The African green monkey (*Cercopithecus aethiops*) as a laboratory model for cyclosporiasis

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DECLARATION

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

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I wish to dedicate this thesis to my husband, Dr James Nguhiu and our children, Sammy Onderi, Grace Wanjiru**,** Samuel Mwangi and Titus Kamanguya**.**

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God bless you all.

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LIST OF ABBREVIATIONS AND ACRONYMS

AGM	African green monkey
AHITI	Animal Health and Industry Training Institute
AIC	African Inland Church
AIDS	Acquired Immunodeficiency Syndrome
Bp	Base pair
BSA	Bovine Serum Albumin
C. cayetanensis	*Cyclospora cayetanensis*
CIOMS	Council for International Organization of Medical Sciences
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FEA	Formalin ethyl acetate concentration technique
Gp	Glycoprotein
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
IPR	Institute of Primate Research
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute

NHP	Non-Human Primates
nm	nanometer
OD	Optical density
OB	Olive Baboon
PAN	*Pabio anubis*
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PVEN	Primate Vaccine Evaluation Network
RNA	Ribosenucleic Acid
SD	Standard Deviation
SIV	Simian Immunodeficiency Virus
Spp	species
SSC	Scientific Steering Committee
TMP-SMX	Trimethoprim-Sulphamethoxazole
VER	Vervet
ZN	Ziehl Neelsen

ABSTRACT

Cyclospora cayetanensis, a coccidian protozoan parasite, that causes diarrhoea in immunocompetent as well as immunocompromised individuals with or without international travel., The biology and epidemiology of *C. cayetanensis* has been complicated by lack of information on the pathogen's origins and if animal reservoirs do exist for this parasite. Morphological and molecular characterization of three new *Cyclospora spp* isolated from East African non-human primates that are very similar to *C. cayetanensis* that infects humans, offer an opportunity to study the biology and pathogenesis of *Cyclospora* species. This study was conducted at the Institute of Primate Research (IPR), Nairobi with a view to developing and testing the suitability of African green monkey (AGM) as a laboratory model for cyclosporiasis in humans. *Pabio anubis* and *Cercopithecus aethiops* collected from the wild by trapping, underwent clinical and parasitological evaluation. Multiple individual faecal samples were collected and processed using the standard formalin ethyl acetate concentration technique. Faecal smear slides were stained with modified Ziehl Neelsen and hot safranin stains, then examined by light microscopy for the presence of *Cyclospora* oocysts and other gastrointestinal parasites. The *Cyclospora* oocysts isolated were used to establish experimental infections in four AGM for pathological evaluation and demonstration of the life cycle stages of *Cyclospora* species. Efficacy of Trimethoprim-Sulphamethoxazole (TMP-SMX) was evaluated in monkeys with natural *Cyclospora* infections.. Hematological analyses were carried out using venipuncture blood from the animals. The Enzyme linked immunosorbent assay was used to probe sera for simian

immunodeficiency virus infections and *Cyclospora* specific antibody responses in the study animals. Nested polymerase chain reaction was used to confirm the identity of isolated *Cyclospora* oocysts from infected non-human primates. Data generated were analysed using Statistical Analysis System where the prevalence of intestinal parasites was computed as the proportion of number of positive observations and the Chi-square statistics was used to determine the associations between parasite species and *Cyclospora* and p values of < 0.05 considered significant. The prevalence of *Cyclospora* infections in non-human primates collected from the wild was 6.2% for the baboons and 64.1% for the monkeys with significantly higher prevalence in adult animals than in juveniles (p value = 0.0310). SIV infection was detected in adult animals, with a prevalence of 30% recorded for baboons, and 64.1% for monkeys (50% in male, 71.5% in female AGM. *Cyclospora*-SIV co-infections occurred in 10% of the baboons and in 30% of the monkeys, though the animals were asymptomatic at the time of faecal sample collection. Experimental *Cyclospora* infections were successfully established in four monkeys. On gross pathology, the juvenile animals had minimal changes at 49 days post-infection, while the adult males showed more severe pathology, with haemorrhages in the stomach and intestine, mildly enlarged and prominent lymph nodes, and the livers had moderate pitting of the surface which was widespread. Histopathology indicated that the stomach and intestines had mild disruptions of the mucosal wall, lymphocytic infiltration, with intracellular parasite vacuoles in the luminal end of the enterocytes. The livers showed periportal lymphocytic infiltration with formation of granulomas in the liver. The mesenteric lymph nodes were hyperplastic with extensive proliferation of the

lymphocytes. TMP-SMX therapy was effective for treatment of *Cyclospora* in AGM. Recurrent asymptomatic *Cyclospora* infection occurred in 33% of the cases which responded to second treatment with TMP-SMX. DNA analysis confirmed the oocysts identified in natural infection used in experimental inoculation study to be *Cyclospora* species with a PCR product of 294 base pairs. In conclusion, and based from this study the *Cyclospora* infections in African green monkey produced pathological lesions similar to those reported in humans with cyclosporiasis. Response to treatment with TMP-SMX was similar to what has been reported for human cases. It appears that the current study has developed the African green monkey as an appropriate model to study cyclosporiasis.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Cyclospora cayetanensis, a coccidian protozoan parasite, is increasingly being identified in faecal smears of immunocompetent persons with or without history of international travel (Berlin *et al*., 1994; CDC, 1996 a,b) as well as in patients with Human Immunodeficiency Virus (HIV) infection or Acquired Immuno Deficiency Syndrome (AIDS) (Berlin *et al*., 1994; Pape *et al.,* 1994). The parasite has been reported from various geographical regions but seems to be endemic in the tropical countries (Ashford, 1979; Ortega *et al.,* 1994; Herwaldt and Ackers, 1997; Nassef *et al*., 1998; Bern *et al.*, 1999; Ceqielski *et al*., 1999; Eberhard *et al.,* 1999a; Kumar *et al.,* 2002).

The stage that is found in the faecal smears of patients infected with *Cyclospora* is the unsporulated oocyst, measuring 8 to 10µm in diameter (Orihel and Ash, 1995; Visvesvara *et al.,* 1997). The oocysts excreted require 5 to 14 days to sporulate in the external environment in vegetation, soil or water (Orihel and Ash, 1995). The sporulated oocyst contains two sporocysts each with two sporozoites. The only known mode of transmission of cyclosporiasis is consumption of contaminated water and/ or food (CDC, 1996a; CDC, 1997; Herwaldt and Ackers, 1997). The oocysts of *C. cayetanensis* are variably acid fast, with staining patterns ranging from no staining or colourless to pink or deep purple, with the extensively used modified Kinyoun's acid-fast stain, which is

commonly used for identification of *Cryptosporidium,* an other protozoan parasite (Orihel and Ash, 1995). This variability in the staining pattern may result in misidentification of the parasite. Visvesvara *et al.* (1997) reported a safranin-based stain that uniformly stains oocysts of *Cyclospora* a brilliant reddish orange, provided that the faecal smears are heated in a microwave oven, prior to staining.

Cyclosporiasis is an emerging gastroenteritis disease which presents as diarrhoea, flatulence, fatigue, and abdominal pain leading to weight loss, and is caused by the coccidian protozoan *Cyclospora cayetanensis* (Ortega *et al*., 1994; Karanja *et al*., 2007). *C. cayetanensis* is associated with diarrhoea among children in developing countries in the Americas, where it is endemic (Eberhard *et al*., 1999a; Bern *et al.*, 2002). *C. cayetanensis* is considered an emerging disease of public health concern and has been identified as the cause of multistate outbreaks of diarrhoea in the United States and Canada, associated with imported produce, notably raspberries (Herwaldt and Ackers, 1997; CDC, 1997). In these countries, *Cyclospora* transmission has been primarily associated with foods imported from developing countries.

Few studies have been carried out to determine the transmission and epidemiology of cyclosporiasis in Africa and its public health impact on the continent. The disease has been reported in humans in Eygpt, Tanzania and Nigeria (Nassef *et al*., 1998; Ceqielski *et al*., 1999; Alakpa and Fagbenro, 2002; Alakpa *et al*., 2002; Alakpa *et al.,* 2003).

In immunocompetent individuals, cyclosporiasis is self limiting, but in immunocompromised individuals and particularly in HIV/AIDS patients, cyclosporiasis results in prolonged diarrhoea that could be life threatening (Kumar *et al*., 2002; Wiwanitkit, 2006).

Studies on morphological and molecular characterization have named three new *Cyclospora* species reported in the East African non-human primates. These are *Cyclospora cercopitheci* sp. n. recovered from the African green monkeys, *C. colobi* sp. n. a parasite from Colobus monkeys, and *C. papionis* sp. n. a species infecting baboons (Eberhard *et al*., 1999b). These three new species plus *C. cayetanensis* which infects humans has increased to four, the recognized species of *Cyclospora* infecting primates. The non-human primate derived *Cyclospora* oocyst and human associated *Cyclospora* species are morphologically similar but there is no cross infection between the different species (Eberhard *et al*., 1999b; Eberhard *et al*., 2000). The Simian species seem to be widespread in Kenya (Eberhard *et al*., 2001). The *Cyclospora* infections in monkeys have been noted to occur throughout the year unlike cyclosporiasis in humans, which tends to show strongly marked seasonality with most infections occuring in the warm months (Bern *et al.,* 2002). Based on available literature, humans may be the only natural hosts for *C. cayetanensis.* Although oocysts of *C. cayetanensis* have been isolated in faeces of dog, monkey, chicken, faeces and milk of buffaloes, natural infection of these animals are yet to be determined (Chu *et al*., 2004). Attempts to

establish experimental *C. cayetanensis* infection in laboratory animals have been unsuccessful (Eberhard *et al.,* 2000).

In epidemiological studies conducted among Peruvian children, cyclosporiasis was associated with ownership of domestic animals, especially birds, guinea pigs, and rabbits (Bern *et al*., 2002). The finding of an association of *C. cayetanensis* with avian hosts though consistent with results of *C. cayetanensis* case-control study in Guatemala, is still unexplained (Bern *et al.,* 1999).

1.2 Problem Statement

Cyclosporiasis is a coccidian infection, worldwide in distribution but the biology of the causative agent and epidemiology of the disease is poorly understood.

1.3 Justification

Very little is known about the epidemiology of the cyclosporiasis due to *Cyclospora cayetanensis,* with the coccidian protozoa being incapable of direct faecal-oral transmission, and with no known zoonotic reservoir (Bern *et al.,* 2002). *Cyclospora* species have been identified in the East African non-human primates, and documented to harbour asymptomatic infections with the parasites (Eberhard *et al*., 2001).

The use of an appropriate animal model to study important gaps in our knowledge of cyclosporiasis is recommended in view of the significance of this disease in human populations especially, in immunocompromised individuals in whom the disease results

in protracted diarrhoea (Sifuentes-Osornio *et al*., 1995). The high prevalence of coccidian parasites, and their potential for associtions on Human Immunodeficiency Virus (HIV) infected individuals, suggests that diagnosis and treatment of infections be considered a component of routine HIV care. The aim of the current study was to develop a laboratory animal model for studying cyclosporiasis.

Given that the African green monkey is a susceptible host, it may be a suitable model for studying *Cyclospora*-like organisms in the laboratory and including association with HIV in the human disease as it harbours Simian immunodeficiency virus (SIV). Simian Immunodeficiency Virus prevalence in the African green monkeys (*Cercopithecus aethiops*) has been shown to be 30-50% and universally in females of reproductive age (Otsyula *et al*., 1996). Antiviral immune mechanisms in the AGM have shown that immune responses in the AGM are similar to those seen in HIV infected humans (Norley *et al.,* 1996). SIV infected AGM are asymptomatic.

1.4 Research Questions

- Is it possible to establishment experimental *Cyclospora* infection in AGM?
- What is the course and pathology of *Cyclospora* infection in AGM?
- Is Trimethoprim-Sulphamethoxazole an efficacious therapy for cyclosporiasis in AGM?
- Do *Cyclospora*-SIV co-infections occur in AGM and how do they present?

1.5 Hypothesis

The study was based on the hypothesis that the prevalence of *Cyclospora* is high in non-human primates, and it is associated with the Simian Immunodeficiency Virus (SIV) infection. In immunocompetent individuals, *Cyclospora* infections cause diarrhoea which is further aggrevated in immunocompromised patients.

1.6 Objectives

1.6.1 General Objective

The general objective of the study was to develop the African green monkey as a laboratory model for *Cyclospora,* and to use the model to elucidate the clinical epidemiology of cyclosporiasis in relation to SIV.

1.6.2 Specific Objectives

i. To determine the prevalence and intensity of gastrointestinal parasites in captive-wild Olive baboons *(Papio anubis*) and African green monkeys (*Cercopithecus aethiops*) held at the Institute of Primate Research (IPR), Karen, Nairobi.

ii. To determine the prevalence of Simian Immunodeficiency Virus (SIV) infections in captive wild Olive baboons and African green monkeys, and its association with *Cyclospora.*

iii. To establish experimental infection with *Cyclospora* in AGM and determine the clinical, hematological and serological characteristics of cyclosporiasis in comparison natural infections of *Cyclospora.*

iv. To determine the gross and histopathological characteristics of cyclosporiasis in AGM experimentally infected with an isolate of *Cyclospora.*

v. To evaluate the efficacy of Trimethoprim-Sulphamethazole (TMP-SMX) combination treatment in AGM with natural infections of *Cyclospora*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Intestinal Parasite Infections in Non-human Primates

Intestinal parasitism is a common phenomenon in non-human primates. Intestinal helminths are common parasites of primates, and numerous protozoan parasites live in the non-human primates with commensals forming the majority of these (Munene *et al*., 1998). Multiple parasite species involving both helminths and protozoans may occur in non-human primates (Kuntz and Myers, 1996; Stoner, 1996; Munene *et al*., 1998; Muriuki *et al.,* 1998; Ocaido *et al*., 2003; Gillespie *et al*., 2004; Muehlenbein, 2006). The most common parasite species involved include *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidia* (among the protozoans), and *Oesophagostomum* species, *Trichuris trichiura*, and *Stronglyloides fullerboni* (among helminths) (Beaver *et al*., 1988; Munene *et al*., 1998; Graczyk *et al.,* 2002; Muehlenbein, 2006; Ekanayake *et al*., 2006; Okanga *et al*., 2006).

Among the protozoa, the cysts are shed in faeces of infected animal and become source of contamination to water sources, food and environment. The cysts are infective for next susceptible host but for some protozoan parasites e.g. *Cyclospora,* cysts require to sporulate before they become infective. Many of the parasitic worms have complex multistage life cycles that may include several hosts. Within the mammalian hosts,

helminth parasites undergo extensive growth and differentiation with the ultimate goal of producing stages intended for transmission to the next host. Overall prevalence of *Trichuris, Strongyloides* and *Oesophagostomum* is high, and hosts experience diarrhoea. Immune responses of the vertebrate hosts to worm infections are remarkably similar, being T helper-2 like with production of significant levels of interleukins (IL-4, IL-5, IL-9, IL-10 and IL-13) and development of strong immunoglobulin E (IgE), eosinophils and mast cell responses (Dunne *et al.,* 1992; Bancroft and Grencis, 1998). Many helminth parasites are long-lived and cause chronic infections. The immune responses that develop often proceed to cause pathological changes that in many helminth infections are the primary cause of disease e.g. liver granulomas. Parasites cause tissue damage and ill health to non-human primates.

2.1.1 Biology of Intestinal Parasites

Many of the protozoa and helminth parasites common to both animals and humans have an indirect life cycle that is interrupted in the laboratory environment and thus limits the zoonotic potential of these organisms. Laboratory housed primates are the most likely source of parasitic infection for animal handlers (Flynn, 1993; Ott-Joslin, 1993; Ghandour *et al*., 1995; Bogers *et al*., 2001). Helminths are potentially zoonotic in the laboratory environment, but they represent a significantly smaller problem than bacterial or viral diseases. Exceptions are those with a direct life cycle. Generally, proper quarantine, surveillance and treatment to decrease endoparasitic burden, routine sanitation to eliminate parasitic ova before they can become infective, and education of

personnel on standard hygiene practices are measures that can be taken to prevent transmission of these diseases.

Most intestinal parasites have a faecal-oral route of transmission via consumption of food or water contaminated with parasite ova or cysts (Fayer *et al*., 2000). Many of the intestinal parasites identified in wild non-human primates can be pathogenic to humans, and non-human primates are susceptible to a variety of human pathogens (Key *et al.*, 1976; Muriuki *et al.*, 1998; Legesse and Erko, 2004; Levecke *et al.*, 2007). Close phylogenic relationship between humans and non-human primates results in high potential risk for pathogen exchange (Beaver *et al.*, 1988; Ott-Joslin, 1993, Bogers *et al*., 2001). Studies have highlighted emerging human diseases, e.g., HIV/AIDS, Ebola, with origins or likely transmission to human that may include non-human primates (Gao *et al.*, 1999; Leroy *et al.*, 2004). Increasing human encroachment into previously non impacted forest areas increases the potential for disease transmission between human and non-human primate populations (Key *et al.*, 1976; Muriuki *et al.*, 1998). This phenomenon is of great concern in terms of public health and non-human primate conservation, hence detailed investigations of primate ecology and parasitology is warranted. Baseline data on patterns of parasitic infections in wild primate populations are critical to providing index of population health and to assess and manage disease risks.

2.1.2 Coccidian parasites

Protozoans are single celled organisms. Many of these organisms are free living in the soil, water and other moist environments while others are obligate or opportunist parasites and cause human disease. Organisms in the phylum Apicomplexa have an apical complex consisting of a series of organelles namely; micronemes, rhoptries and conoid. Members of the class sporozoa reproduce by by both sexual and asexual processes. The intestinal coccidian of medical importance include, *Cryptosporidium, Cyclospora, Isospora, Toxoplasma* and *Sarcocystis.* Oocysts which have a thick wall contain infective sporozoites which are shed in the faeces of infected animals and humans (Orihel and Ash, 1995).

2.2 *Cyclospora* Infections

2.2.1 History

The first report of *Cyclospora cayetanensis* (*C. cayetanensis*) in humans was published by Ashford (1979), who found unidentified *Isospora*-like coccidian parasites in the faeces of three individuals in Papua New Guinea. Ortega *et al* (1994) published a complete morphological description of the parasite and validated the name *Cyclospora cayetanensis.* To date, *C. cayetanensis* transmission in developed countries is through food- and waterborne outbreaks but the transmission and risk factors in endemic tropical countries are poorly understood (Herwaldt and Ackers, 1997; CDC, 1997; Alakpa and Fagbenro-Beyiouku, 2002; Alakpa *et al.,* 2003; Bern *et al.,* 2002; Kumar *et al*., 2002; Shields and Olson, 2003; Goncalves *et al.,* 2005; Karanja *et al.,* 2007).

Cyclospora cayetanensis, a coccidian parasite, has increasingly been recognized as a cause of gastrointestinal tract illness (Levine, 1988; Hoge *et al.*, 1993; Huang *et al.*, 1995). The clinical features and risk factors for illness in a foodborne outbreak of *Cyclospora cayetanensis* at a wedding in a Boston restaurant, USA were described by Fleming *et al* (1998). *Cyclospora* infections have been recognized as emerging parasitic infections (Soave, 1996; Herwaldt and Ackers, 1997; Stephanie, 1997).

In Eastern Africa, *Cyclospora papionis* and *Cyclospora cercopitheci* have been described in non-human primates in Olive baboons and African green monkeys in Tanzania, Ethiopia and Kenya (Smith *et al*., 1996; Legesse and Erko, 2004; Eberhard *et al.*, 2001). *Cyclospora* infections have also been reported in non-human primates have also been reported in Sri Lanka (Ekanayake *et al*., 2006).

2.2.2 Taxonomy, Biology and Life Cycle of *Cyclospora*

The characteristics of Coccidia (Phylum, Apicomplexa) are that the sporozoites are within a sporocyst, have a membrane-bound nucleus and micronemes (Goodgame, 1996). *Cyclospora* are intracellular pathogens of the small bowel, where they undergo sexual and asexual reproduction (Orihel and Ash, 1995). *Cyclospora cayetanesis* is an obligate intracellular coccidian protozoan parasite, whose life cycle involves a single host (Ortega *et al*., 1994). Phylogenetically, *Cyclospora* belongs to Eimeriadade (Relman *et al.*, 1996; Pieniazek and Herwaldt, 1997).

Cyclospora oocysts are spherical, 8-10µm in diameter with a smooth outer wall that autofluoresces in ultraviolet light. The oocysts are shed in faeces unsporulated and require 5-14 days to sporulate and become infectious for the next host. Transmission is by ingestion of contaminated food or water (Orihel and Ash, 1995). The infective sporulated oocyst contains 2 sporocysts each containing 2 sporozoites (Orihel and Ash, 1995). Once inside the gut of the host, the sporozoites escape from the sporocyst and penetrate epithelial cells along the small intestines usually in the jejunum. The process of schizogony begins with the formation of a trophozoite that grows to a mature schizont; micro and macrogamete form a zygote, which in turn develops into an oocyst. The oocyst passes into the environment in the faeces as a nonsporulated noninfectious oocyst (Sun *et al.*, 1996; Nhieu *et al*., 1996; Ortega *et al*., 1997).

