- ❖ Chicken _____ how many _____
- ❖ Any other _____ how many _____

8 Which of the above animals sleep in the same house as you do?

_____

9 Where do you get your water?

- ❖ River
- ❖ Well
- ❖ Spring
- ❖ Bore hole
- ❖ Rain
- ❖ Municipal water works

10 How do you treat your water before drinking?

- ❖ Boil
- ❖ Sieve
- ❖ Add chemical
- ❖ None of the above

10 **DIARRHEA HISTORY**

10.1 Is the patient having diarrhea

- ❖ Yes { }
- ❖ NO { }

If yes, for how has it been there?

- ❖ 1 week
- ❖ 1-2 weeks
- ❖ 2weeks

10.2 Have taken any medication?

- ❖ Yes
- ❖ No

If yes, did the condition improve? Yes { } No { }

10.3 Is there anybody in your household with the same problem?

- ❖ Yes
- ❖ No

If yes,

What is the relationship with the patient? _____

10.4. What are the other complaints of the patient?

- ❖ Fever
- ❖ coughing
- ❖ skin rash
- ❖ headache
- ❖ vomiting
- ❖ abdominal pains

Any other _____

10.5. Do you know you HIV status?

- ❖ Yes
- ❖ No

If yes, what is the status

- Positive _____ what is the CD4 count_____
- Negative

If no are you ready to take the test

- Yes
- No

APPEDIX 5

MODIFIED ZN STAINING PROTOCOL

1. Wet smears were made on slides using a drop of normal saline and left to air dry completely.
2. The dry smears were fixed by dipping them 6 times in absolute alcohol and left to dry.
3. The slides were then placed in Carbol fuschin for about 7 minutes after which they were washed in running tap water till the water off the slide was clear.
4. The slides were then decolourize in 3% acid alcohol, until no more colour of the stain came out, followed by washing of the slide in running tap water.
5. The slides were then counterstained using malachite green for 7-10 minutes then washed and left to air dry.
6. The slides were then observed at a magnification of X1000 under oil immersion.
7. *Cryptosporidium* oocysts were seen as intense pink-reddish rounded bodies against a green background.

APPENDIX 6

DNA EXTRACTION PROTOCOL USING QIAAMP®

STOOL MINI KIT:

1. Pea-size amount of formed stool or 200 µL of diarrheic stool was aliquoted into a 1.5 ml eppendroff tube. Then the samples were cleaned by adding 800 µL of distilled water, mixed well with an applicator stick, vortexed for 15 seconds and then centrifuged at 13000 rpm (16.1g) for 5 minutes. The supernatant was discarded and another 800 µl of distilled water added, mixed using an applicator stick, vortexed and centrifuged as above. This procedure was repeated four times.
2. After the final wash 1.4 ml of ASL extraction buffer from Qiagen was added to each sample followed by vortexing for 1 minute. The suspension was then heated for 15 minutes in a water bath set at 75^{o}C. After this it was vortexed and frozen at -80^{o}C for 30 minutes. The heating and freezing steps were repeated four times. After the last heating the samples were vortexed and centrifuged at 13000 rpm for one minute to pellet the stool particle.
3. 1.2 ml of the supernatant from the step above was added to a 2 ml collecting tube containing the InhibitEX tablet (proprietary composition, tablet supplied with the kit) immediately vortexed continuously for 1 minute or until the tablet was completely suspended. The suspension was then incubated for 1 minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
4. The samples were then centrifuged at 13000 rpm for 3 minutes to pellet stool particles and inhibitors bound to InhibitEX. The supernatant was then transferred to a 1.5 ml micro-centrifuge tube and the pellet discarded. The supernatant was

further centrifuged at 13000 rpm for 3 minutes to pellet any residues of the inhibitor tablet.