Table 2.2.2 Taxonomical classification of *Cyclospora*

***Cyclospora* species**	**Host species**	**Location where collected**	**Reference**
Undescribed coccidian	Man	Papua new Guinea	Ashford. 1979
Cyclospora cayetanensis	Humans	Peru	Ortega *et al*., 1993 Ortega *et al*., 1994
Cyclospora cercopitheci	African green monkeys	Ethiopia/ Kenya	Eberhard *et al*., 1999
Cyclospora colobi	Colobus monkeys	Ethiopia/ Kenya	Eberhard *et al*., 1999
Cyclospora papioni	Olive baboons	Ethiopia/ Kenya	Eberhard *et al*., 1999

The life cycle begins when the human host ingests infective sporulated oocysts in contaminated food or water. Once inside the gut of the host, the sporozoites escape from the sporocyst and penetrate epithelial cells along the small intestines usually in the jejunum. The endogenous stages of *Cyclospora* species are intracytoplasmic and contained within a vacuole (Ortega *et al*., 1997). The process of schizogony begins with the formation of a trophozoite that grows to a mature schizont that contains 8-12 merozoites. These are released to invade other epithelial cells and are type 1 meronts which are asexual forms. After several cycles of type 1 schizogony, type 2 meronts (sexual forms) develop, with each cell containing 4 merozoites. After invading epithelial cells, some of these form single macrogametes and others divide multiple times to form microgametes. When released, a microgamete fertilizes a macrogamete to form a zygote, which in turn develops into an oocyst. The oocyst passes into the environment in the faeces as a non sporulated non infectious oocyst (Sun *et al.,* 1996; Nhieu *et al*., 1996; Ortega *et al*., 1997). In the environment, the oocysts sporulate becoming infectious for humans. The sporont divides into two sporocysts, each containing two sporozoites. The sporulation takes a few days to weeks depending on the ambient temperature (Orihel and Ash, 1995). Contamination of food and drinking water with infectious oocysts are the source of transmission to human with the infectious innoculums being very low. Consequently human-to-human transmission does not occur.

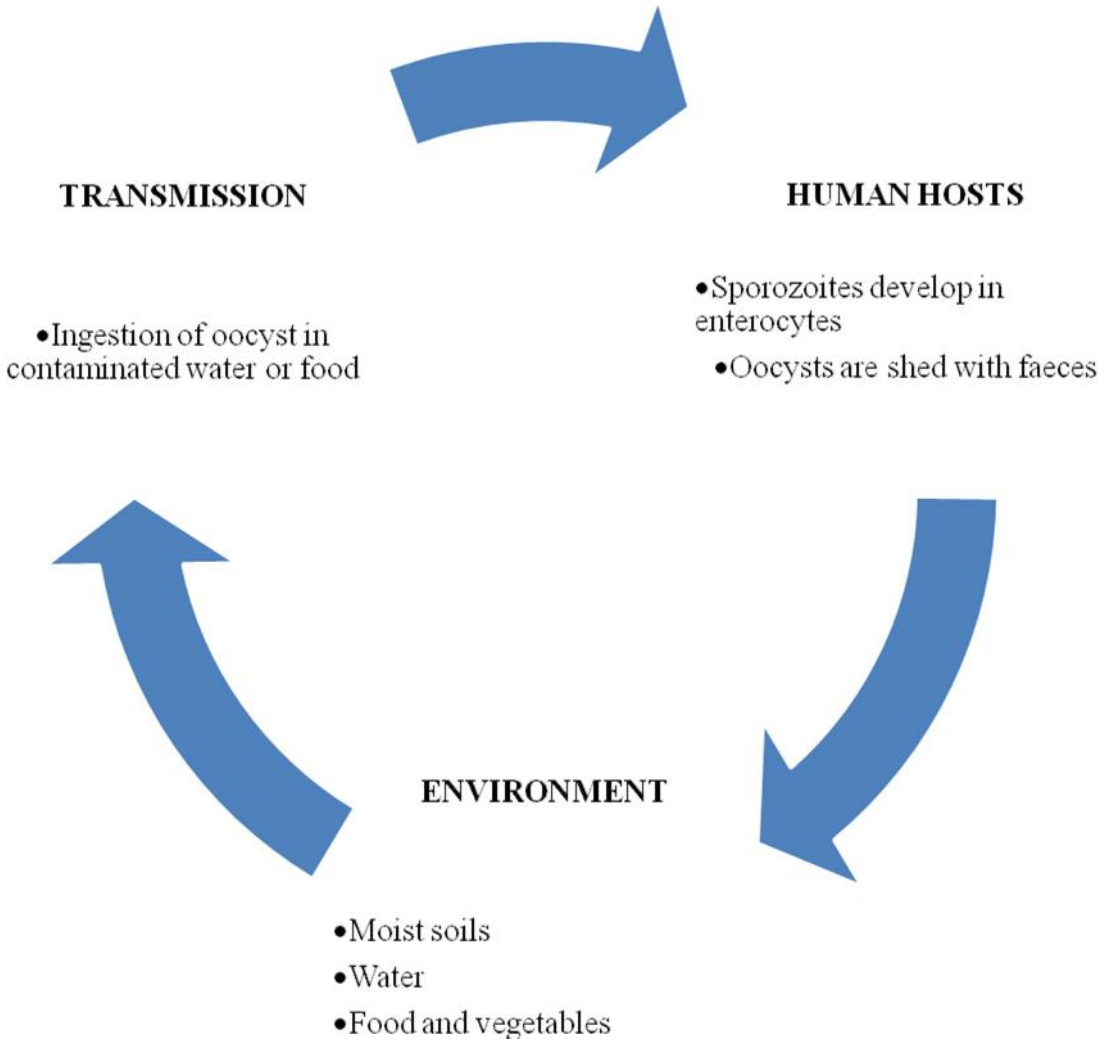


Figure 2.2.2 Schematic representation of transmission cycle of *Cyclospora*

2.2.3 Epidemiology of *Cyclospora*

C. cayetanensis infection occurs only in humans, there is no known animal reservoir and attempts to establish experimental infections in laboratory animals has been unsuccessful so far (Eberhard *et al.*, 2000; Chu *et al.,* 2004). Isolation of *C. cayetanensis* in poultry has been reported by Garcia-Lopez *et al* (1996). First record of *C. cayetanensis* in buffalos (*Bubalus bubalus*) in Neinava Governorate, Iraq in faeces and milk were only reported in Lugol's iodine and Ziehl Neelsen methods and no polymerase chain reactions were done (Abbassa and Rahemo, 2006). Li *et al* (2007) characterized *Cyclospora* like organisms in dairy cattle. Since its description in 1986, *Cyclospora cayetanensis,* a coccidian parasite, has been increasingly recognized as a cause of diarrhoeal illness (CDC, 1991; Bendall *et al*., 1993). *C. cayetanensis* is emerging worldwide in immunocompetent and in AIDS patients (Ortega.*et al*., 1993; Wurtz *et al.*, 1993; Hale *et al.,* 1994; Ooi *et al*., 1995).

Cyclospora infections are endemic in Latin and South America countries: Haiti, Peru, Brazil, Guatemala (Ortega *et al.*, 1994; Bern *et al*., 1999; Eberhard *et al*., 1999 a; Bern *et al*., 2002), South East Asia, China, India, Nepal, Thailand (Fryauff *et al*., 1996; Kumar *et al.,* 2002; Kimura *et al*., 2005) and in Africa: Eygpt, Tanzania and Nigeria (Nassef *et al*., 1998; Ceqielski *et al*., 1999; Alakpa *et al*., 2003). In endemic countries, the prevalence is from 1-15%, and varies with seasons with usually high prevalence in the warm seasons. Children ≤9 years of age account for 70% of these infections (Eberhard *et al*., 1999a; Bern *et al*., 2002). Some adults in endemic areas have

asymptomatic infections and excrete few oocysts. Soil contact is an important risk factor for children younger than 2 years in endemic areas (Bern *et al*., 2002). *Cyclospora* oocysts survive in water for 2 months at 4 ^{o}C and are resistant to chlorine disinfection at standard water treatment levels (Rabold *et al.*, 1994). Ownership of animals or living in homesteads with animals was found to be associated with *Cyclospora* infections (Bern *et al.*, 2002).

In the United States of America (USA), sporadic cases of *Cyclospora* infection have been reported among immunocompromised individuals or those with a history of recent travel (Ortega *et al*., 1994; CDC, 1996 a,b; Herwaldt and Ackers, 1997). However, the prevalence of reported *Cyclospora* infection has been as low as 1-3% (Herwaldt and Ackers, 1997). Before 1996, only 3 *Cyclospora* outbreaks had been reported in North America, with drinking water implicated epidemiologically in 2 cases and bare-handed contact with soil in the third. In May and June 1996, more than 850 cases of laboratory-confirmed *Cyclospora* infection, including both sporadic and event-related clusters, were reported in 20 states east of the Rocky Mountains and 2 Canadian provinces (CDC, 1996 b; Koumons *et al*., 1996; Herwaldt and Ackers, 1997).

Cyclospora infections in England and Wales (United Kingdom) are sporadic cases associated with travel abroad, and although similar foods are imported to UK and USA from endemic tropical countries, no outbreaks have occurred so far (Cann *et al.*, 2000; Chalmers *et al*., 2000).

Cyclospora infections tend to occur throughout the year in non-human primates without sex or age differences (Eberhard *et al.*, 2001). The infections tend to be widespread in forest and savannah grassland non-human primates (Eberhard *et al*., 2001; Legesse and Erko, 2004; Ekanayake *et al*., 2006).

2.2.4 Public Health Significance of *Cyclospora cayetanensis*

C. cayetanensis has been identified as the cause of foodborne outbreaks, waterborne disease, traveler's diarrhoea and opportunistic infection.

C. cayetanensis has been identified as the cause of foodborne outbreaks in the United States, Canada, Germany and Mexico (Conner and Shlim, 1995; Carter *et al*., 1996; CDC, 1997; Conner, 1997; Herwaldt and Ackers, 1997) where the outbreaks were secondary to consumption of imported fruit and vegetables from endemic tropical countries (CDC, 1996a,b; CDC, 1997; Herwaldt and Ackers, 1997). Several types of fresh produce imported from various countries have been implicated as vehicles of transmission of *C. cayetanensis* which include fresh raspberries, snow peas and mesclun lettuce (Bern *et al*., 2002). A large outbreak of *Cyclospora* infection that occurred following a May 1996 wedding reception at a restaurant in Boston Mass is described in which 57 out of the 101 respondants had cyclosporiasis after eating pudding containing raspberries (Fleming *et al.*, 1998). The objectives of that report were to identify risk factors associated with illness and to describe the clinical course and spectrum of illness.

Studies suggest that *Cyclospora* is a waterborne infection. An outbreak among staff and employees in a hospital dormitory in Chicago was associated with ingestion of water, *Cyclospora* oocysts were detected in stools of 11 out of 21 persons (Wurtz *et al.*, 1993). Among British soldiers in a military camp in Nepal, 12 out of 14 persons who developed diarrhoea and *Cyclospora* oocysts were detected from 6 out of 8 patients (Rabold *et al*., 1994). A study on the role of water in the transmission of cyclosporiasis has been reported in Eygpt (el Karamany *et al.*, 1995). Sturbaum *et al.* (1998) detected *C. cayetanensis* in wastewater lagoons in Peru.

C. cayetanensis infections have been reported in various countries as a cause of travelers' diarrhoea after international travel among expartriates (Long *et al.*, 1990; Shlim *et al*., 1991; Hoge *et al*., 1993; CDC, 1996 a; CDC, 1997; Crucifti *et al*., 1999; de Gorgolas *et al*., 2001; Egloff *et al*., 2001).

Intestinal parasites have been reported as a common occurrence in many countries with or without diarrhoea (Eberhard *et al*., 1999b; Awole *et al.*, 2003; Cranedonk *et al.*, 2003; Lopez *et al.*, 2003) but *Cyclospora* an enteric protozoa has been described as opportunistic infection among HIV patients with protracted disease symptoms (Lindo *et al.*, 1998; Lebbad *et al*., 2001; Gupta and Razaida, 2004; Conner *et al.*, 2007; Dwivedi *et al*., 2007).

2.2.4 Mortality, Morbidity and Symptoms associated with *Cyclospora*

Cyclospora infection is not considered a fatal disease. No reported deaths have been directly attributed to it. The greatest risk is critical dehydration due to the protracted diarrhoea. Infants are at risk of critical dehydration due to protracted diarrhoea. A protracted course of several weeks to months with diarrhoea, dehydration and weight loss can produce significant morbidity (Koru *et al*., 2006). It is more severe in immunologically naïve, such as expatriates and travelers (Long *et al*., 1990; Hoge *et al.*, 1993). If exposed to *Cyclospora*, patients with HIV who are not taking Trimethoprim-Sulphamethoxazole for prophylaxis have a significant risk of developing chronic and debilitating diarrhoea (Peterson, 1998).

Both symptomatic and asymptomatic states of cyclosporiasis have been described. In endemic countries, children often have asymptomatic infections and adults are infrequently infected (Ortega *et al*., 1997). No racial or sex predilections exist in cyclosporiasis (Shoff, 2007). The symptoms begin to appear about a week after ingestion of contaminated food or water with *C. cayetanensis* oocysts and the incubation period ranges from 2-11 days (Soave, 1996). Infection may persist for a month or more. Mild infections produce few or no clinical signs.

Clinical manifestations in immunocompetent patients are watery diarrhoea sometimes alternating with constipation, substantial weight loss, fatigue, abdominal pain, vomiting, nausea, and/ or low-grade fever (Zerpa *et al.*, 1995). The disease is usually self-limiting

and is typically not fatal, although there can be relapse after a month (Hale *et al.*, 1994; Huang *et al.*, 1995; Goodgame, 1996; Soave, 1996; Turk *et al*., 2004). Among immunocompromised hosts, *Cyclospora* infection has occurred primarily in HIV infected individuals who are predisposed to more frequent and prolonged infections with spore forming intestinal protozoa which cause severe diarrhoea (Berlin *et al*., 1994; Sifuentes-Osornio *et al*., 1995; Goodgame, 1996; Fleming *et al.*, 1998; Mohandas *et al.*, 2002).

After ingestion, *Cyclospora* oocysts excyst in the gastrointestinal tract and invade small bowel epithelia where they undergo asexual division followed by sexual division to produce mature oocysts that are shed in stool (Orihel and Ash, 1995). Grossly, moderate to severe erythema of the distal duodenum is observed in patients with *Cyclospora* infection (Connor *et al.*, 1993). Distal duodenal and jejunal aspirates of patients with *Cyclospora* infections had the following histopathological findings: acute and chronic inflammation, reactive hyperemia with vascular dilatation and villious capillary congestion, parasitophorous vacuoles that contain both asexual and sexual forms, crypt hyperplasia, epithelial disarray, and partial villous atrophy (Pape *et al.*, 1994; Ortega *et al*., 1997; Shoff, 2007). On electron micrographs, intracellular particles similar to sporozoites have been demonstrated (Ortega *et al*., 1997).

C. cayetanensis infections have been associated with the onset of various extra-intestinal complications that may accompany prolonged infection, especially in HIV

immunocompromised patients. The infections include: alcalculous cholecystitis, biliary disease, Gullian-Barre Syndrome, and reactive arthritis (Zar *et al*., 2001; Sifuentes-Osornio *et al*., 1995; Richardson *et al.*, 1998; Connor *et al*., 2001; Pinge-Suttor *et al*., 2004). One case of *C. cayetanensis* oocysts in the sputum of a 60 year old HIV-negative male with a history of successfully treated tuberculosis (TB) has been reported in Argentina (Di Gliulio *et al*., 2000). Similar case was reported in Egypt where a 45 year old HIV-negative male with TB history presented oocysts in sputum in active TB reaction (Hussein *et al*., 2005).

Cyclospora infections are widespread in non-human primates with prevalence ranging from 9 to 25% (Eberhard *et al*., 2001; Legesse and Erko, 2004). However, no mortalities have been associated with *Cyclospora* infections. *Cyclospora* infections in non-human primates are asymptomatic (Smith *et al*., 1996; Eberhard *et al*., 2001).

2.2.5 Diagnosis of *Cyclospora* Infections

Clinical diagnosis of cyclosporiasis is an illness of variable severity caused by protozoa *C. cayetanensis* and commonly characterized by watery diarrhoea, loss of appetite, weight loss, abdominal bloating and cramping with increased flatus (Hoge *et al.*, 1993; Zerpa *et al*., 1995; Ortega *et al.*, 1997).

Parasitological diagnosis is based on morphological identification of *Cyclospora* cysts in faecal samples by examination of formal ethyl acetate (FEA) concentration sediments

unstained and modified Ziehl Neelsen and hot safranin stained smears. The stage found in faecal samples is the unsporulated cyst, spherical with well defined cell wall, morula like inclusion and measures 8-10µm in diameter (MAFF, 1979; Orihel and Ash, 1995; Eberhard *et al.,* 1997; Visvesvara *et al*., 1997). Unsporulated oocyst is non refractile, double walled sphere, measuring 8-10µm in diameter (Orihel and Ash, 1995). In fresh stool specimen, the double wall envelopes refractile granules giving the aspect of a morula with greenish shade (Ortega *et al*., 1994). Oocysts are autofluorescent and appear neon blue circles when examined by microscopy in ultra violet light (Hoge *et al.,* 1995). The oocysts on a modified Ziehl Neelsen acid-fast stain, stain variably while uniform staining has been achieved hot safranin stain (Orihel and Ash, 1995; Visvesvara *et al*., 1997). Sporulated oocysts have two sporocyts with two sporozoites in each (Ortega *et al*., 1994; Orihel and Ash, 1995).

Genomic deoxyribonucleic acid (DNA) isolation involves membrane solubilization and protein denaturation, amplification of short sequence of DNA by use of polymerase chain reaction (PCR) and visualization of PCR product on agarose gel stained with ethidium bromide. Molecular characterization techniques targeting single-nucleotide in the 18S rRNA gene using a nested polymerase chain reaction for *Cyclospora* specific species have been developed (Jinneman *et al.,* 1996; Relman *et al*., 1996; Yoder *et al*., 1996; Orlandi *et al*., 2003).

Pathological evaluation of cyclosporiasis was by gross and histopathology. Grossly, moderate to severe erythema of the distal duodenum has been observed in patients with *Cyclospora* infection (Connor *et al.*, 1993; Orihel and Ash, 1995). Hematoxylin-eosin stained tissue smears are examined on light microscopy for detection of tissue stages of *Cyclospora cayetanensis* and for histopathology of small intestine due to infection has been reported by other researchers (Orihel and Ash, 1995; Nhieu *et al.*, 1996). Tissue stages of *Cyclospora* have been demonstrated by light microscopy (Nhieu *et al*., 1996) and transmission electron microscope (Ortega *et al*., 1997).

2.2.6 Prevention and Control of *Cyclospora* Infections

Immunocompetent adult patients have been successfully treated with trimethoprim-sulphamethoxazole at dosage level of 160mg and 800mg per os twice a day for 7 days (Hoge *et al.,* 1995; Fryauff *et al.*, 1996). Children treated in Peru with a 3-day course had a significant decrease in the duration of oocyst excretion (Madico *et al*., 1997). Patients with AIDS were treated at dosage level of 160mg trimethoprim-800mg sulphamethoxazole four times a day for 10 day period and a prophylaxis treatment given three times a week to prevent relapse (Pape *et al.*, 1994).

There is no vaccine for cyclosporiasis. Prevention should be directed to avoidance of contamination of food and water. Observance of personal hygiene and proper hand washing is of utmost importance in preventing food borne infections. Food handlers should be meticulous about hand washing. Public waters should be guarded to reduce

risk of contamination of fountains, pools and recreational water facilities. Water should be properly treated to eliminate *Cyclospora* oocysts. Fruits should be washed properly; vegetables should be washed or cooked before serving them. Investigations to identify cases, source of illness and to institute disease control to prevent further spread are recommended.

To date, no record was available on treatment of *Cyclospora* infection in non-human primates. The treatment regimen used in this study was based on treatment success achieved in human patients using Cotrimoxazole® {(Trimethoprim 160mg and sulphamethoxazole 800mg) (Pape *et al*., 1994; Fryauff *et al.,* 1996; Donald *et al*., 1997; Madico *et al*., 1997; Verdier *et al*., 2000).

Based on the fact that *Cyclospora* infections are transmitted through consumption of contaminated water or food, avoidance of faecal contamination would aid in the prevention of such infections. There are no documented control methods for *Cyclospora* infections in non-human primates currently.

2.3 Human and Simian Immunodeficiency Virus Infections

2.3.1 The viruses

Human and Simian Immunodeficiency viruses (HIV and SIV) are retroviruses with ribose nucleic acid (RNA) as genetic material. Molecular structure of SIV and HIV is a dense cylindrical core surrounded by lipid envelope which is bilipid with two viral

glycoproteins gp 41 and gp120 (Janis, 1992). Replication of these viruses is characterized by presence of reverse transcriptase enzyme and maturation outside host cell. HIV-1, HIV-2 and SIVs are lentiviruses (Desrosiers *et al*., 1989). These viruses cause wasting disease, neurological disorders, immunodeficiency syndromes, induction of certain cancers and are associated with long incubation persistent chronic infections characterized by opportunistic infections (Cann and Chen, 1990; Desrosiers *et al.*, 1989).

Simian Immunodeficiency Viruses have been isolated in different simian species (Garner, 1996) SIVagm (*Cercopithecus aethiops,* Ohata *et al*., 1988), SIVmnd (*Pabio mandrillus*, Tsujimoto *et al.*, 1988), SIVsmm (*Cercopithecus atys*, Hirsch *et al.*, 1989), SIVsky (*Cercopithecus mitis*, Emmau *et al*., 1991), SIVwcm (*Cercocebus torquartus*, Tomonaga *et al*., 1993) and SIVcpz (*Pan troglodytes,* Peters *et al.*, 1989). SIVagm is endemic in wild African green monkeys (*C. aethiops,* AGM) with seroprevalence of 30-50% (Otsyula *et al.*, 1996). Studies indicate genetic relatedness between SIV, HIV-1 and HIV-2 (Baier *et al.*, 1989; Gao *et al.,* 1999; Joy *et al*., 2001).

2.3.2 Immunology of SIV Infections in the African green monkey

Immunological investigation of SIV infected monkeys indicate that response to SIVagm in naturally infected monkeys was similar as seen in HIV-infected humans (Norley *et al*., 1990). The African green monkey lack antibody response to non denatured gag core proteins (Gicheru *et al.,* 1999). Baier et al. (1989) reported that molecularly cloned

simian immunodeficiency virus SIVagm, is highly divergent from other SIVagm isolates and is biologically active in vivo and in vitro.

SIV is thought to be non pathogenic in several natural hosts, including the AGM, never the less many SIV strains induce a profound immunodeficiency virtually identical to HIV-1 induced in humans when administered to Asian macaque species such as *Macaca mulatta* (Joy *et al*., 2001). Joy *et al*. (2001) have described SIV infections in vervet monkeys (*Clorocebus aethiops*) at an Australian zoo with the animals developing clinical symptoms of persistent profuse watery diarrhoea which progressed to emaciation and dehydration. SIV can also cause AIDS after cross species transmission among African non-human primates {(NHP) (Jones, 2004)}. The origin of Human immunodeficiency virus type 2 is believed to have been in the chimpanzee *Pan troglodytes* (Gao *et al.,* 1999) and SIV has been described as a model for HIV (Norley and Kurth, 1997). Locher *et al*. (2003) reported increased viral replication and virulence after serial passage of HIV-type 2 in baboons. Non-human primates are susceptible to immunodeficiency and viral infections that are lymphoproliferative. The livers of SIV-infected monkeys showed mild hepatitis, with predominantly CD8+ T lympocytes infiltration in to the periportal fields and sinusoids comprised of a high percentage of SIV-specific cytotoxic T lymphocytes (CTLs) (Schmitz *et al*., 2000). Similar SIV-specific CTLs were found in gastrointestinal tissues of chronically SIV-infected rhesus monkeys (Schmitz *et al*., 2001). Rhesus monkeys infected with SIV manifested

immunosuppression and central nervous disease that was pathological (Rausch *et al*., 1999).

2.3.3 Association of *Cyclospora* with HIV Infections

One of the major health problems among HIV seropositive patients is superimposed infections due to the defective immunity. Intestinal parasite infections are common in tropical countries and have high prevalence among HIV-infected patients (Wiwanitkit, 2001). Coccidian protozoa *Isospora belli*, *Cryptosporidium* species and *Cyclospora cayetanensis*, which are opportunistic infections, are all causes of chronic severe diarrhoeal diseases in the context of HIV interactions (Ooi *et al.,* 1995; Sifuentes-Osornio *et al.,* 1995; Peterson, 1998; Muthal *et al*., 2004; Karp and Anwaerter 2007; Meamer *et al*., 2007). Several studies have recorded co-infections of *Cyclospora* with HIV (Pape *et al*., 1994; Sifuentes-Osornio *et al*., 1995; Verdier *et al*., 2000).

2.3.4 Association of *Cyclospora* with SIV Infections

Two rhesus monkeys experimentally infected with SIVmac 239 strain prior to inoculation with *C. cayetanensis* had absolute CD4 counts of 172 (11%) and 373 (11%) cells/mm^3 which is significantly below normal values of 800-1,100 cells/mm^3 (40-50%), with a level of opportunistic infections observed. However, these monkeys did not establish *C. cayetanensis* infection although they were immunodeficient (Eberhard *et al*., 2000). To date, no record of *Cyclospora*/ SIV co-infections has been documented in non-human primates.

2.4 The Role of Non-human Primates in Biomedical Research

The use of experimental models to investigate aspects of human disease involves highly specific considerations in order to use appropriate model framework. The purpose of biomedical research involving non-human primates is to improve diagnostic, therapeutic, prophylactic procedures, understanding etiology and pathogenesis and to assess the likely risks and benefits to human health. It is often necessary to use non-human primate (NHP) in development and evaluation of new compounds prior to clinical trials with humans. Research with non-human primates plays a role in advancement of human and animal health.

Non-human primates share 98% of human genes, reflect the anatomical, physiological and behavioral make up of humans, hence provide irreplaceable bridge between laboratory studies and clinical use (www.ca-biomed.org). Non-human primates have continued to play a critical role in medical progress on a wide variety of diseases (Kuntz, 1973; Nyindo and Farah, 1990; Olobo *et al.,* 2002). Rhesus monkeys are prime models for HIV treatments and potential vaccine testing (Rausch *et al.,* 1999).

2.4.1 Sources of Non-human Primates

Most of the non-human primates used in research are normally wild-trapped as described by Moinde *et al* (2004), with unknown genealogy, past history and present health status. Research centres can also breed their research animals. Captive-bred

primates have an advantage over wild-trapped in that their genealogy, history and health status are known.

The general guidelines on animal experimentation on care and use in biomedical research are as stipulated by the Private Vaccine Evaluation Network PVEN, (Poole and Thomas.The principles of animal experimentation conform to generally accepted principle recommendations based on Council for International Organizations of Medical Sciences, (CIOMS, 1983) and the World Medical Association of Helsenki (WMA, 1992) modified to apply to non-human primates and Russel Burch (1959) Three "R" humane principles of reduced numbers of animals to a minimum and to replace the need for animals with organs and cells whenever possible.

2.4.2 Current Status of Models for Human Disease Using Non-human Primates in Kenya

Currently many models for specific pathogens have been developed. *Cercopithecus aethiops* has been identified in Kenya as a natural host for *Leishmania major* and transmission has been achieved by insect vectors (Olobo *et al.*, 2002). *Pabio cynocephalus* and *Cercopithecus aethiops* have been recorded as natural hosts of *Schistosoma mansoni* in Kenya and the baboon is currently being used in immunological studies (Nyindo and Farah, 1990; Muchemi, 1992). There is evidence that Simian Immunodeficiency Virus infections occur in several species of monkeys,

the rhesus monkey has served as valuable model for Human Immunodeficiency Virus infections (Rausch *et al*., 1999).

2.4.3 Relevance of African Green Monkey (*Cercopithecus aethiops*) as an Animal Model for *Cyclospora* and *Cyclospora*-HIV Co-infections

Cyclospora infections have only been documented in humans and non-human primates. *Cyclospora* infections have been characterized in African non-human primates with prevalence of 9- 25% (Smith *et al.*, 1996; Lopez and Manglicnot, 1999; Eberhard *et al*., 2001). The natural infections are asymptomatic. SIV infections are widespread in African green monkey, and the infections are asymptomatic even in seropositive individuals (Otsyula *et al.*, 1996). *Cyclospora* infections in humans are asymptomatic or present as self limiting diarrhoea while the infections in immunocompromised individuals particularly HIV/ AIDS patients have protracted diarrhoea (Berlin *et al*., 1994; Pape *et al.,* 1994).

Very little is known about the epidemiology of the cyclosporiasis due to *Cyclospora cayetanensis*. The use of an appropriate animal model to study aspects of the cyclosporiasis is recommended in view of the significance of this disease in human populations especially, in immunocompromised individuals. The development of a laboratory animal model for studying cyclosporiasis would improve our understanding of this parasite.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out at the Institute of Primate Research (IPR), Karen, Nairobi, Kenya which is a World Health Organization (WHO) Collaboration Centre.The Institute of Primate Research (IPR) is situated in Oloorua Forest, which is an indigenous forest in Nairobi, and is located 15 km southwest of Nairobi city. The Institute holds non-human primates captured in the wild from various locations within Kenya. The non-human primates used in this study were captured from Lemuria Forest Reserve, Aberdares, the Arboretum, Nairobi and from Nguruman in Kajiado district. Some of the animals included in this study were colony-born at IPR. Figure 3.1 is a map of Kenya showing the various locations from where the study animals were trapped.

Aberdare Forest is located in Central Kenya, about 150 kilometers north of Nairobi and lies between longitude 36^0 41' 57" East and latitude 0^0.22' 48" South. The park altitude ranges 2100 to 4300 m above sea-level, and covers an area of 766 sq kms. The vegetation cover is varied and includes moorland, bamboo forest and an equatorial rain forest. The equatorial rain forest receives rains throughout the year, with peak periods being in the months of April and July. Lemuria Forest Reserve is part of the Aberdares National Park and borders the Solio Ranch. The communities living near the Lemuria

Forest Reserve are small-holder farmers practising subsistence horticultural farming and keeping of livestock. Other activies in the area include tourism. Prior to the animal trapping, Kenya had experieesnced a long dry spell and cattle had moved into the forest which also affected this area. Apart from primates, the wildlife found in the area includes lions, leopards, baboons, zebras, warthogs, giraffes and gazelles. The Ewaso Ngiro River is the source of drinking water for humans, domestic livestock and the wildlife.

The Arboretum is a man made forest situated in the Nairobi and lies between longitude 37^0 10' 0" East and latitude 0^0 22' 48" South. The rainfall is usually in two seasons the year with peak periods in the months of April and November. The forest neighbours a built up residential area, and it is used for recreation and as a picnic site.

Nguruman is a rural reserve, 50 kilometers south of Lake Magadi in Kajiado District, and lies between longitude 36^0 1' 0" East and latitude 1^0 45' 0" South. This is a semi arid area with annual rainfalls of less than 200mm with peak periods in March and November. The area has two seasonal rivers which local farmers use for canal irrigation for horticultural farming, and also serve as source of water for humans, livestock and wildlife. The surrounding area is savanna grassland with small acacia bushes. The wild animals found in the savanna grassland include baboons, monkeys, giraffes, zebras and lions.

3.1.1 Climatic Data for the Study Area

The total rainfall recorded at Nanyuki weather station to represent Aberdares area for the year 2007 and 2008 was 695 and 549mm, respectively. The rainfall season run through out the year with peak rainfall in April 2007 and November 2008. The Dagoretti weather station representing the Arboretum and Oloorua forest (where IPR is situated) recorded total rainfall of 1587 and 749 during 2007 and 2008, respectively. In the year 2007, there was rainfall throughout the year but in the year 2008, there were dry spells in May, August and December. The total rainfall recorded at Narok weather station to represent Nguruman area for the year 2007 and 2008 was 711.6 and 616.1mm, respectively. The rainfall season run through out the year with peak rainfall in February 2007 and March 2008. The rainfall patterns for Nanyuki, Dagoretti and Narok for 2007 and 2008 are presented in Figure 3.1.2.

The study areas experienced varying temperatures during the study period, with peak temperatures in the months of February-March (27.2 ^{o}C) for Nanyuki, and March (26.1 ^{o}C) for Dagoretti. Average daily maximum for Nanyuki was 25.0 ^{o}C and daily minimum of 10.5 ^{o}C. Average daily maximum for Dagoretti was 23.8 ^{o}C and daily minimum 13.8 ^{o}C. No weather data was available for Nguruman (Kajiado District) as there is no weather station in the area, but the weather recording for Narok Weather Station which is in the same zone was used. The Narok area experienced varying temperatures during the study period, with peak temperatures in the month of December (27.5 ^{o}C). Average daily maximum for Narok was 23.6 ^{o}C and daily minimum of 10.4 ^{o}C (Figure 3.1.3).

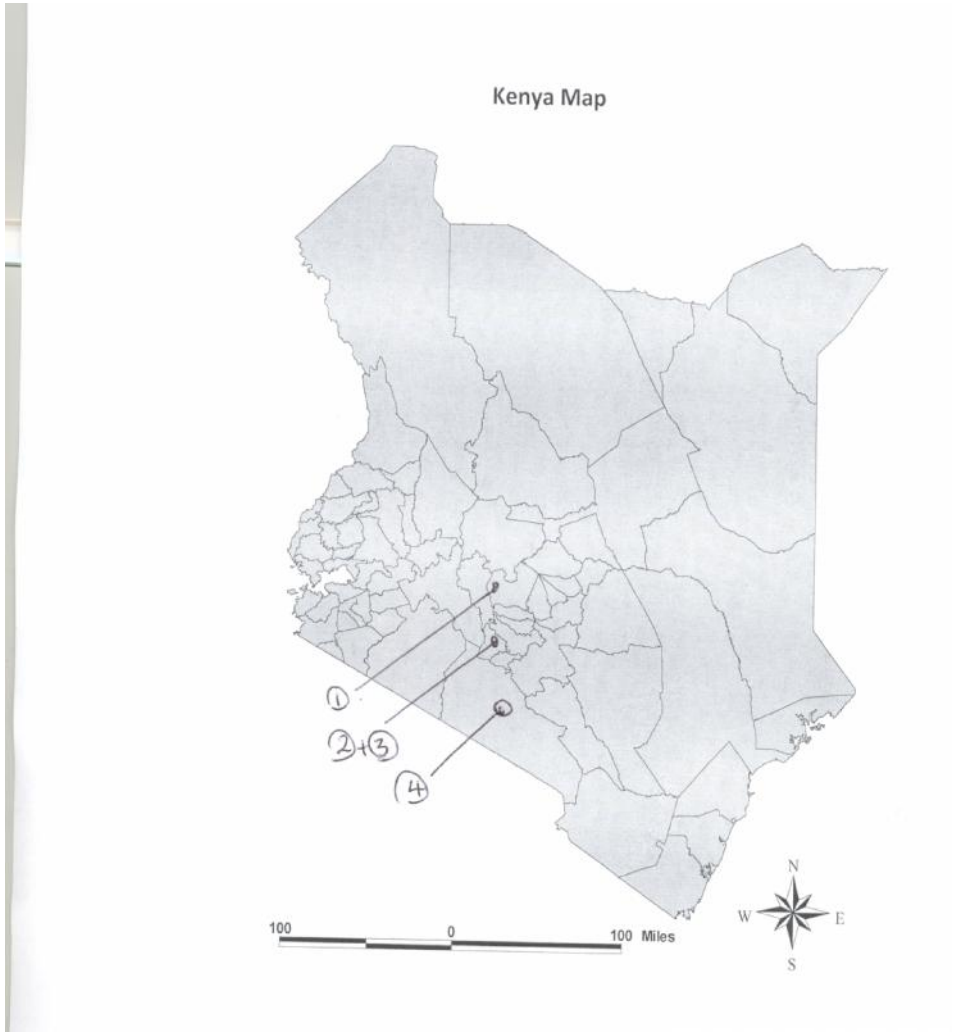


Figure 3.1.1: A map of Kenya showing the location of the areas where the study animals were trapped in 2008.

Key:

1- Lemuria Forest reserve, Aberdares; 2-Nguruman, Kajiado district; 3- State House Nairobi, near the Aboretum and 4- Institute of Primate Research, Nairobi

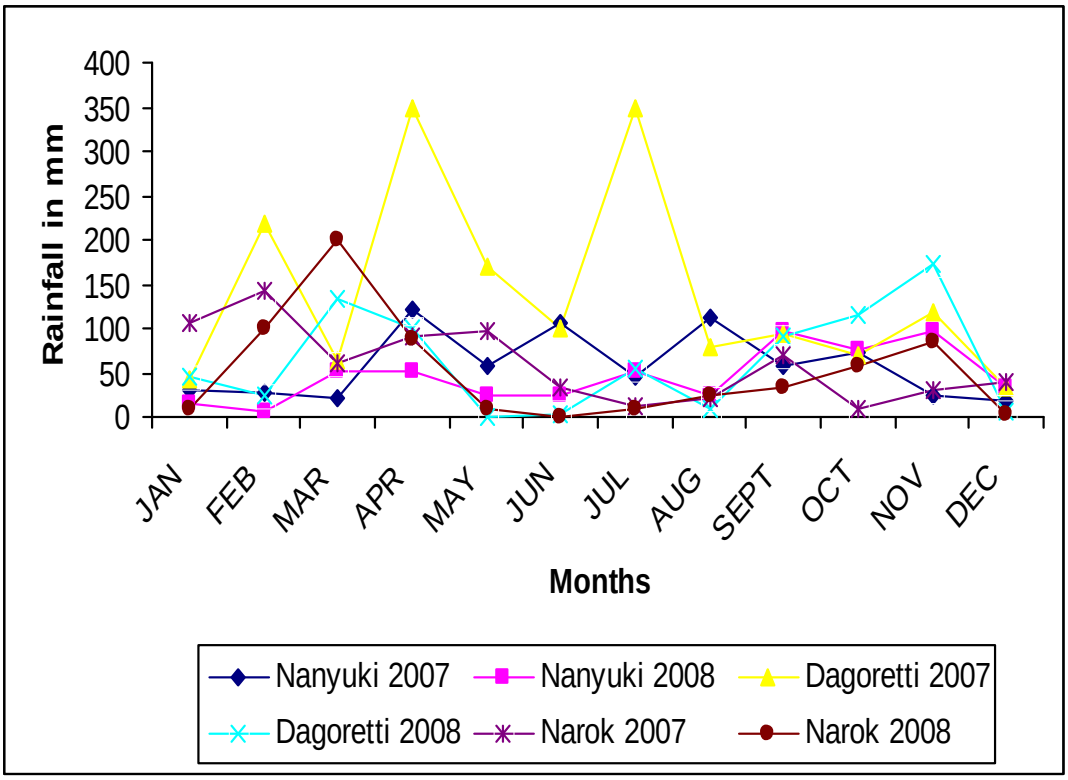
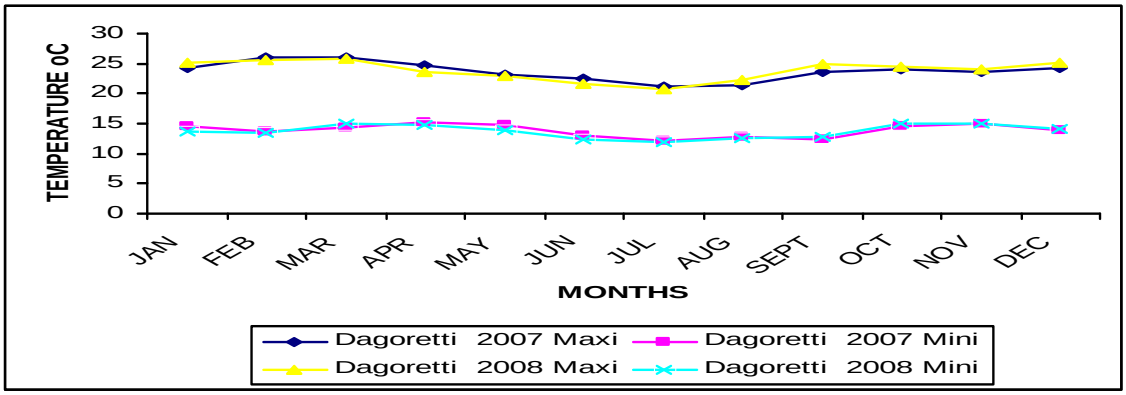
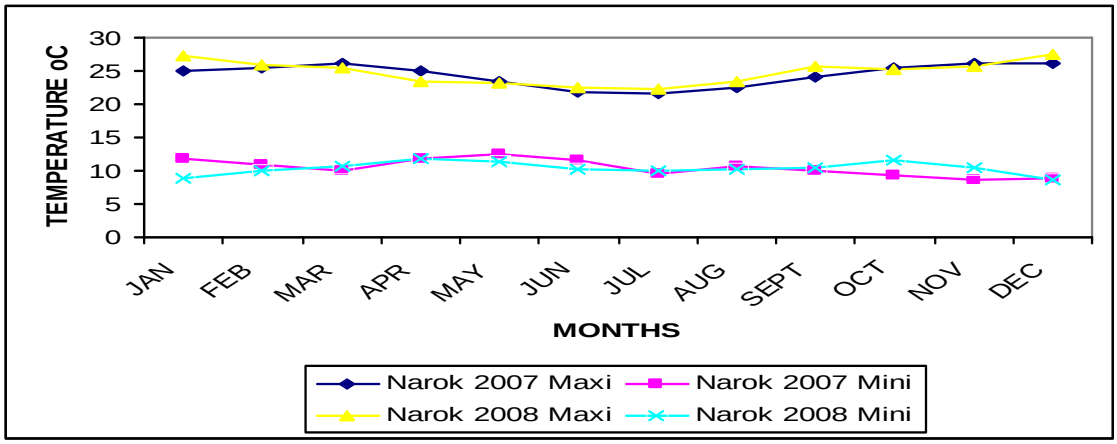


Figure 3.1.2: Rainfall Pattern for Nanyuki, Dagoretti and Narok areas as recorded during the period January 2007 –December 2008.