5. Then into a 1.5 ml microcentrifuge tube containing 15 µL of proteinase K, 200 µL of the supernatant was added followed by 200 µL of AL buffer and vortexed for 15 seconds. The samples were then incubated at 70^{o}C for 10 minutes.
6. After incubation 200 µL of 96-100% ethanol was added to the lysate and mixed well by vortexing. Then all the lysate was applied into a QIAamp spin column that had been appropriately labeled and placed in a 2 ml collecting tube. The cap of each tube was closed and samples centrifuged at 13000 rpm for 1 minute. The spin columns were placed in a new 2 ml collection tubes and the tube containing the filtrate was discarded.
7. Into the QIAamp spin column 500 µL of wash buffer AW1 was added and centrifuged at 13000 rpm for 1 minute. Each spin column was then transferred to a new 2ml collecting tube and the tube containing the filtrate discarded.
8. Into the QIAamp spin column 500 µL of wash buffer AW2 was added and centrifuged at 13000 rpm for 3 minutes. The tube containing the filtrate was discarded.
9. The QIAamp spin column was transferred to a new, labeled 1.5 ml microcentrifuge tube. 60 µL of buffer AE (10 mM tris- HCl, 0.5 mM EDTA pH 9.0) was directly pipetted into the QIAamp membrane, incubated for 1 minute at room temperature and then centrifuged at 13000 rpm for 1 minute to elute DNA. The eluted DNA was transferred back to the spin column and spun at full speed for 1 minute. The spin column was then discarded and the DNA stored at -20^{o}C awaiting molecular analysis.

APPENDIX 7

CONTENTS OF QIAamp® DNA MINI KIT

1 QIAamp Mini Spin Columns (50)

2 2 ml collection Tubes (200)

3 InhibitEX® Tablets (50)

4 Buffer ASL (proprietary composition) 140 ml.

5 Buffer AL (proprietary composition) 33 ml

6 Buffer AW1 (proprietary composition) concentrate 19 ml. Before being used for the first time, 25 ml ethanol (96–100%) was added into the concentrate to make the working solution.

7 Buffer AW2 (proprietary composition) concentrate 13 ml. Before being used for the first time, 30 ml ethanol (96–100%) was added into the concentrate to make the working solution.

8 Buffer AE (10 mM tris- HCl, 0.5 mM EDTA pH 9.0) 12 ml.

9 proteinase K 1.4 ml

APPENDIX 8

PREPARATION OF A 50 µL MASTER MIX FOR THE PRIMARY REACTION

1. 25.75µL of distilled water was put in a sterile 1.5 ml microcentrifuge tube
2. 5 µL of the 10X Perking Elmer PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) was added
3. Then 8 µL of the 1.5mM dNTPs was added
4. 1.25 µL of each 10 µM primer (forward and reverse) was added
5. Then to the master mix 3 µl of 25 mM $MgCl_2$ was added
6. 2 µL of 10% BSA was then added
7. 0.25 µL of Taq DNA polymerase was added and the mix was vortexed briefly for homogeneous mixing
8. 0.5 µL of the template DNA was added into 0.2 ml containing the mix, vortexed briefly and placed into a thermocycler.

APPENDIX 9

GEL ELECTROPHORESIS PROTOCOL

This was done to separate the PCR products. The materials and reagents for this procedure included

1. Electrophoresis horizontal gel tanks
2. Well forming combs
3. Power pack
4. Polaroid Camera
5. Polaroid Films
6. Micro-wave
7. 6X loading dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 60% glycerol, 60 mM EDTA)
8. 20X TBE buffer (1.780 M Tris-borate 1.78 mM boric acid 0.04 M disodium EDTA dehydrate, final pH 8.3)
9. 1 kb molecular weight maker
10. Agarose analar grade
11. Double distilled deionised water
12. Ethidium bromide (10 mg/ml)
13. Sterile measuring cylinders
14. Sterile conical flasks
15. Magnetic stirrer

The procedure and performance for gel electrophoresis was carried as follows

1. 1.5 g of agarose was put into a clean conical flask and 100 ml of 1X TBE buffer.