(Data Source: Kenya Meteorological Department)





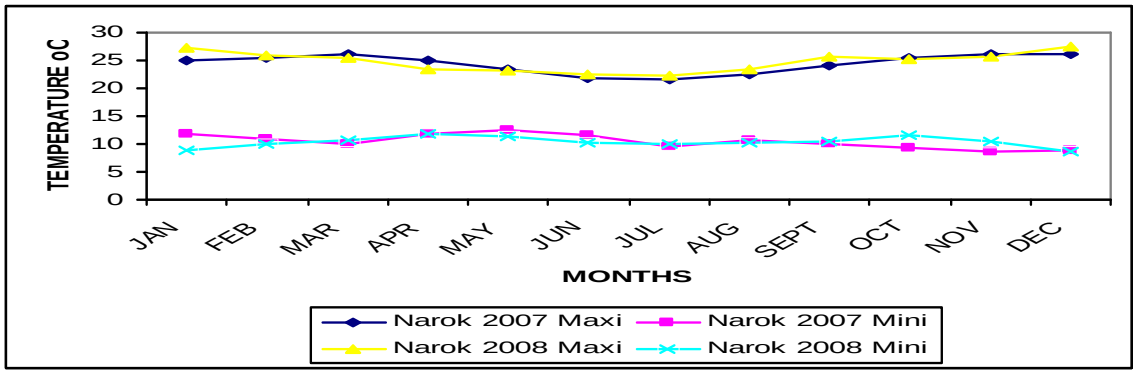


Figure 3.1.3: Daily Minimum and Maximum Temperatures for Nanyuki, Dagoretti and Narok areas as recorded during the period January 2007 –December 2008

(Data Source: Kenya Meteorological Department)

3.2 Study Animals

The study animals, wild captured from October 2008 to February 2009 were transported to IPR where they were housed in cages. The animals were stratified by species (Olive baboons and monkeys), age groups (juveniles and adults) and sex (males and females). The criteria for determining age of animals are summarized in Table 3.2.1. The animals included 65 Olive baboons (*Papio anubis*) and 64 African green monkeys (*Cercopithecus aethiops*). All the animals used for the study were individually housed in stainless steel cages measuring 60x70x160cm. The animal room was temperature controlled and was maintained at 25 $\pm 1^0$ C, relative humidity 50%, was supplied with fresh air, and provided with insect screened windows, a 12 light hour period from 6 AM to 6 PM and a 12 dark hour period from 6 PM to 6 AM. The animals were fed with commercial monkey pellets (UNGA Feeds Ltd, Nakuru), fresh vegetables (Kale), and fruits (bananas and oranges). Fresh clean water was offered *ad libitum*. The animals were tested for tuberculosis every fortnight during the 90 day Quarantine period. The animals were dewormed when all the faecal samples needed for detection of gastrointestinal parasites and *Cyclospora* species had been collected.

Table 3.2.1: The criteria used for determining the age of non-human primates studied

AGE CLASS	**JUVENILES**		**ADULTS**	
Characteristic	**Olive baboons**	**African green Monkey**	**Olive baboons**	**African green monkey**
Teeth	Decidous	Decidous	Permanent	Permanent
Age in years	4	4	4	4
Body weight in kg	4	4	4	2

Data Source: Eley *et al*., 1989

3.3 Sample Size Estimation

The sample size for the animals needed was determined based on results of previous studies in which the prevalence of *Cyclospora* species in Kenyan non-human primates was established to be 25% (Eberhard *et al*., 2001). The sample size was also based on practical guidelines for general surveys of primate parasites in which an assumed prevalence of 5% applies resulting in a minimum requirement of 60 independent faecal samples (Gillespie, 2006). Sample size estimations were calculated using the formula provided by Kirkwood, 1988 as follows:

$$n \geq \frac{Z^2\,\alpha/2 \,.\, \theta^2}{d^2}$$

$$= \frac{Z^2\,\alpha/2 .\, p(1-p)}{d^2} = \frac{1.96^2 .45(1-45)}{1^2}, \text{ or } = \frac{1.96^2 .\, 25(1-25)}{1^2}$$

$$= \frac{3.8416x45(-44)}{1^2}, \text{ or } = \frac{3.8416x25(-24)}{1^2} = 50 \text{ or } 23$$

where: n = minimum sample size

Z = normal deviate =1.96

α = two tailed test = 0.05

d = precision indicated by investigator = 1

θ^2 = Population variance

p = Population with cyclosporiasis / Total population in which cyclosporiasis could occur = 45% or 25% (Eberhard *et al.*, 2001)

with 95% Confidence Interval and 5% significance level.

For the experimental case control studies, a minimum of four (4) individuals per study group was adopted to enable the data generated to be statistically viable.

3.4 Study Design

Cross-sectional surveys of gastrointestinal parasites, *Cyclospora* and Simian Immunodefiency Virus infections were conducted in the captive Olive baboons and African green monkeys held at IPR. Case control studies were also used to follow up natural and experimental *Cyclospora* infections in captive animals, and to evaluate of Trimethoprim-Sulphamethoxazole therapy for *Cyclospora* infections in African green monkeys.

3.5 Laboratory Procedures

3.5.1 Examination of Primates for Gastrointestinal Parasites

Olive baboons and African green monkeys, captured in the wild were transported to the IPR, Nairobi Kenya, where they were screened for gastrointestinal parasites using parasitological means. A total of sixty five *Pabio anubis* (Olive baboons) and sixty four *Cercopithecus aethiops* (African green monkeys, AGM) were used in this study. Table 3.5.1 illustrates the species, sex, age and location collected for the study animals. Freshly defecated individual faecal samples were collected in small clean, plastic bags and labeled with a number corresponding to each individual animal's number and date of sample collection. The faecal samples were transferred to the laboratory within 30-60 min of collection and were processed for parasitological examination in a fume hood

cabinet. Four faecal samples were collected from each individual animal twice a week (Mondays and Thursdays) between 9-10 AM over a period of two consecutive weeks. A total of 260 Olive baboon and 256 African green monkey faecal samples were collected.

Table 3.5.1: Distribution of study animals by species, age, sex and location trapped used in gastrointestinal parasite species survey at IPR, Nairobi from 2008 to 2009

Species	Number	Male Adults	Female Adults	Male Juveniles	Female Juveniles	Location Trapped
Olive Baboons	65	0	24	22	19	**Aberdares**
African green monkeys	14	5	6	2	1	**Arboretum**
	48	21	10	6	11	**Nguruman**
	2	0	0	0	2	**IPR colony**

3.5.1 Faecal Sample Processing and Examination

The faecal samples were examined for texture and characterised as formed or loose, and were also examined for presence of adult or larval worms. Both the direct faecal smear and McMaster techniques were used to process the faecal samples for parasitological examination.

For the direct method, a small pea size portion of faecal sample from an animal taken from larger faecal sample obtained from an individual animal and placed on a clean microscope slide, emulsified with two drops of 0.85% sodium chloride (saline), cleared of debri and spread into a thin smear using a wooden applicator stick (Cheesbrough, 2005). The wet smear was covered with a 15x15 cover slip and examined by light microscopy at X100 and X400 magnification to screen for parasite ova and cysts using Nikon Optiphot microscope (Sciex EA Ltd Nairobi, Kenya). Representatives of parasites observed on direct wet smear were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp, Japan).

In order to concentrate the parasite ova and cyst, the formalin ethyl acetate concentration technique as described by Orihel and (Ash, 1995) was used. One gram of faeces from each individual faecal sample was processed and the sedient smears from each sample were made and subjected to various staining techniques for different parasites. The smear slides were examined systematically to include the entire area

under cover slip. Higher magnification was used to identify structures of interest and their detailed morphology. Smears of sediments from each sample were made and subjected to various staining techniques for different parasites.

In order to identify the acid fast parasites, the modified Ziehl Neelsen staining technique was used (Cheesbrough, 2005). A wet smear from the sediment of each individual faecal sample collected was made on the labelled slide using a drop of normal saline then left to dry completely. The smear was fixed by dipping it 6 times in absolute alcohol and again left to dry. Afterwards the slide smears were placed in dilute Carbol fuschin for about 7 minutes, after which they were washed in gentle running tap water. The slide smears were decolourized in 3% acid alcohol, until no more colour was coming out. The slide smears were washed in gentle running tap water. The slide smears were counterstained in 1% Malachite green for 10 minutes, after which they were washed in gentle running tap water and left to dry. The slide smears were observed under X400 and X1000 magnification for acid-fast staining intestinal coccidian parasites (Cheesborough, 2005). Representatives of parasites observed on Ziehl Neelsen (ZN) stained smears were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp, Japan).

To further differentiate *Cyclospora* from *Cryptosporidium* the modified safranin staining technique as described by Visvesvara *et al* 1997 was used. The two prepared slide smears per individual faecal sample were allowed to dry completely before

staining. The slides smears were placed in a Coplin jar containing acidic alcohol (3% vol/vol HCl in methanol) and allowed to stand for 5 minutes. The excess acidic alcohol was washed with cold tap water and the slide smears placed in a Coplin jar containing (1% wt/vol) safranin solution in acidified water (pH 6.5). The Coplin jar was placed on a glass plate so that the overflowing stain [because of boiling] could be caught in the tray and not on the microwave oven floor. The Coplin jar with safranin stain and slide smears was placed in a microwave at full power (650 W) for 1 minute. The excess stain was washed with tap water and the slide smears were placed in a Coplin jar containing 1% wt/vol aqueous solution of malachite green and left to stand for 1 minute, and then rinsed gently with tap water and left to dry. The slide smears were examined by microscopy at X400 and X1000 magnification. Fresh staining solution was used after a cycle of 10 microwave heatings (Visvesvara *et al.,* 1997). From positive slide smears the presenting protozoa were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp, Japan).

To quantify the parasite ova, the Mac Master technique was used (MAFF, 1979). One gram of faecal sample from an individual animal was placed in a 15ml tube and 14ml of super-saturated salt solution was added into the tube. The tube contents were thoroughly mixed using an applicator stick and strained through 400µm sieve into a second 1 ml tube. Using a Pasteur's pipette, the suspension was transferred into the two chambers of the MacMaster slide and allowed to stand for 5 minutes to allow eggs to settle. The MacMaster slide was examined by light microscopy at X100 magnification. The

parasites detected were identified based on morphological features and counted in the two chambers (MAFF, 1979). The number of various ova enumerated in the individual faecal samples was recorded on a specially designed data sheet for this purpose and used to calculate parasite egg intensities.

The Harada Mori technique (1995) was used to cultivate helminth larva for identification and differentiation between *Oesophagostomum* and *Strongyloides* for faecal samples that were positive for Strongyle eggs. A faecal smear was made on a 1.5cm strip of filter paper, placed in a 15ml test tube with 4ml distilled water and corked. The test tube was kept in a warm room at ambient temperature 23-25 ^{0}C and observed at two day intervals, for a period of 14 days for larvae using a dissecting microscope (Cheesborough, 2005). Representations of different larvae observed were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp, Japan).

Determining size of ova or oocyst can help in parasite identification. A stage micrometer and eyepiece were used in measuring parasite sizes. Micrometry involves the measurement of objects using a calibrated eyepiece scale. The eyepiece micrometer to fit into X10 eyepiece is divided into 50 divisions. The stage graticule is engraved on glass slide and measures 2mm each large division measuring 0.1mm (100µm). Objective Micrometer (Graticules Ltd Tenbridge Kent, England) was used to calibrate the stage micrometer of the microscope. The procedure used for calibration of the

microscope was as described by Cheesbrough (2005). The eyepiece micrometer disc was inserted into upper lense of X10 eyepiece. The stage graticule slide was placed on the stage. Using X10 objective lense, the field was adjusted until the 0 line of eyepiece aligned with 0 line of calibration scale. On the scales the point at which the line of eyepiece aligned exactly with calibration scale was measured. Each small division measures 0.01mm and a count was made of the small divisions in the alignment. The same procedure was repeated using the X40 objective lense. Using X10 lense, 1 unit (10 small divisions) ocular corresponded to 1 unit stage micrometer which is equivalent to 100µm while with X40 objective lense, 4.1 units ocular corresponded to 1 unit stage which was equal to 100µm, therefore 1 unit ocular was equivalent to 24.39µm. The sizes of parasite eggs and protozoa cysts were measured using this calibrated microscope. Parasite sizes were important characteristics in distinguishing between different parasite species.

3.5.2 Identification of Parasites

Protozoan cysts were detected on wet FEA sediment smears using their characteristics of shape, size, presence of refractile structures and number of nuclei as described by others (Ash and Orihel, 1995; Cheesborough, 2005). The morphological features used to identify *Cyclospora* oocysts in stained slides was their spherical shape, sizes varying from 8 to 10µm in diameter, a smooth outer wall and characteristic uniform reddish orange in safranin based stains. Representatives of protozoan cysts encountered were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp,

Japan). Worm eggs were detected on wet mounts using characteristics of size, shape, stage of development, thickness of egg shell, colour and presence of operculum dependent on the species of helminth parasites present as described by others (Sloss *et al.*, 1994; Cheesborough, 2005). The *Strongyloides* positive faecal smears, the eggs had thin egg shells and were larvated. Representatives of helminth ova were photographed using digital camera (Sony Cybershot 7.2 Mega Pixels, Sony Corp, Japan).

3.5.3 Preservation of Faecal samples

Various techniques were used to preserve the faecal samples for future processing as per procedures required. Preservation of faecal samples in 10% buffered formalin for faecal samples to be used for *Cyclospora* oocyst harvesting. Using a wooden stick applicator and a weighing scoop, 3gm of fresh faecal sample from each individual faecal sample collected were placed in a well labeled 50ml centrifuge tube containing 30ml of 10% buffered formalin. The faecal sample was stirred and brought to a suspension and tube closed with a rubber stopper. The buffered formalin preserved faecal samples were kept at room temperature. These were labeled with the individual animal identification number and date of faecal sample collection to be used later for harvesting *Cyclospora* oocysts.

Preservation of faecal samples in 2.5% potassium dichromate ($K_2Cr_2O_7$) was done for faecal samples to be used for harvesting *Cyclospora* oocysts. A portion of the 3gm of faecal sample collected was washed in clean water, allowed to settle, the sediment

passed through a 400µm sieve and resuspended in clean water. The supernatant was discarded and the sediment mixed with 2.5% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$) solution in a 3:1 ratio and allowed to settle. The supernatant was discarded, and fresh potassium dichromate added at ratio of 3:1 and the mixture kept at room temperature (Eberhard *et al*., 1997), to be used for harvesting *Cyclospora* oocysts.

3.5.4 Harvesting of *Cyclospora* Oocysts

The harvesting of *Cyclospora* oocysts was done using two techniques, namely sugar and salt floatation techniques. The Sheather's sugar floatation procedure was used for harvesting *Cyclospora* oocysts from positive faecal samples that would be used in animal inoculation trials and DNA analysis while the oocysts harvested by modified salt floatation technique were used for protein preparation for ELISA (Cheesborough, 2005).

Sheather's sugar solution was prepared by adding 500g of sucrose to 320ml of distilled water and boiled. A portion of 0.5g of Phenol was melted in a water bath at 56 ^{0}C and added to sucrose mixture and stirred gently. This step was performed in a fume hood as a safety measure. The solution was allowed to cool to room temperature. A thick faecal suspension was made using tap water. A 1.5ml of the suspension was placed in a 15ml conical tube, Sheather's sugar solution added to the level of 9ml mark on the tube and the contents of the tube were stirred using an applicator stick until properly mixed. The remaining portion of the tube was filled with Sheather's sugar solution leaving only

about 1.5cm open at the top. The tube was loosely covered with cup and was centrifuged at 450g for 10 minutes. Several loopful of material were removed from the top layer in the tube using a dropper and a wet preparation smear was made with this material and the slide smear was examined under light microscopy at X400 magnification for presence of *Cyclospora* parasites. The *Cyclospora* oocysts harvested were preserved in 2.5% potassium dichromate and used in further tests in animal inoculation and DNA extraction.

To harvest *Cylospora* oocysts using the modified salt floatation technique, a composite faecal sample from the *Cyclospora* oocyst positive AGM were emulsified passed through a sieve to remove coarse particles and the suspension was sedimented by centrifugation at 450g for 2 minutes. The sediment was emulsified, the tube filled with saturated salt solution, inverted several times. Salt solution was added to the tube until a meniscus appeared and an 18x18 mm cover slip was placed on the tube and the mixture centrifuged at 200g for 2 minutes. The cover slip was removed with a deliberate upward movement, placed on a slide and examined by light microscopy at X400 magnification for presence of *Cyclospora* oocysts. The *Cyclospora* oocysts harvested were washed into a clean 15ml centrifuge tube using saline solution and used for protein preparation for *Cyclospora* specific enzyme linked immunosorbent assays (ELISA).

3.5.5 Sporulation, Purification and Preservation of *Cyclospora* Oocysts

To sporulate oocysts, a simple culture was made by emulsifying 1gm of faecal sample with 2.5% potassium dichromate, straining it through a 400µm sieve to form a shallow layer in a Petri dish. The *Cyclospora* oocysts were incubated and allowed to sporulate at ambient room temperature of 23-25 ^{0}C. They were examined at two day intervals until sporulation was complete in two weeks (Smith *et al*., 1997). *Cyclospora* oocyst structure was observed under a microscope at X400 and X1000 magnification.

Cyclospora oocysts were then purified using discontinuous sucrose gradient concentraton technique whereby the sample was layered between two sucrose densities of 2.103 and 1.110g/l centrifuged at 450g for 25 minutes and *Cyclospora* oocysts harvested (Ortega *et al.,* 1993; FDA, 1997; Graczyk *et al*., 1998). The purified *Cyclospora* oocysts were mixed with a 2.5% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$) solution for preservation at room temperature (Eberhard *et al.,* 1997). The oocysts were used for animal inoculation experiments and oocyst sporulation assays. Some of the purified oocysts were mixed with distilled water and stored at -20 ^{0}C for DNA extraction and analyses.

To prepare *Cyclospora* soluble protein for coating 96 well microtitrer used with ELISA, *Cyclospora* oocysts were harvested by the modified salt floatation technique and frozen at -20^{o} C (Cheesbrough, 2005). The frozen parasite oocysts were thawed to room temperature on ice, were then disrupted by probe sonication for 10X for 1 minute with

rest intervals using vibra cell sonicator (Soniprep 150) at 25 watts. The mixture was transferred to clean eppendorf tubes and centrifuged at 14000rpm for 1 hour at +4 ^{0}C. The BIO-RAD protein assay (Bio-Rad Laboratories GMbH, Munchen) according to manufacturer's instructions was used to quantify the amount of soluble protein. The principle for this microassay is based on the observation that absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs. The standard used is Bovine serum albumin {(BSA) (Sigma Chemical Company, USA)} sealed in Nitrogen. This is a microassay procedure in which the 1-20µg protein in BSA less than or equal to 25µg/ml protein can be assayed. The test soluble protein had a concentration of 1µg/ml and was stored at -20 ^{0}C until use.

3.5.7 Blood Sample Collection and Processing

Heparinized blood and serum samples were routinely collected at the beginning of 90 day quarantine period after the capture of the primates. Animals were immobilized with 10mg/kg bwt Ketamine (Ketaset$^{\text{œ}}$, Fort Dody Iowa) plus 6 mg/kg bwt Xylazine (Chanazine, Ireland) at the ratio of 5:3 for sampling. One blood sample was collected from each study animal. Weights of the individual animals were recorded during anesthesia. A blood sample (5ml) was collected through venupuncture of the femoral vein (Figure 3.5.7).

A 2ml blood sample from each individual animal was placed in labeled vacutainers containing heparin as anticoagulant for hematology. These samples were analysed for complete blood cell count using hematological analyzer (Coulter counter, Beckman Coulter, Miami, USA). The remaining blood sample of 3ml per individual animal was allowed to stand at room temperature for a minimum of 2 hours to form a clot, stored in the refrigerator at 4 ^{o}C and allowed to stay overnight. The sample tubes were centrifuged at 450g for 10 minutes. The clear supernatant, serum was retrieved using a Pasteur pipette and transferred to a clean epperndorf tube and stored at -20 ^{o}C until used in serological tests.

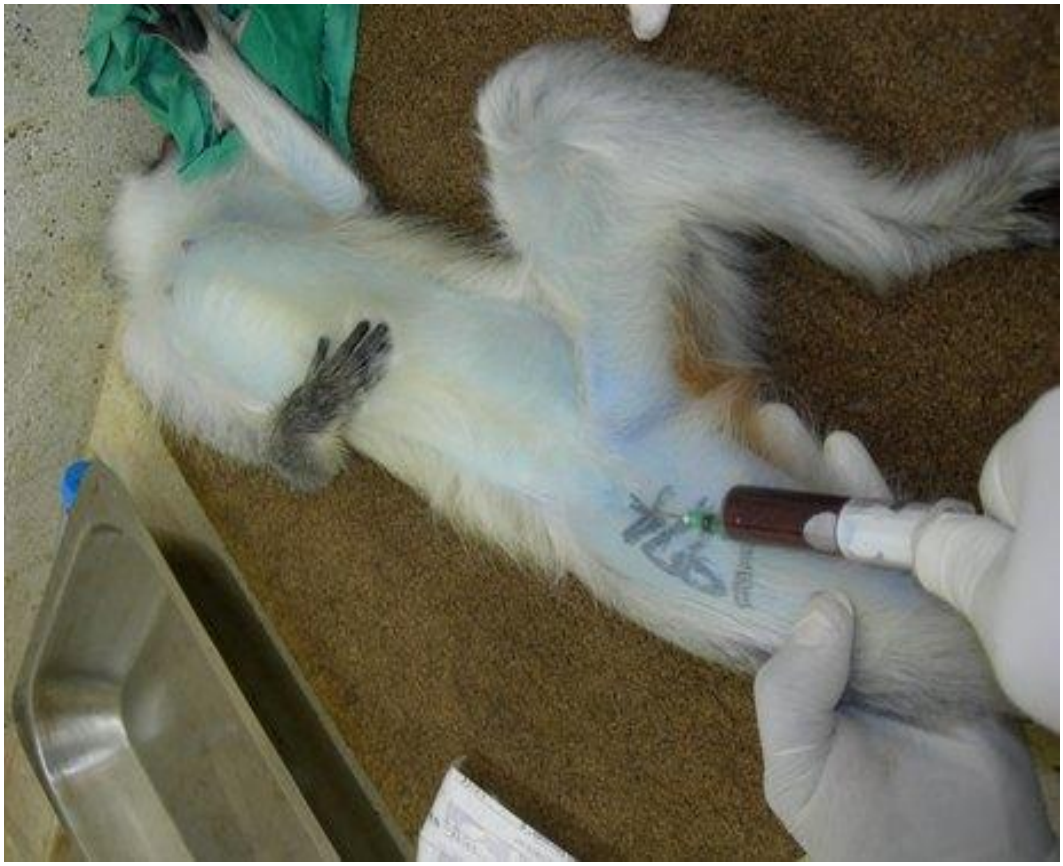


Figure 3.5.7: Blood sample collection by venupucture of the femoral vein at IPR, Nairobi in 2009

3.5.8 Enzyme Linked Immunosorbent Assay (ELISA)

3.5.8.1 *Cyclospora* Specific ELISA

A cohort of 12 AGM with natural *Cyclospora* infection were followed up for one year. Two *Cyclospora* naïve colony-bred AGM were used as negative controls, Blood for serology was collected at the commencement and end of study period.

To perform the ELISA test, the *Cyclospora* soluble protein prepared and assayed was allowed to thaw slowly at +4 ^{0}C, was diluted to 10µg/ml in bicarbonate buffer at pH 9.6, and volume of the 100µl of diluted soluble protein was used to coat wells of a 96 well microtitre ELISA plate (Nunc Roskilde, Denmark), and incubated over night at +4^{0}C. Further reaction was blocked with 3% BSA in phosphate buffered saline (PBS) and incubated for 1 hour at 37 ^{0}C. The plates were washed with 0.05% Tween 20 in phosphate buffered saline (PBS) using ELISA plate washer. The test and control serum harvested were diluted 1:100 in 1% BSA PBS-Tween 20 solution (dilution buffer). A 100µl per well of test sera was added in duplicates into each well and incubated for 1½ hours at 37 ^{0}C. Human anti monkey IgG conjugated to horse radish peroxidase (Sigma Immunochemicals Mo, USA) was diluted to 1:2000 in dilution buffer and 100µl added to each well. The plates were incubated for 1 hour at 37 ^{0}C. A volume of 100µl TMB microwell peroxidase substrate (SureBlue™, Garthersburg, USA) was added to each well and the plates incubated for 30 minutes at ambient temperature in the dark. The reaction was stopped by adding dilute sulphuric acid and absorbance read at 630nm

using Dynex Technologies ELISA microplate reader (Dynatech Laboratories, UK). Positive samples were defined as samples with optical density (OD) greater than two standard deviations (2SD) of the mean negative control.

3.5.8.2 Simian Immunodeficiency Virus Antibody ELISA

Ten Olive baboons and thirty three African green monkeys were screened for Simian Immunodeficiency Virus (SIV) infections in July 2009. The study animals included: ten female adult Olive baboons and thirty three African green monkeys comprising of 10 male adults, 7 female adult, 3 male and 11 female juveniles (all wild-trapped) and 2 female juveniles that were colony-bred.

A 96 microtitre ELISA plate was coated with 21 amonoacid SIVmac synthetic peptide (ELISA kit, Coulter^{œ} Coporations, Hiatech, Florida) 0.4µg/ well in bicarbonate buffer. The coated plate was sealed, and stored overnight at 4 ^{0}C. The plate was washed with ELISA washer and excess fluid drained on blotting paper. Blocking buffer was added to the microwells and incubated for I hour at 37 ^{0}C. The plate was washed and test sera and controls added at 1:100 dilutions to the wells in duplicate, the plate sealed and incubated for 3 hours at 37 ^{0}C. The plate was washed and 100µl of 1:2800 diluted goat anti monkey Immunoglobulin G (IgG) conjugated to horse radish peroxidase (Nordic Laboratory, CA) was added and the plate incubated for 2 hours at 37 ^{0}C. Plate was washed and O phenylenediamine (Sigma Chemicals, St Louis Mo) as chromogen and hydrogen peroxide as substrate added and incubated for 30 minutes at ambient room

temperature in a dark place. The reaction was stopped by adding 50µl of diluted sulphuric acid. Optical densities were read on spectrophotometer at 630nm filter (Dynatech Max). Positive samples were defined as samples with optical density (OD) greater than two standard deviations (2SD) of the mean negative control.

3.5.9 Establishment and Evaluation of Experimental *Cyclospora* in *Cercopithecus aethiops*

Cyclospora infections were established in four (two female juvenile and two male adult) AGM by introduction of oocysts into the stomach using a catheter. Two adult males and two female juveniles AGM were used in this study. *Cyclospora* clean AGM were purposefully selected for inoculation experiment with *Cyclospora* oocysts. The choice of two juveniles was on the basis that they have a naïve immune system and two adult AGM were *Cyclospora* clean but SIV seropositive. The assumption was that the two SIV seropositive adults were immunosuppressed and would therefore support *Cyclospora* infections.

Cyclospora oocysts harvested from *Cyclospora* positive AGM and preserved in 2.5% $K_2Cr_2O_7$ were used to inoculate the test AGM. The animals were anaesthetized using Ketamine and Xylazine as described in section 3.5.7. Inoculation was by introduction of 1000 *Cyclospora* oocysts in a solution of distilled water into the stomach using a catheter. Three slide smears using 50µl of the inoculum each were prepared and the *Cyclospora* oocysts on each of the the slide smears counted under the microscope to

determine the number of oocysts per milliliter. The average number of oocysts was 100 per ml and 10ml of the inoculum was administered to each study animal.

Physical examination was carried out in the anaesthetized animals and body weight recorded. Daily clinical observations were carried and any change in animal behaviour, demeanor, and lack of appetite and character of faeces passed was noted during the seven week experiment period. Faecal samples were collected, processed and examined for *Cyclospora* oocysts twice weekly for the seven week period. Blood for haematology was collected at baseline, 30 and 49 days post inoculation. These samples were analysed for complete blood cell count using hematological analyzer (Coulter counter, Beckman Coulter, Miami, USA). Blood for serological studies was collected and processed at baseline. *Cyclospora* specific antibody responses were assayed on each of the serum samples collected while anti-SIV antibody responses were assayed on only the set of blood samples collected at baseline.

In order to evaluate pathological changes, the experimentally infected AGM were sacrificed and humanely put to sleep using ketamine at 10mg/kg, and euthanized with 60mg/Kg bodyweight of Pentobarbitone sodium 200mg/ml (Sagatal®, May and Baker Ltd., England). On post mortem, gross pathological lesions were noted and recorded. The tissues collected for histological examination included liver, stomach, small intestine (jejunum) and lymph nodes (mesenteric and inguinal). The tissues were fixed in 10% formalin.

For histological evaluation, the tissues were sectioned into 1-2mm pieces and dehydrated through ascending concentrations of absolute isopropyl alcohol starting from 80% to 100%. The tissues were cleared in xylene, embedded in paraffin wax, blocks on wooden chunks, and fixed on the microtome. The tissues in paraffin wax blocks were cut into pieces of 5µm thickness. Four sections were made from every block specimen. These tissue sections were then dewaxed in xylene and hydrated through graded alcohol (100 to 50%), cleared in xylene, alcohol and washed in water. The tissues were subsequently stained with haematoxylin and eosin (H&E). After dehydration, the tissues were mounted on microscope slides and cover slips using Destrene 80, dibutyl Phthalate and Xylene (DPX) mountant (Luna, 1968; Leeson *et al*., 1985). The sections were examined under the light microscope using x10, x40 and x100 objective lenses using discussion microscope (Leitz Laborlux 12). The results were recorded and where necessary, photomicrographs were taken using a digital camera (Sony Cyber shot 8.1 mega pixels, Sony Corp, Japan).

3.5.10 Evaluation of Trimethoprim-Sulphamethoxazole for the Treatment of *Cyclospora* Infections in *Cercopithecus aethiops*

The efficacy of Trimethoprim (TMP) 160mg-Sulphamethoxazole (SMX) 800mg combination for the treatment of cyclosporiasis in African green monkeys was evaluated.Twelve juvenile African green monkeys were purposefully selected for this study comprising of two males and ten females. Ten of the animals had natural *Cyclospora* infections and two female juveniles were *Cyclospora* naïve. Two positive

male juveniles and four *Cyclospora* positive female juveniles were treated with trimethoprim 160mg and sulphamethoxazole 800mg (TMP-SMX) twice daily for 7 days offered in ripe banana, while four *Cyclospora* positive female juveniles and two *Cyclospora* negative were left untreated as positive and negative controls.

Fresh individual faecal samples were collected twice weekly, on Mondays and Thursdays from each of the study animals for an eight week period from beginning of TMP-SMX treatment, processed for parasitological examination by standard FEA concentration technique, prepared sediment smears were stained with hot safranin and examined for *Cyclospora* oocysts. The identified oocysts were enumerated as total slide parasite counts. Blood samples were collected at the baseline and 30 days post-treatment from each of the study animals, processed for hematology and serology. Complete blood cell counts were performed using a haematological analyzer (Coulter counter) to show haematological trend post TMP-SMX treatment, and *Cyclospora* specific antibody responses were assayed by ELISA.

3.5.11 Molecular Characterization of *Cyclospora* Species

Specific identification of *Cyclospora* is prerequisite for studying the epidemiology, biology and is central to the diagnosis and control of cyclosporiasis. To confirm that the parasites identified in natural and experimental infections were *Cyclospora*, conventional nested PCR amplifications were performed using *Cyclospora* specific primer pairs F1E- R2B and F3E- R4B targeting small subunit ribosomal RNA (SSU-

rRNA) coding region of the genome (Relman *et al.,* 1996; Eberhard *et al*., 1999 b). The PCR product visualized on 1.5% agarose gel stained with ethidium bromide. The expected size of the primary amplicon is 636 base pairs (bp) while the expected secondary amplicon is 294 base pairs.

Total genomic deoxyribonucleic acid (DNA) was extracted from oocyst harvested from *Cyclospora* positive faecal samples, nested polymerase chain reaction (PCR) and visualization of PCR product examined on 1.5% agarose gel stained with ethidium bromide. Faecal samples from AGM with natural and experimental *Cyclospora* infections were collected processed and *Cyclospora* oocysts harvested. The *Cyclospora* oocysts were characterized by light microscopy. The *Cyclospora* oocysts were purified by sucrose density floatation at sites physically separate from the laboratory where further molecular studies were performed. The *Cyclospora* oocysts were stored at -20 ^{0}C until use in a separate room from that in which PCR amplicons were handled.

DNA was extracted by adding 500µl of 8 M urea and sodium dodocyl sulphate to the *Cyclospora* oocysts, enzymatic digestion was by incubating the mixture in a water bath (Model 181 Precision Scientific Incorporated, Illinois, USA) at 56 ^{0}C for 3 hours. To the mixture, 500µl of phenol chloroform in the ratio of 1:1 was added, shaken thoroughly (TPM-2 Sarstedt Shaker) then centrifuged (Eppendorf centrifuge 5415 C) at 1000rpm for 5 minutes. The supernatant was transferred to clean collecting tube and rewashed in phenol chloroform. A volume of 500µl of chloroform iso amyl alcohol in

the ratio of 24:1 was added to the supernatant, shaken thoroughly and centrifuged at 10000rpm for 3 minutes. The supernatant was transferred to new collecting tube and rewashed in chloroform iso amyl alcohol. The supernatant was transferred to new collecting tube, 45µl of sodium acetate and 1000µl of ice cold absolute alcohol were added and the mixture incubated at -20 ^{0}C over night. The mixture was centrifuged at 14000rpm for 10 minutes, the supernatant poured off to remain with a pellet. The pellet was dried on a heat block (Dri Bath Thermolyne 16500) for 15 minutes to remove any excess ethanol. DNA was eluted using Tris EDTA.

Conventional nested PCR amplifications were performed using *Cyclospora* primer pairs F1E-R2B and F3E-R4B as described in earlier studies (Jinneman *et al.,* 1996; Relman *et al*., 1996; Yoder *et al*., 1996; Orlandi *et al*., 2003; BAM, 2004). PCR primers were sourced from (Eurofinis mwg/operon), and their sequences were as tabulated (Table 3.5.2). Primary amplifications were performed in a total volume of 100µl containing 2.0mM MgCl, 200µM of each dNTP, 0.2µM of CYCF1E and CYCR2B (Table 3.5.3). All PCR amplifications were performed in a Peltier Thermal Cycler-200 DNA Engine (MJ Research, Waltham, MA). The amplification program began with an initial activation at 95 ^{0}C for 5 minutes. The cycling program consisted of 45 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 52 ^{0}C for 30 seconds and a primer extension at 68 ^{0}C for 90 seconds. A final extension cycle at 72 ^{0}C for 10 minutes was followed by soaking at 4 ^{0}C. The nested PCR was performed in a total volume of 100µl using a master mix containing 2.0mM $MgCl_2$, 200µM of each dNTP, 0.2µM of

CYCF3E and CYCR4B (Table 3.5.4). Five microliters of primary PCR product was used as template for secondary nested PCR. Cycling parameters were identical to first round amplification with the exception of annealing temperature, which was increased to 60 ^{o}C.

Table 3.5.2: The *Cyclospora* primers used in PCR amplification for *Cyclospora* oocysts isolated from *Cercopithecus aethiops* studied at IPR, Nairobi in 2009

Primer	**100 pmol/µl**	**TM ^{0}C**	**Sequence 5^{1}-3^{1}**	**Use**
CYCF1E	196	52.0	GGAATTCCTACCCAATGAAAA CAGTTT	1^{ST} Forward
CYCR2B	126	61.8	CGGGATCCAGGAGAAGCCAAG GTAGG	1^{ST} Reverse
CYCF3E	216	65.5	GGAATTCCTTCCGCGCTTCGCT GCGT	2^{ND}Forward
CYCR4B	252	59.4	CGGGATCCCGTCTTAAACCCC CTACTG	2^{ND} Reverse

Table 3.5.3: First PCR reaction conditions for amplification for *Cyclospora* oocysts isolated from *Cercopithecus aethiops* studied at IPR, Nairobi in 2009

	Component	µl	Final concentration
1	D H_2O	64.7	
2	10X PCR Buffer	10	50 mM KCl, 10mM Tris HCl, Ph 8.3, 1.5 mM $MgCl_2$
3	dATP, dCTP, dGTP, dTTP mix	8.0	200µM each dNTP
4	$MgCl_{2;}$ 25Mm	2.0	0.5mM (total 2.0mM)
5	Primer CYCF1E 4 Mm	5.0	0.2 µM
6	Primer CYCR2B	5.0	0.2 µM
7	Ampli Taq Polymerase	0.3	1.5 units
8	Template DNA	5.0	
	Total	**100.0**	

Table 3.5.4: First PCR thermal cycling parameters used for amplification for *Cyclospora* oocysts isolated from *Cercopithecus aethiops* studied at IPR, Nairobi in 2009

	STEP	Temperature ^{0}C	Duration
1	Initial Inactivation/ Denaturation: 1 cycle	95	5 min
2	Amplification: 45 cycles		
3	Denaturation	94	30 sec
4	Annealing	52	30 sec
5	Extension	68	90 sec
6	Final Extension: 1 cycle	72	10 min
7	Hold	4	

To visualize PCR product a 1.5% solution, 1.5g Agarose (Ultrapure™, Invitrogen®) was added to 100ml Tris Acetic Acid EDTA (TAE), the agarose was dissolved by heating in a microwave at 80% for 3 minutes. A volume of 5µl of ethidium bromide was added to the solution and the mixture was poured into an agarose block and allowed to solidify. The PCR product was loaded onto the agarose block using paraffinol strip to mix 50µl of loading dye (Bromothymol blue, Xylene cyamol plus glycerol) with equal amount of PCR product suspension onto wells. The electrophoresis tank, a double wide Midi gel unit (CBS Scientific Co, California) whose power supply was Ephortec ™ was set at 100 ^{0}C for 30 minutes. The PCR product was visualized after electrophoresis on 1.5% agarose gel and staining with 0.2µg/ml of ethidium bromide using a Benchtop UV Transilluminator (Upland CA, USA), the images captured and processed (BioDoc-H Imaging system, Upland, CA, USA) and stored on a memory card for computer.

3.6 Data Handling and Data Management

For most of the parameters, the data collected indicated presence or absence of a particular parasite species and were recorded as “1” (meaning “Yes” for presence of a parasite species), and “0” (meaning “No” for absence), while “2” (meaning parameter “Not Determined”). Data was recorded and managed per individual animal (Olive baboon or African green monkey) in the cross-sectional, case control, and cohort studies. The data were stored in Microsoft Office Excel 2003 (Microsoft Corporation, 2003). The data was validated and verified to be correct as per the entries from manual

record sheets. The data was imported to SAS © 2002-2003 (SAS Institute Inc, Cary, NC, USA). Descriptive statistics and test of association were computed for each data set.

Prevalence of intestinal parasites was computed as proportion of the number of positive observations of each parasite species to the number of non-human primates examined. Chi-square (χ^2) statistics was used to determine associations between parasite species and *Cyclospora* at the level of $p < 0.05$. Odds ratio (O.R.) was also calculated by 2 x 2 contingency tables for prediction of probability of occurrence of *Cyclospora* against some parasites (Kirkwood, 1988).

Stepwise multiple logistic regressions were done using SAS by forward selection and backward elimination of eligible variables in order to model for occurrence of *Cyclospora* against the potential risk factors. The logistic modeling controlled the effects of confounding and interactions between the risk factors being considered. The factors that were found to be significant were retained in the model while the rest were eliminated (Kirkwood, 1988).

3.7 Quality Control Analysis

Collection of faecal and blood samples, processing and examination of the samples was carried out carefully and professionally. Reagents and chemicals were sourced from reputable suppliers and stored according to manufacturer's recommendation. As far as

possible, freshly reconstituted reagents were used. Cold room and refrigerator temperatures were checked regularly. The animal handlers and laboratory technicians were well trained, certified and competent for their assignments. All study protocols and data were recorded in a laboratory workbook. Data sheets were then checked and entered carefully into a computer. The computer data sheets were double checked for errors.

3.8 Ethical and Scientific Review

This research reviewed and approved by the Scientific and Ethics Committee of Kenya Medical Research Institute (KEMRI) as SSC. No. 1417, and the Institutional Review Committee (IRC) of Institute of Primate Research (IPR), Karen.This study was carried out at IPR. The procedures used on the non-human primates were humane, taking into account all aspects of animal welfare. The safety of the people involved in the experiments (animal handlers, technician and researcher) and environmental safety were ensured.

CHAPTER FOUR

4.0 RESULTS

4.1 Gastrointestinal Parasite Infections in Pabio anubis and Cercopithecus aethiops

4.1.1 Identification of Gastrointestinal Parasite Species

In the survey of gastrointestinal parasite species in 65 wild-trapped *Pabio anubis* (Olive baboons) and 64 African green monkeys (*Cercopithecus aethiops*) from Lemuria Forest Reserve, Aberdares, Kenya, both helminth and protozoan parasites were observed in the faecal samples. A total of twelve parasite species were identified based on the presence of characteristic ova for helminths or cysts for protozoa. The helminth species identified were *Oesophagostomum, Trichuris, Strongyloides, Trichostrongylus* and *Enterobius* while the protozoan species included *Entamoeba, Iodamoeba, Paramecium* Cyclospora, *Cryptosporidia* and *Isospora* (Table 4.1.1).

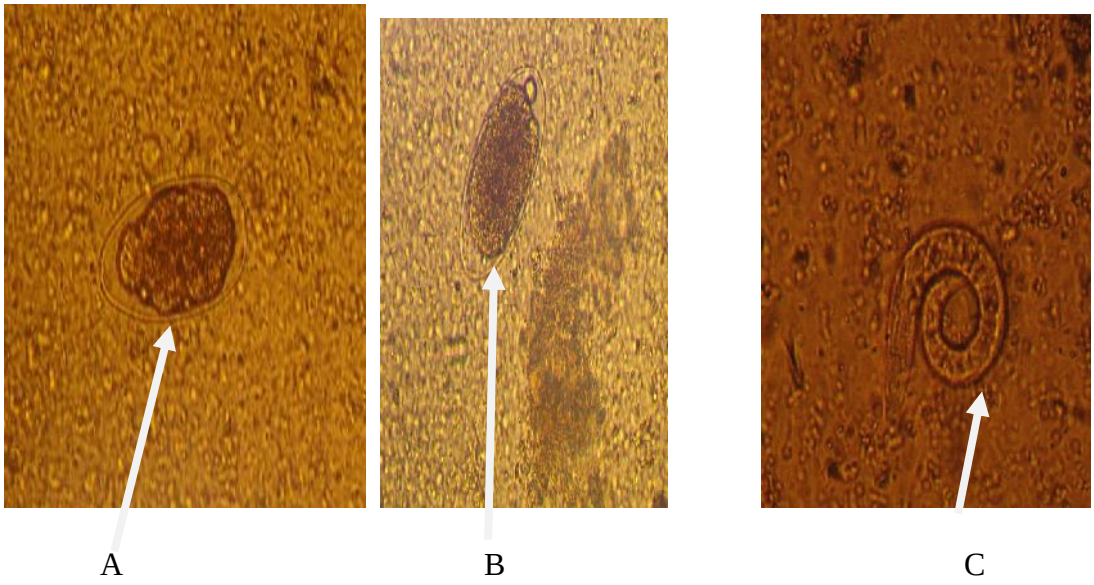
Oesophagostumum species were identified by elliptical, unlarvated egg measuring 74.9 x 44.5µm (Figure 4.1.1.A) and verified by cultured third stage larvae which were filariaform with a sheath and long tail. The average length of the larvae was 840µm. *Strongyloides* species were identified by oval, thin shelled and larvated eggs measuring 48.6 x 30.8µm (Figure 4.1.B) and verified by cultured third stage larvae which were filariaform with no sheath. The average length of the larvae was 480µm (Figure

4.1.1.C). *Trichuris* species were identified by characteristic barrel shaped, yellow to brown, bipolar eggs measuring 56.2 x 27.5µm (Figure 4.1.1.D). *Trichostrongylus* species were identified by ovoid, more pointed at both ends eggs measuring 85-115µm (Figure 4.1.1.E) and verified by cultured third stage larvae which were filariaform, with large anterior sheath and a short tail. The average length of the larvae was 795µm. *Enterobius* species were identified by oval, flattened on one side, larvated eggs measuring 55 x 30µm.