2. The agarose was melted in a micro-wave and allowed to cool with constant stirring.
3. Then 2.5 µL of ethidium bromide (10 mg/ml) was added into the 100 ml gel as it cooled.
4. Then a gel forming plate was placed on to the electrophoresis tank in an orientation that sealed the ends and well forming combs were then placed in position.
5. The gel was then poured into the plate and left to set at room temperature and the combs were carefully removed.
6. 1X TBE buffer containing ethidium bromide (0.25 - 0.5 mg/ml) was poured into the electrophoresis tank to submerge the gel.
7. 2 µL of the 6X loading dye was mixed with 10 µL of a secondary PCR product on a parafilm.
8. 5 µL of the 1kb molecular weight maker mixed with 6X loading dye and distilled water in a ratio of 1:1:4 respectively was loaded into the first well.
9. The samples were loaded into the individual slots and electrophoresed at 100 V for 1 -2 hours
10. The Gel was then placed on a UV trans-illuminator and photographed using a Polaroid camera

APPENDIX 10

MATERIALS AND PROTOCOL FOR RFLP REACTIONS

1. Secondary PCR product
2. *Ssp*1 restriction enzyme (Promega) in storage buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol)
3. 10X *Ssp*1 restriction buffer (60 mM tris-HCl, 60 mM $MgCl_2$, 1M NaCl, 10 mM DTT)
4. *Vsp*1 restriction enzyme (Promega) in storage buffer (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.2 mg/ml BSA, 50% glycerol)
5. 10X *Vsp*1 restriction buffer (60mM tris-HCl, 60mM $MgCl_2$, 1.5M NaCl, 10mM DTT)
6. Double distilled deionised water
7. Sterile 0.2ml eppendorf tubes
8. Water bath set at 37^{o}C

The procedure and performance of RFLP was as follows

1. 10 µl of the secondary PCR product were pipetted into a sterile 0.2 ml tube in two sets one, for *Ssp*1 and another *Vsp*1
2. 4 µl of the respective restriction buffer was added into the respective tubes.
3. 2 µl (20 units) of *Ssp*1 and 1 µl (8-12 units) *Vsp*1 were added into the respective set of tubes.
4. 22 µl and 24 µl of distilled water was added into the *Ssp*1 and *Vsp*1 tubes respectively.
5. The samples were vortexed briefly and incubated at 37^{o}C overnight.

6. 20 µl of the digest were analysed on 2% agarose gel stained with ethidium bromide. The *Ssp*1 restriction products were run on the upper lane for species identification while the *Vsp*1 restriction products were run on the lower lane of the gel for genotyping
7. The gel was run for 1 hour at 100V, then photographed on UV trans-illuminator using a Polaroid camera

APPENDIX 11

PURIFICATION OF DNA FROM GEL USING THE QIAquick KIT

1. Before the extraction the gel slice was weighed in the colorless 1.5 ml microfuge tube.
2. Three volumes of buffer QG (Proprietary composition) were added to one volume of the gel (1 mg of gel is equivalent to l µL). This was incubated at 50^{o}C for 10 minutes with vortexing every 2-3 minutes to dissolve the gel. After dissolving the gel, one gel volume of isopropanol was added and gently mixed so as to increase the yield of DNA.
3. A QIAquick spin column was placed in a 2 ml collection tube, 800 µL of the sample was added and centrifuged at 1300 rpm for 1 minute.
4. The flow through was discarded and the spin column was reloaded with the sample that may have remained but not exceeding 800 µL and then centrifuged as above.
5. Then to the column 0.5 ml of buffer QG was added, allowed to stand for one minute and then centrifuged at 13000 rpm.
6. The column was washed by adding 0.75 ml of buffer PE (Proprietary composition) into it and centrifuged at 13000 rpm. The flow through was discarded and the Qiaquick column was centrifuged for an additional 1 minute to remove any residual ethanol.
7. The column was then placed in an appropriately labeled 1.5 ml microfuge tube.
8. To elute the DNA, 40 µL of buffer EB (10 mM Tris.HCl, pH 8.5) was added to the centre of the QIAquick membrane, allowed to stand for 1 minute and then centrifuged at 13000 rpm.

9. The DNA was then kept at -20^{o}C.

To ascertain the yield 5 µL of the DNA was electrophoresed in 2% agarose, visualized under UV and photographed before sequencing.