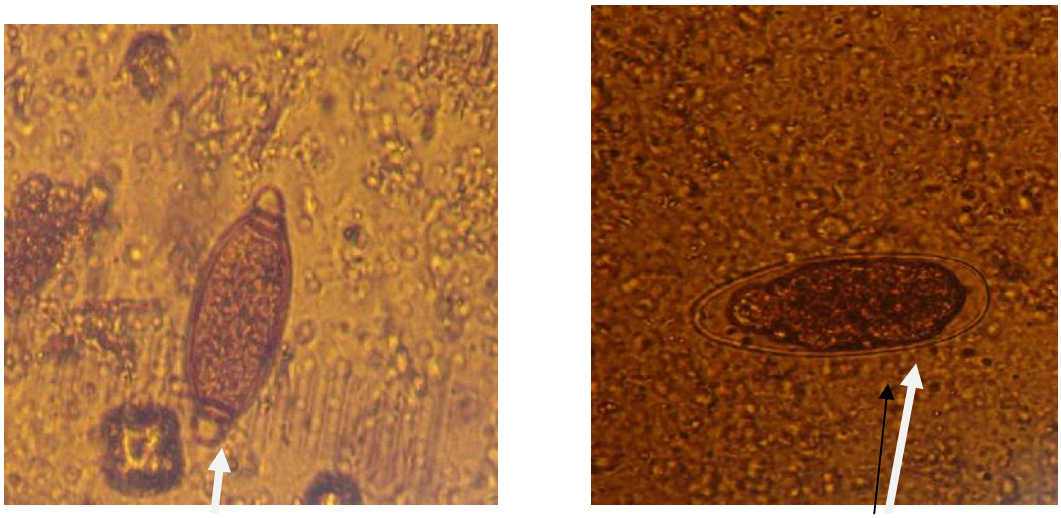
Entamoeba coli cysts found in faecal smears were large spheres, containing more than 4 nuclei, and measured 15-18µm (Figure 4.1.2.A), whereas *Entamoeba histolytica /Entamoeba dispar* species were small round cysts, with 2 or 4 nuclei and measured 12-15µm (Figure 4.1.2.B). *Iodomoeba butschlii* species were large ovoid cysts, had a single nucleus and a distinct glycogen vacuole on Lugol's iodine stain and measured 9-15µm in diameter (Figure 4.1.2.C). The *Cyclospora* oocysts were spherical, measuring 8-10µm in diameter (Figure 4.1.2.D). *Cryptosporidim* species were uniformly red staining spheres, measuring 4-6µm in diameter (Figure 4.1.2.E). *Isospora* species were elliptical, red staining organisms, measuring 20-33 x 10-19µm (Figure 4.1.2.F).

Figure 4.1.3.A is a *Cyclospora* oocyst observed from a wet mount of positive faecal samples from the Olive baboons and AGM. The oocyst was spherical with distinct cyst wall and a morula and measured approximately 9.96µm in diameter. Figure 4.1.3.B was a *Cyclospora* oocyst stained in hot safranin showing uniform reddish staining spheres.

On examination of Ziehl Neelsen stained smears, *Cyclospora* oocyst stained variably. Figure 4.1.3.C was unstained *Cyclospora* oocyst in Ziehl Neelsen stained smear which was spherical and had a distinct cyst wall. Figure 4.1.3.D was stained *Cyclospora* oocyst which stained red in ZN. Figure 4.1.3.E was stained *Cyclospora* oocyst in ZN which stained greenish yellow.



A B C



D E

Figure 4.1.1: Helminth species ova and larva observed in wild-trapped *Pabio anubis* and *Cercopithecus aethiops* at IPR, Nairobi from 2008 to 2009.

A- *Oesophagostomum*, B & C *Strongyloides*, D- *Trichuris* and E- *Trychostrongylus*. X400

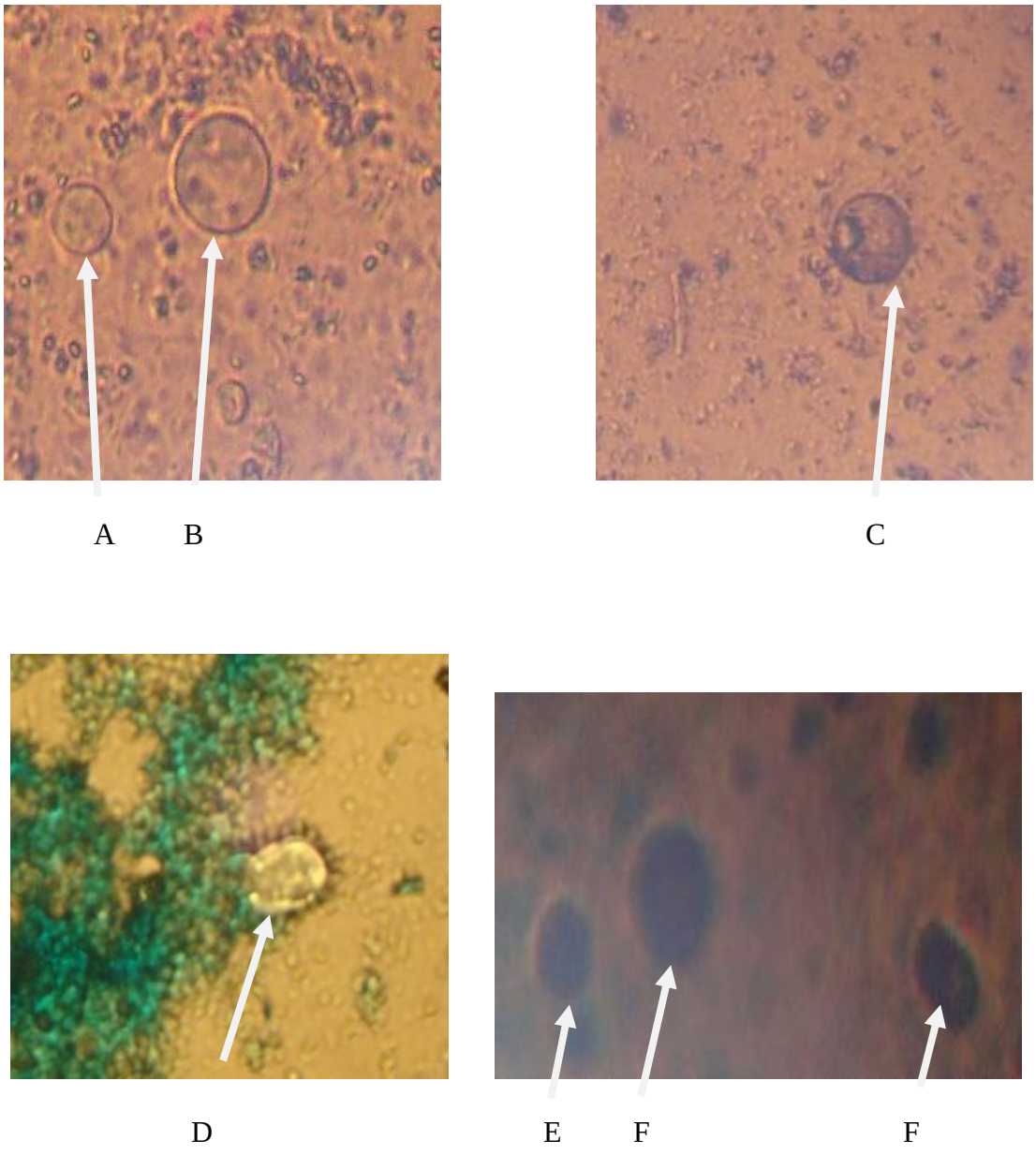
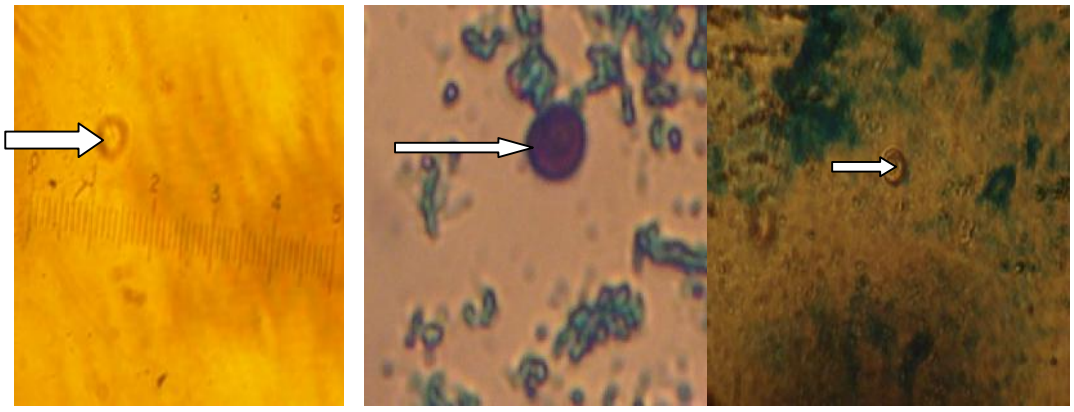
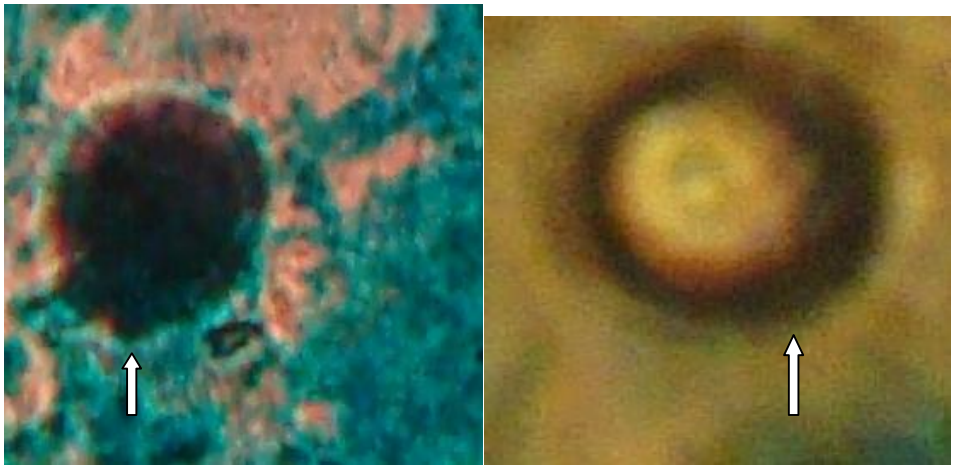


Figure 4.1.2: Protozoan cysts observed in wild-trapped *Pabio anubis* and *Cercopithecus aethiops* at IPR, Nairobi from 2008 to 2009.

A- *Entamoeba histolytica/dispar*, B-*Entamoeba coli*, C-*Iodamoeba butschilii*, D-*Paramecium*, E-*Cryptosporidium*, F-*Cyclospora*, G-*Isospora*. X400



A B C



D E

Figure 4.1.3: *Cyclospora* oocysts observed in wild-trapped *Pabio anubis* and *Cercopithecus aethiops* studied at IPR, Nairobi from 2008 to 2009.

A-Unstained (Wet mount), B- Stained- (Ziehl Neelsen), C- Unstained (Zehl Neelsen), D- Stained (Hot Safranin), E- Unstained (Ziehl Neelsen).

(Size on stage micrometer 4 small divisions=9.96µm. A-C, X400; D and E, X1000

4.1.2 Prevalence of Gastrointestinal Parasite Species

Gastrointestinal parasite species detected in faecal samples of the 65 wild-trapped *Pabio anubis* (Olive baboons) and 64 *Cercopithecus aethiops* (African green monkeys) collected from Lemuria Forest Reserve, Aberdares, and Nguruman, Kajiado District, Kenya, included both helminth and protozoan parasites. Table 4.1.1 shows the species present in the primates. There were 5 helminth and 7 protozoan genera. The prevalence of intestinal parasite infections was derived from faecal smear slides examination after standard formol ether (FEA) concentration technique of the faecal samples. The prevalence of gastrointestinal parasite species was defined as percentage of the number of individual animals that were positive for the particular parasite.

For the Olive baboons, among the helminth present included *Oesophagostomum* spp, the most common followed by *Trichuris* spp and *Strongyloides* species in that order (Table 4.1.1). *Trichostrongylus* spp typically a parasite of ruminant and interestingly, the human healminth *Enterobius vermicularis* were also present. Prevalence of *Trichostronglyus* and *E. vermicularis* was however, less 10% and 1.5% (Table 4.1.1). For the African green monkeys, the helminth present included, *Trichuris* spp, the most common followed by *Strongyloides* and *Oesophagostomum* in that order (Table 4.1.1). *Trichostrongylus* and *Enterobius* were also present. However, the prevalence of *Trichostrongylus* and *Enterobius* was less than 5% (Table 4.1.1).

The protozoan parasites present among the Olive baboons, *Entamoeba coli* was the most common, with prevalence of 78.4%. The other gastrointestinal protozoan parasites detected were *Entamoeba histolytica/ dispar* (23.1%), *Cyclospora* spp (6.2%), *Cryptosporidium* spp (4.6%), *Isospora* (3.1%) and *Iodamoeba butschilii* (1.5%). Similarly among the African green monkeys, the protozoan parasites, *Entamoeba coli* was the most common, with prevalence of 78.1% followed by *Cyclospora* spp (64.1%). The other protozoan parasites detected were *E. histolytica/ dispar, Cryptosporidium* spp and *Isospora* spp with prevalence of 23.4%, 6.3% and 1.6%, respectively (Table 4.1.1). *Paramecium* trophozoite with a low prevalence of 1.6% was observed in the faecal sample of one animal studied (Table 4.1.1).

Majority of the baboons examined harboured at least one of the parasite species, with protozoan parasites being the most common. On the other hand, less than 40% of the animals had helminth infections with *Oesophagostomum* species being the most commonly occurring species (Table 4.1.2). Single parasite species infections were observed in 20.8% of the animals examined, while multiple parasite species infections were observed in 69.2% of the animals (Table 4.1.2). Majority of those with multiple species infections (59.7%) had 2-3 parasite species per individual, and less than 10% had more than 3 parasite species, and infection with 6 parasite species was rare (Table 4.1.2). Majority of the African green monkeys examined harboured at least one of the parasite species with the protozoan parasites being the most common. On the other hand,

less than 50% of the animals had helminth infections with *Trichiuris* being the most commonly occurring species (Table 4.1.2).

The prevalence of each parasite investigated for the Olive baboons in relation to age and sex of the host animals was as presented in Table 4.1.2. In general, the juveniles showed consistently higher prevalence for both helminth and protozoan parasite species (Table 4.1.2). Statistically significant higher prevalence for juveniles was observed for *Strongyloides* species and *Entamoeba coli* with $p < 0.0001$ in both cases. The other observations in prevalence were not statistically significant with p values > 0.05. Overall, the female animals consistently showed higher parasite prevalence than males, but these differences were not statistically significant with $p > 0.05$. Only *Enterobius vermicularis* had significant prevalence higher in males (1.5%) than females (0%), with $p < 0.05$.

Among the African green monkeys, the prevalence of each parasite investigated in relation to age and sex of the host study animals were as presented in Table 4.1.2. In general, the adult monkeys showed consistently higher prevalence for the helminth parasite detected with *Trichuris* being the most common. However, these differences were not statistically significant with $p > 0.05$. Among the protozoan parasite species detected, statistically significant higher prevalence in adults than juveniles was observed for *Cyclospora* species with p value of 0.031. The other protozoa parasite species detected showed no statistical difference with $p > 0.05$. Among the helminth infections,

the differences in prevalence observed between the females and males were not statistically significant with $p > 0.05$. Among the protozoa, statistically significant difference in prevalence was observed in males for *Cyclospora* species infections with p value of 0.0028. The other protozoan parasite species identified in the study had no sex-related statistical significant differences in their prevalence with $p < 0.05$.

Cyclospora oocysts were recorded in 4 of the 65 Olive baboons screened using safranin stained smears, with an overall prevalence of 6.2%. The prevalence of *Cyclospora* was higher in the juveniles (4.6%) than in adults (1.5%) and also higher in females (4.6%) than males (1.5%). However, the differences in prevalence were not statistically significant with $p > 0.05$. *Cyclospora* oocysts were recorded in 41 out of the 64 AGM tested using safranin stained faecal smears, with an overall prevalence of 64.1%. The prevalence was higher in the adults (46.4%) than in the juveniles (15.7%), and also higher in the males (39.1%) than in the females (25.2%). These differences in prevalence were statistically significant with p values < 0.05. On comparison of the prevalence of *Cyclospora* infections among baboons and AGM, the prevalence in the AGM (64.1%) was higher than in the Olive baboons (6.2%). Statistically significant higher prevalence was observed in the AGM than in the Olive baboons with p value of 0.0386. Statistically significant higher prevalence for *Cyclospora* infections were observed in the adults than in the juveniles with p value 0.0002. The *Cyclospora* infections showed statistically higher prevalence in the males than in the females.

Table 4.1.1: Prevalence of gastrointestinal parasite species observed in 65 wild-trapped *Pabio anubis* and 64 *Cercopithecus aethiops* studied at IPR, Nairobi from 2008 to 2009

Host species	***Pabio anubis***	***Cercopithecus aethiops***
Parasite Species	**Number (%) Positive**	**Number (%) Positive**
Helminths		
Oesophagostomum spp	22 (33.8)	22 (10.9)
Trichuris trichiura	20 (30.8)	30 (46.9)
Strongyloides spp	17 (26.1)	17 (15.6)
Trichostrongylus spp	6 (9.3)	6 (3.1)
Enterobius vermicularis	1 (1.5)	1 (1.6)
Protozoa		
Entamoeba coli	51 78.4)	50 (78.1)
Entamoeba histolytica/ E. dispar	15 (23.1)	15 (23.4)
Cyclospora spp	5 (6.2)	41 (64.1)
Cryptosporidia spp	3 (4.6)	4(6.3)
Isospora spp	2 (3.1)	1 (1.6)
Iodamoeba butschilii	1 (1.5)	0 (0)
Paramecium spp	0 (0)	1 (1.6)

Table 4.1.2: The number of gastrointestinal parasite species per individual animal among 65 wild-trapped *Pabio anubis* and 64 African green monkeysstudied by age and sex at IPR, Nairobi from 2008 to 2009

Number of Parasite species	***Overall Prevalence %***		***AGE***				***SEX***			
	OB	**AG**	**OB**	**AG**	**OB**	**AG**	**OB**	**AG**	**OB**	**AG**
			Adults %		**Juveniles %**		**Males %**		**Females %**	
0	10.0	4.3	5.2	0.1	4.8	4.2	4.1	2.6	5.9	1.7
1	20.8	15.5	13.8	4.3	7.0	11.2	6.6	4.3	14.2	11.2
2	34.4	34.4	26.8	8.5	7.6	25.9	12.9	8.3	21.5	23.2
3	25.3	32.0	20.7	6.5	4.6	25.5	15.2	9.8	10.1	22.2
4	7.9	10.8	7.2	2.3	0.8	8.5	5.5	3.6	2.4	7.2
5	1.2	1.8	0.8	0.3	0.4	1.5	0.9	0.8	0.3	1.0
6	0.4	1.2	0.4	0.3	0	0.9	0.4	0.3	0	0.9

OB for Olive baboon, AG for African green monkey

4.1.3 Intensity of Gastrointestinal Parasite Species

Infection intensity expressed in egg counts per gram of faeces (EPG) for the four commonly occurring helminth parasites, namely *Trichuris, Oesophagostomum, Strongyloides* and *Trichostrongylus* present in the baboons and monkeys ranged from 0 to 2350 EPG. Most baboons had light infections with parasite intensities of 1-199 epg with *Trichuris* being the most frequently encountered parasite species. Less than 6% of the animals had moderate parasite infection intensities and only about 4% of the animals were heavily infected with intensities ≥ 400 EPG. Only one of the animals examined had an egg count of 2350 EPG. Among the AGM, most of the helminth infections were low with parasite intensities of 1-199 epg with *Trichuris* being the most frequently encountered parasite species. Less than 5% of the animals had moderate parasite infection intensities. None of the animals had heavy parasite infections.

When infection intensity was examined on animal age, the adult Olive baboons had a mean egg count of 65.1 ±7.1 while the juveniles had a mean intensity of 61.5 ±11.8 p value 0.9934 for *Trichiuris* (Table 4.1.3.1). The mean egg counts were highest in *Trichiuris* followed by *Oesohagostomum*, *Strongyloides* in that order. However, the age differences in infection intensity were not statistically significant ($p > 0.05$) (Table 4.1.3.2). Similar patterns were observed for infections with *Oesophagostomum, Strongyloides* and *Trichostrongylus* (Table 4.1.3.2). When the EPG were examined between male and female there were sex-related difference, with males having a slightly

higher EPG than females, but these differences were not significant for *Trichuris*, *Oesophagostomum*, *Strongyloides* or *Trichostrongylus*. The mean EPG for African green monkeys were not examined as majority of the infections were of low intensity

Table 4.1.3.1: Intensity of infection with gastrointestinal parasite species observed in different sex classes of *Pabio anubis* and their statistical differences studied at IPR, Nairobi from 2008 to 2009

Parasite species	Adults		Juveniles		Statistic			RM
	Mean	SE	Mean	SE	DF	(χ^2) value	P value	
***Trichuris* spp**	65.1	7.1	61.5	11.8	54	31.6858	0.9934	N.S.
***Oeophagostomum* spp**	16.3	2.4	39.9	11.4	27	15.9295	0.9430	N.S.
***Strongyloides* spp**	13.4	2.8	24.1	4.3	24	30.8198	0.1590	N.S.
***Trichostrongylus* spp**	4.4	1.3	5.4	1.3	9	8.8812	0.4483	N.S.

DF- Degrees of freedom, SE- Standard Error, RM- Remarks, N.S. = Not statistically significant, p value >0.05

Table 4.1.3.2: Intensity of infection with gastrointestinal parasite species observed in different age groups of *Pabio anubis* and their statistical differences studied at IPR, Nairobi from 2008 to 2009

Parasite species	Males		Females		Statistic			RM
	Mean	S E	Mean	SE	DF	(χ^2) value	P value	
***Trichuris* spp**	67.434	10.761	58.783	7.999	18	19.3131	0.3725	N.S.
***Oeophagostomum* spp**	31.304	10.971	22.635	5.673	9	9.3825	0.4027	N.S.
***Strongyloides* spp**	19.047	3.743	15.912	3.375	8	5.3962	0.7145	N.S.
***Trichostrongylus* spp**	5.844	1.531	3.885	1.0595	3	2.613	0.7400	N.S.

SE- Standard Error, DF- degrees of freedom,RM= Remark,

N.S. = Not statistically significant, p value > 0.05

4.2: Screening for *Cyclospora* and Simian Immunodeficiency Virus Co-Infections in Captive baboons and monkeys

A total often Olive baboons and thirty three African green monkeys were tested. Each animal was examined for *Cyclospora*, SIV and *Cyclospora*- SIV co-infections. Diagnosis of *Cyclospora* was based on microscopic detection of parasite oocysts in a faecal sample. SIV-specific ELISA was used to detect SIV antibodies. Optical density values of > 0. 500 were considered positive and values < 0.500 negative. None of the animals had clinical symptoms of cyclosporiasis at the time of faecal sampling.

Table 4.2 summarises the results of of examination of baboons and monkeys for *Cyclospora*, SIV and co-infections. Prevalence of of *Cyclospora* or SIV was generally very low in the female adult Olive baboons examined, and co-infections of *Cyclospora*-SIV were also low. These results suggest that baboons are less susceptible to *Cyclospora* or SIV. On the other prevalence for *Cyclospora* and SIV was high with an overall prevalence of 70% for *Cyclospora*, 30% for SIV and 15% for co-infections. While nearly 69% of the juvenile monkeys had *Cyclospora*, none of them was infected with SIV. Interestingly, about a similar proportion (70%) of adult monkeys were also infected with *Cyclospora*,but about 59% of these tested positive for SIV, and nearly 30% of them had *Cyclospora*- SIV co-infections (Figure 4.2).

These results suggest that the African green monkey is susceptible to infections with both *Cyclospora* and SIV, and could potentially be a suitable model for studies with cyclosporiasis.

Table 4.2: Prevalence of *Cyclospora,* SIV and *Cyclospora*-SIV Co-infections in Olive baboons and African green monkeys collected from field locations in Kenya and held captive at IPR, Karen in 2009.

Animal species	Description	No. Examined	No. infected with *Cyclospora* (%)	No. infected with SIV (%)	No with *Cyclospora*-SIV co-infection (%)
Baboons	Adult female	10	1 (10)	3(30)	1 (10)
African green monkeys	Juvenile, female	13	9 (69)	0 (0)	0 (0)
	Juvenile, male	3	2 (67)	0 (0)	0 (0)
	Adult female	7	4 (57)	5 (71.5)	2 (28.5)
	Adult male	10	8 (80)	5 (50)	3 (30)

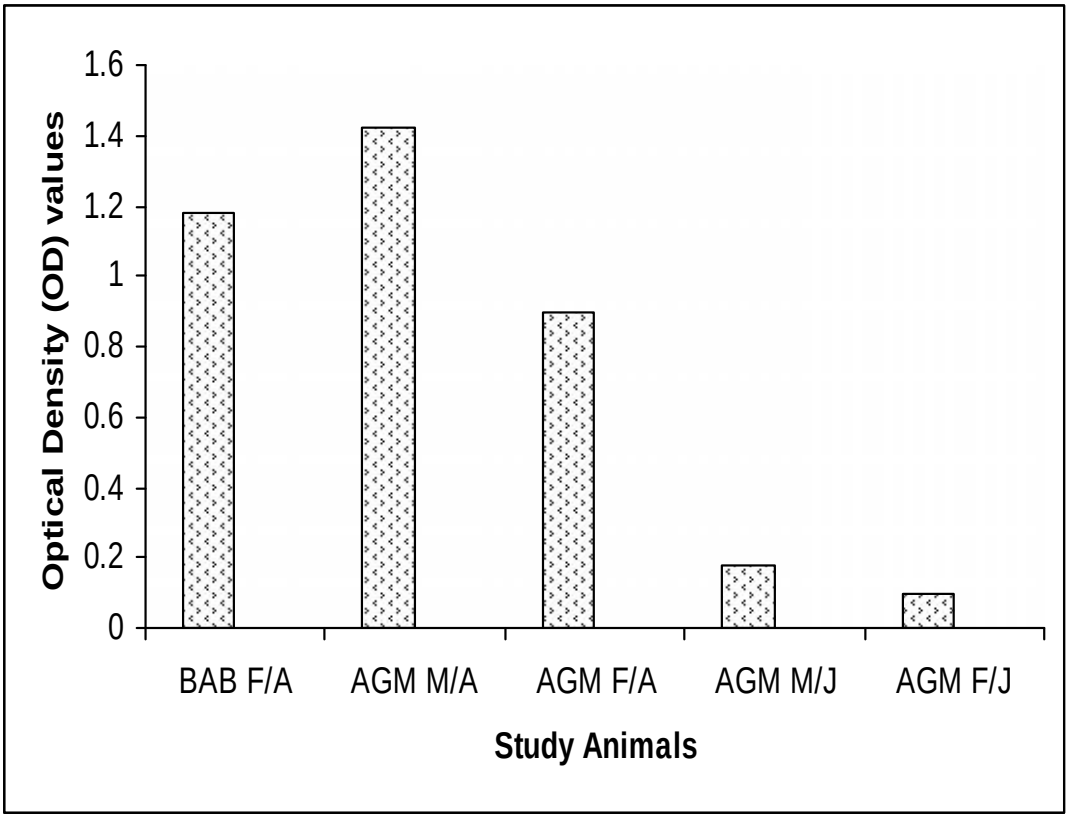


Figure 4.2: The mean Anti-SIV antibody responses in 10 *Pabio anubis* and 33 *Cercopithecus aethiops* studied by species, age and sex at IPR, Nairobi in 2009.

Key:

BAB Baboon, AGM African green monkey, M Male, F Female, A Adult and J Juvenile

4.3 *Cyclospora* Cohort Study in *Cercopithecus aethiops*

An AGM cohort comprising of 4 male adults, 4 female adults and 4 female juveniles was monitored periodically over a 12 month period to elucidate the course of cyclosporiasis. Faecal samples were collected every three months from individual study animals, processed and stained with hot safranin and examined by microscopy. Table 4.3.1 presents the characteristics of the study animals, source and the *Cyclospora* results at each examination time point.

Initially, 4 AGM were *Cyclospora* negative and 8 were positive. Three of the *Cyclospora* negative AGM remained negative throughout the study period (Table 4.3.1). The 8 AGM that were *Cyclospora* positive remained positive at the 3- and 6- month sample time. At the 9- month sampling time, 4 AGM had lost the infection and only 4 AGM (CC-5, CC-6, CC-8 and CC-10) remained positive. At the 12- month sampling time the 4 AGM had lost the infection. However, two of the AGM that had lost infection at the 9- month had recurrent infections by the 12-month sampling point (CC-2 and CC-3). One AGM that was wild trapped, and *Cyclospora* negative, remained negative at 3-, 6-, and 9- month sampling points but tested positive for *Cyclospor*a infection at the 12-month sampling point. The two colony-bred AGM remained *Cyclospora* negative at all sampling points (Table 4.3.1.1).

Cultures of *Cyclospora* oocysts from five (5) *Cyclospora* positive monkeys made in potassium dichromate and maintained at ambient temperatures were examined by microscopy periodically over a two week period. Sporulation of the *Cyclospora* oocysts was observed from day 4 post-incubation and continued thorough out the two week study period by which time most of the *Cyclospora* observed were sporulated. The total number of *Cyclospora* oocysts present both sporulated and unsporulated was determined by microscopic examination of slide smears made from the cultures at each sample point (Table 4.3.1.2, Figure 4.3.1). The rate of sporulation of the varied between the different animals examined at each sample point. For VER 2062, low number of *Cyclospora* oocysts were sporulated at the day 4 (3) sampling point with maximum number of oocysts sporulated at day 10 (9) sampling time. For VER 2072, low numbers of *Cyclospora* oocysts were sporulated at the day 4 (3) sampling time and then the number of sporulated oocysts increased to 6 (day 7, day 10) and 7 (day 14). VER 2080 showed maximum sporulation at day 10 (15) while VER 2081 had maximum sporulation at day7 (17). VER 2082 generally showed low number of *Cyclospora* oocysts (3-6) sporulated at all the sampling points (Table 4.3.1.2, Figure 4.3.1). Sporulated oocysts are the infective stage of the parasite.

Table 4.3.1.1: Characteristics of study animals and *Cyclospora* oocyst shedding at different sampling points over 12 month period in *Cercopithecus aethiops* examined at IPR, Nairobi from 2008 to 2009

Study Animal Characteristics			Sampling Time in Months				
AnimID	**Sex/Age**	**Location trapped**	**Baseline**	**3 -**	**6**	**9 -**	**12**
CC-1	M/A	Aboretum	0	0	0	0	0
CC-2	F/A	"	1	1	1	0	1
CC-3	F/A	"	1	1	1	0	1
CC-4	F/J	"	0	0	0	0	1
CC-5	F/A	Nguruman	1	1	1	1	0
CC-6	F/A	"	1	1	1	1	0
CC-7	M/A	"	1	1	1	0	0
CC-8	M/A	"	1	1	1	1	0
CC-9	M/A	"	1	1	1	0	0
CC-10	F/J	"	1	1	1	1	0
CC-11	F/J	IPR Colony	0	0	0	0	0
CC-12	F/J	"	0	0	0	0	0

Key: CC-1 to CC-12 for *Cyclospora* cohort study animals 1-12, M Male, F Female, A Adult, J Juvenile,

1 *Cyclospora* positive, 0 *Cyclospora* negative.

Table 4.3.1.2: The number of sporulated and unsporulated *Cyclospora* oocysts at different sampling times in 5 *Cercopithecus aethiops* studied at IPR, Nairobi in 2009.

AnimID	Sex	Age	Sample Time	Sporulated	Unsporulated	Sporulation %
	F	A	4 days	3	11	
VER 2062						21.4
VER 2062	F	A	7 days	7	4	63.6
VER 2062	F	A	10 days	9	3	75.0
VER 2062	F	A	14 days	6	1	85.7
VER 2072	F	A	4 days	3	7	30/0
VER 2072	F	A	7 days	6	3	66.7
VER 2072	F	A	10 days	6	3	66.7
VER 2072	F	A	14 days	7	0	100.0
VER 2080	M	A	4 days	4	8	33.3
VER 2080	M	A	7 days	9	4	69.2
VER 2080	M	A	10 days	15	6	71.4
VER 2080	M	A	14 days	7	1	87.5
VER 2081	M	A	4 days	9	12	42.9
VER 2081	M	A	7 days	17	9	65.4
VER 2081	M	A	10 days	7	3	70.0
VER 2081	M	A	14 days	8	3	72.7
VER 2082	M	A	4 days	3	3	50.0
VER 2082	M	A	7 days	6	3	66.7
VER 2082	M	A	10 days	4	2	66.7
VER 2082	M	A	14 days	6	1	85.7

Key: AnimID animal identification with unique individual numbers for the study animals whose faecal samples were observed for sporulation, M males, F Females and A Adults.

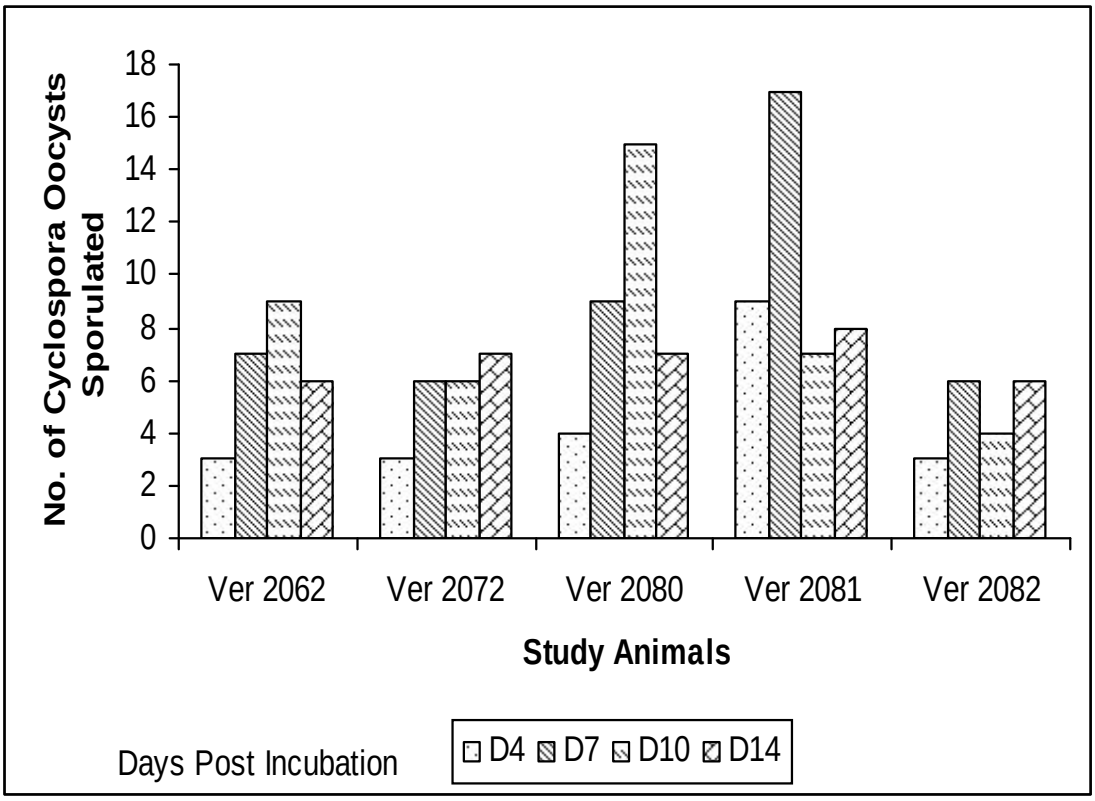


Figure 4.3.1: The number of sporulated *Cyclospora* oocysts at different sampling times in the 5 *Cercopithecus aethiops* examined at IPR, Nairobi in 2009.

D represents days post-incubation

4.3.2 Haematological Parameters

The haematological parameters of normal AGM studied at IPR, were measured to establish baseline values and to show any changes that might occur in case of *Cyclospora* infections. On haematological analysis the erythrocyte values were generally higher only in the adults. The red blood cells ($x10^6/\mu l$) varied between 5.28-5.44 for female adults, 5.2-6.69 for male adults, 3.7-5.14 for juvenile females and 3.29-5.14 for male juveniles. Similarily, the hemoglobin (g/dl) values had similar trend with range between 10.9-14.2 for male adults and 8.7-12.7 female juveniles (Table 4.3.2.1). The platelets ($x\ 10^3/\mu l$) varied from 110-653 with lowest values recorded in male juveniles and highest in female adults. The white blood cells ($x10^3/\mu l$) varied among the study animals range 2.9-8.7 with highest values recorded in male adults. The neutrophils ranged from 22-42% with higher values in male juveniles. Lymphocytes ranged from 52-78% with highest levels observed in male adults. Few monocytes and eosinophils were recorded in the study animals while no basophils were detected. The total plasma protein ranged from 6.6-7.5g/dl while plasma fibrinogen ranged from 0.2-0.4 g/dl (Table 4.3.2.1).

The haematological values for African green monkeys with natural *Cyclospora* infections were analysed and presented in Table 4.3.2.2. Generally, the red blood cell counts decreased marginally in all sex-age categories with marginal drop in female adult monkeys at the 42 day sampling point. The haemoglobin levels had only minor changes.

The white blood cell counts showed marked variations, with a decrease in female juveniles (3.3-7.3 to 1.4-3.5) and male adults (3.9-8.7 to 1.5-5.3) while an increase in the female adults (4.3-7.1 to 5.4-8.6) and male juveniles (5.9-8.1 to 8.8-17.7) was observed. The percent neutrophils were increased in all sex-age categories with a range of 17-33% while the lymphocyte percentages were decreased with a range of 2-43% and the biggest variation was observed among the male adults (Figure 4.3.2).

4.3.3 *Cyclospora* Specific ELISA in *Cercopithecus aethiops* with Natural *Cyclospora* Infections

Sera were probed for *Cyclospora* specific antibody responses using soluble *Cyclospora* proteins by ELISA in seven AGM with natural *Cyclospora* infections at 30 day and 49 day time point. Positive *Cyclospora* responses were observed in all the study animals. Higher optical density (OD) values were observed in the male than in the female adults. The OD values observed in all study animals tested were about 0.560 with mild increase at the 49 day sample time except for female juvenile (F/J CE-2) which remained constant (Figure 4.3.3).

Table 4.3.2.1: The normal red blood and white blood cell values of *Cercopithecus aethiops* studied at IPR, Nairobi from 2008 to 2009

	Adults		Juveniles	
Blood Parameter[1]	**Females Mean ±SD (Range)**	**Males Mean ±SD (Range)**	**Females Mean ±SD (Range)**	**Males Mean ±SD (Range)**
RBC	5.36±0.8 (5.28-5.44)	5.95±0.75 (5.2-6.69)	4.4±0.7 (3.7-5.14)	4.22±0.93 (3.29-5.14)
Hb	11.7±2.0 (8.7-12.7)	12.55±1.7 (10.9-14.2)	10.7±2.0 (8.7-12.7)	12.4±0.8 (11.6-13.2)
HCT	33.4±4.8 (28.6-38.1)	32.1±9.5 (22.6-41.5)	33.4±4.8 (28.6-38.1)	27.6±5.4 (22.2-32.9)
MCV	70.6±2.0 (68.6-72.6)	71.9±2.8 (69.1-74.7)	80.5±6.5 (74.0-87.0)	71.5±1.5 (70.0-73.0)
MCH	24.2±1.1 (23.1-25.3)	27.3±1.0 (26.3-28.3)	27.1±1.0 (26.1-28.1)	24.8±1.2 (23.6-26.0)
MCHC	25.8±5.3 (20.5-31.1)	30.0±1.1 (28.9-31.1)	32.6±1.7 (31.1-34.4)	30.6±0.5 (30.0-31.1)
Plt	528±125 (403-653)	489±127 (362-616)	586±65 (521-650)	215±105 (110-320)
WBC	5.7±1.4 (4.3-7.1)	6.3±2.4 (2.9-8.7)	5.3±2.0 (3.3-7.3)	7.0±1.1 (5.9-8.1)
Neu	35.5±5.5 (30-41)	32±10 (22-42)	36.5±4.5 (32-41)	40.5±0.5 (40-41)
Ly	61.5±8.5 (53-70)	67±11 (56-78)	62±5 (58-67)	52.5±0.5 (52-53)
Mo	0	0	1	0
Eo	0	2	2	3
Ba	0	0	0	0
TPP	7.5	7.4	6.6	6.6
PF	0.4	0.2	0.4	0.4

1 Units of measurement as follows:
Red blood cells RBC, ($x10^6/\mu l$), Hemoglobin Hb, (g/dl), Hematocrit (%), Mean corpuscular volume MCV, (fl): Mean corpuscular hemoglobin MCH, (µµg): Mean corpuscular hemoglobin concentration MCHC, g/dl: White blood cells WBC, ($x10^3/\mu l$): Platelets Plt, ($x\ 10^3/\mu l$): Neutrophils Neu, (%): Lymphocytes Ly, (%): Monocytes Mo, (%): Eosinophils Eo, (%): Basophils Ba, (%): Total Plasma Protein TPP, (g/dl): Plasma Fibrinogen PF, (g/dl).

Table 4.3.2.2: Red blood and white blood cell values of *Cercopithecus aethiops* with Natural *Cyclopsora* Infection studied at IPR, Nairobi from 2008 to 2009

	ANIMAL Sex/Age Class	**Baseline haematological values**	**Values 42 days post exposure to *Cyclospora***
Red blood cells X10^6 /µl	Female/ Adult	5.28-5.44	5.06-5.28
	Male /Adult	5.2- 6.99	4.4-5.06
	Female/ Juvenile	3.7-5.14	2.8-5.6
	Male/ Juvenile	3.29-5.14	3.1-4.5
Haemoglobin g/dl	Female/ Adult	8.7-12.7	9.8-10.3
	Male /Adult	10.9-14.2	14-14.4
	Female/ Juvenile	8.7-12.7	12.0-12.2
	Male/ Juvenile	11.6-13.2	10.1-11.0
White Blood cells X10^3	Female/ Adult	4.3-7.1	5.4-8.8
	Male /Adult	3.9-8.7	1.5-5.3
	Female/ Juvenile	3.3-7.3	1.4-3.5
	Male/ Juvenile	5.9-8.1	8.8-17.7
Neutrophils %	Female/ Adult	30-41	50-63
	Male /Adult	22-42	57-68
	Female/ Juvenile	32-41	55-67
	Male/ Juvenile	40-41	57-60
Lymphocytes %	Female/ Adult	53-70	46-68
	Male /Adult	56-78	25-35
	Female/ Juvenile	58-67	48-52
	Male/ Juvenile	52-53	38-42

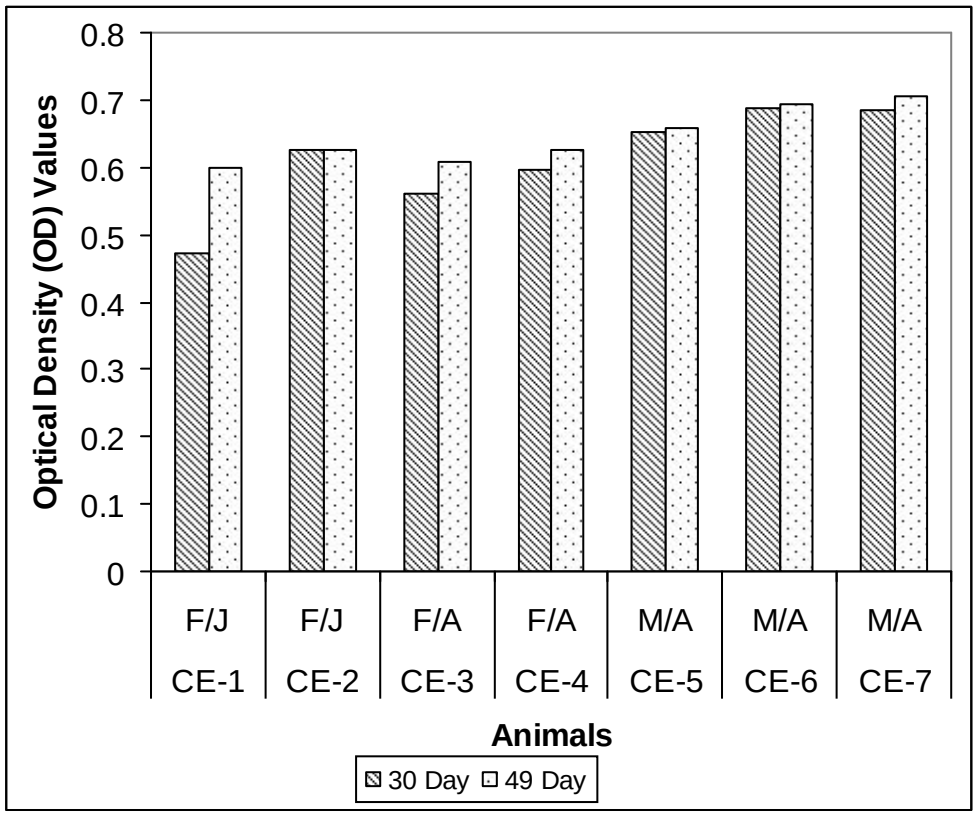


Figure 4.3.3: *Cyclospora* specific antibody responses observed in 7 *Cercopithecus aethiops* with natural *Cyclospora* infections at 30 day and 49 day sample time studied at IPR, Nairobi in June 2009.

Key:

CE-1 to CE-7 for *Cyclospora* ELISA assays animals 1-7, M Male, F Female, A Adult and J juvenile.

4.4: Experimental *Cyclospora* Inoculation in *Cercopithecus aethiops*

Animal inoculation tests were carried out to establish experimental *Cyclospora* infections to elucidate clinical and pathological changes in cyclosporiasis. Experimental *Cyclospora* infections were established in two female juvenile and two male adult African green monkeys which were not shedding *Cyclospora* at the time of inoculation. Infections were by inoculation of 1000 *Cyclospora* oocysts directly into the stomach using a catheter in each study animal.

Faecal samples were collected twice weekly for each individual study animal, processed by FEA technique, prepared slide smears stained by hot Safranin and slide smears were examined microscopically for *Cyclospora* oocyst. Based on morphology and staining characteristic, *Cyclospora* oocysts indistinguishable from those identified from naturally infected animals were shed from the experimentally infected animals from day 14 post inoculation. The two female juveniles continued to shed low numbers of *Cyclospora* oocyst*s* in their faeces until they were sacrificed at 49 days post inoculation. On the other hand, the two male adults shed high numbers of *Cyclospora* oocysts with maximum numbers shed on day 22 and then a sharp decline in the numbers shed on day 24 and then shedding ceased by day 34 up to end of study period (Figure 4.4.1.1). The study animals remained asymptomatic through out the study period.

Sera were probed for *Cyclospora* specific antibody responses using soluble *Cyclospora* proteins by ELISA in the four AGM with experimental *Cyclospora* infections (VER

1974, VER 2009, VER 2065 and VER 2092) and one positive control animal (VER 2048) at different sampling times. VER 1974 and VER 2009 were female juveniles while VER 2065 and VER 2092 were male adults. Positive *Cyclospora* responses were observed in all the study animals and absorbance values > 0.500 were considered positive. The observed OD values varied in the animals at different sampling times.

VER 1974 started with an OD value of 0.45 at baseline which increased marginally to 0.50 and then rose to peak value of 0.700 at 42 day followed by decline at day 49. VER 2009 started with high OD value which declined at 14 day sampling time and then increased to peak OD values at 49 day sampling time. VER 2065 had high optical density (OD) values of > 0.78 which remained fairly constant at all sampling times. Ver 2092 had high OD values which decline marginally at the 49 day sapmling time. The positive control animal (VER 2048) had OD values of about 0.57 which remained fairly constant through out the study period (Figure 4.4.1.2).

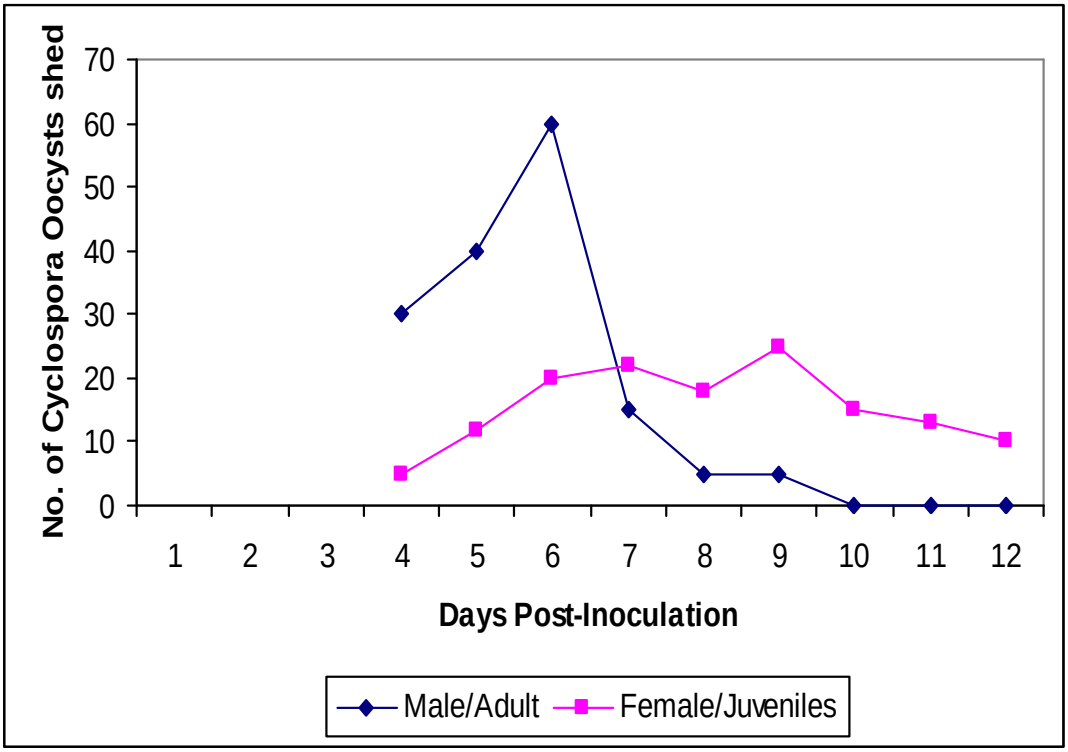


Figure 4.4.1.1: Shedding pattern of *Cyclospora* oocysts from 4 *Cercopithecus aethiops* post- experimental *Cyclospora* inoculation studied at IPR, Nairobi in 2009.

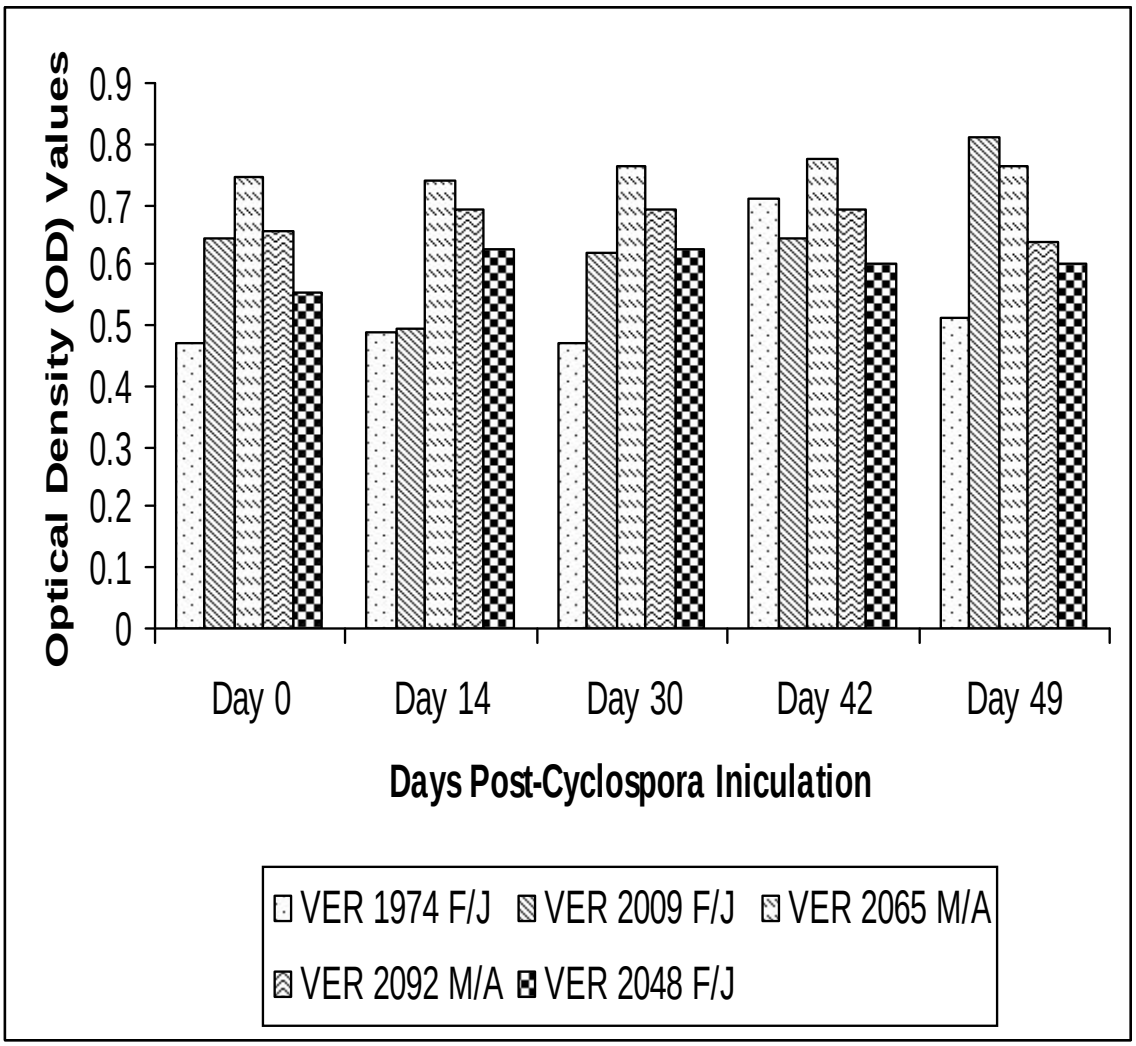


Figure 4.4.1.2 *Cyclospora* specific antibody responses of experimentally infected *Cercopithecus aethiops* assayed at different sampling times studied at IPR, Nairobi in 2009.

Key:

VER 1974, VER 2009, VER 2065 and VER 2092 *Cyclospora* ELISA assay animals, while VER 2048 was positive control animal, VER = African green monkey monkey, D = Days.

4.4.2 Post Mortem Evaluation of *Cercopithecus aethiops* (African green monkeys) with Experimental *Cyclospora* Infection

The two female juvenile and two male adult AGMs with experimental *Cyclospora* infections were humanely sacrificed, 49 days post-inoculation. Slight weight gains of 0.1-0.3kg were recorded in three of the study animals during the 7 week study period but the VER 2065 showed a slight decline from 6.2 to 5.9 kg (Table. 4.4.2.1).

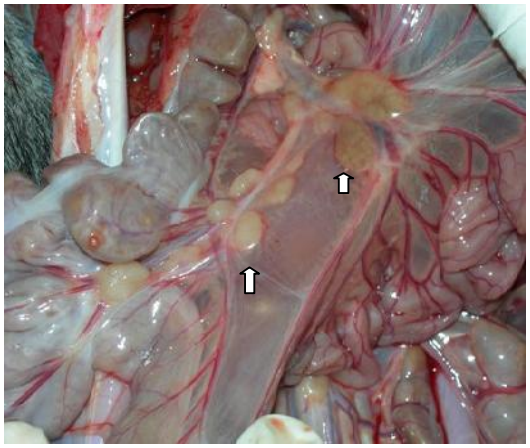
On gross examination, no abnormalities were detected in the juvenile animals except that VER 1974 had slightly enlarged mesenteric lymph nodes (Table 4.4.2, Figure 4.4.2.1.A). The male adults displayed more pathology. VER 2065 had prominent and mildly enlarged mesenteric lymph nodes (Table 4.4.2.1 and Figure 4.4.2.1.B). It also had haemorrhages on the surface of the stomach which spread to the small and large intestines (Figure 4.4.2.1 C). In addition, VER 2065 liver showed mild pitting of the surface which was widespread and also had a congested and enlarged spleen (Figure 4.4.2,1 .D). VER 2092 showed no abnormalities in the stomach, the intestinal wall was rough and thickened and the mesenteric lymph nodes were slightly enlarged. The liver had moderate focal necrotic foci of about 1mm in diameter spread all over the surface (Table 4.4.2.1).

Table 4.4.2.1: Body weight and gross pathological findings in the 4 *Cercopithecus aethiops* with Experimental *Cyclospora* infections studied at IPR, Nairobi, from to 2009

Animal ID	Weight in kg		Stomach	Intestines	Lymph nodes	Liver
	At CI	At PM				
VER 1974 F/J	1.8	1.9	NAD	NAD	Mesentric slightly enlarged	NAD
VER 2009 F/J	1.7	1.9	NAD	NAD	NAD	NAD
VER 2065 M/A	6.2	5.9	Haemorrhages	Haemorrhagic mucosal surface which was focal but spread along the whole intestine	Prominent and mildly enlarged	Mild to moderate pitting of the surface wich was widespread
VER 2092 M/A	4.1	4.4	NAD	Wall moderately thickened and rough	Mesenteric lymph nodes mildly enlarged	Moderate focal necrotic foci about 1mm in diameter spread all over the liver surface

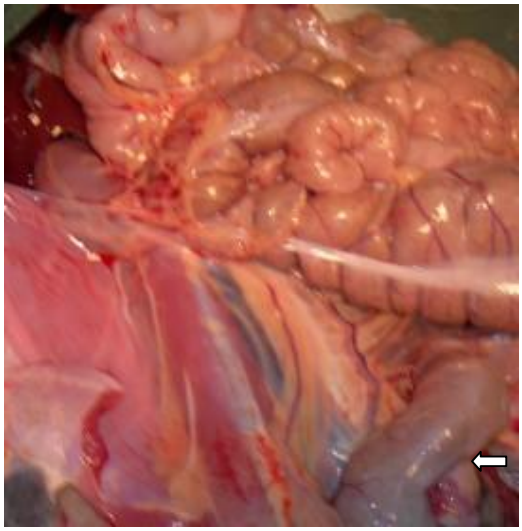
Key: Animal Identification of four Experimental *Cyclospora* inoculated study animals comprising of 2 female juvenile and 2 male adult African green monkeys. VER African green monkey, F Female, M Male, A Adult, J Juvenile, CI *Cyclospora* Inoculation, PM Post Mortem and NAD no abnormality detected.

A



B

C



D

Figure 4.4.2.1: Grosss pathological lesions observed in *Cercopithecus aethiops* with experimental *Cyclospora* infection, studied at IPR, Nairobi in 2009.

A Intestinal wall- no pathology B Mesenteric lymph nodes- enlarged

C Intestine- haemorrhages D Spleen- congested

4.4.3 Histopathological Evaluation of *Cercopithecus aethiops* with Experimental *Cyclospora* Infection

In general, on microscopic examination of the haematoxylin eosin (H&E) stained tissue sections, significant histopathological findings were observed in both the female juveniles and male adults African green monkeys examined (Table 4.4.3.1 and Figures 4.4.3.1 to 4.4.3.4). VER 1974 which was a female juvenile, showed congestion of the blood vessels in the stomach and intestinal mucosa, hyperplastic lymph nodes and periportal lymphocytic infiltration of the liver while VER 2009 which was the female juvenile AGM had no observable defects in the stomach, intestine, mesenteric lymph node and liver tissues (Table 4.4.3.1). The lesions ranged from mild inflammatory reaction of the stomach and intestinal wall of the animals to intense cellular infiltration. The male adults had parasite vacuoles in their enterocytes (Figure 4.4.3.2). The livers showed lymphocytic infiltration which was both diffuse and periportal, destruction of the hepatocytes and granuloma formation (Table 4.4.3.1 and Figure 4.4.3.3).

Figure 4.4.3.1 illustrates various sections of the stomach wall. Figure 4.4.5.1.A & B are sections of normal stomach mucosawhile Figure 4.4.3.1.C was a section sowing mild lymphocytic infiltration. Figure 4.4.3.2 illustrates various sections of the intestinal wall. Figure 4.4.3.2.A is a section of intestinal wall showing congested capillaries and mild lymphocytic infiltration. The infiltrating cells had dense nuclei and varying sizes. Figure 4.4.3.2.B. and C were sections from intestinal wall showing mild lymphocytic infiltration, increased goblet cells and parasite vacuoles in the enterocytes. The

developmental stages of *Cyclospora* were numerous, occurred in vacuoles in the cytoplasm near the luminal end which differentiates them from another coccidian parasite species, *Cryptosporidium* where the parasites are localized within the brush borders of the enterocyte. Significant cellular reactions were observed in three of the AGM examined except in one female juvenile (VER 2009) where no abnormalities were detected. Parasites in the enterocytes were observed only in the two male adults while no parasites were detected among the female juveniles.

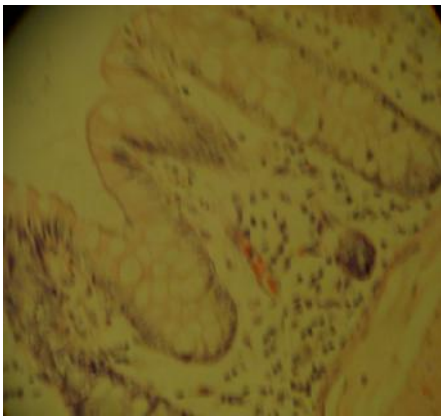
The liver showed varied pathology ranging from mild lymphocytic infiltrations to well organized mature granulomas. The histopathological findings were in three of the AGM and collaborated the gross findings. VER 2009 which was a female juvenile AGM had no appreciable liver changes detected (Figure 4.4.3.3). Figure 4.4.3.3 A. is a normal liver section. Figure 4.4.3.2.B is a liver section showing with mild lymphocytic infiltration that was periportal while Figure 4.4.3.3.C. was a section showing diffuse lymphocytic infiltration with necrosis of the hepatocytes, haemosiderin deposits and. loss of demarcation of the liver lobules. The liver sections had numerous granulomas at different stages of development. Some of the granulomas were diffuse aggregates of lymphocytic cells which displaced the hepatocytes while others started at the portal triad and extended into the liver tissue (Figure 4.4.3.3.B & C). As the granulomas progressed to mature, there was complete destruction of hepatocytes and no clear demarcation of the liver lobules (Figures 4.4.3.3.D & E).

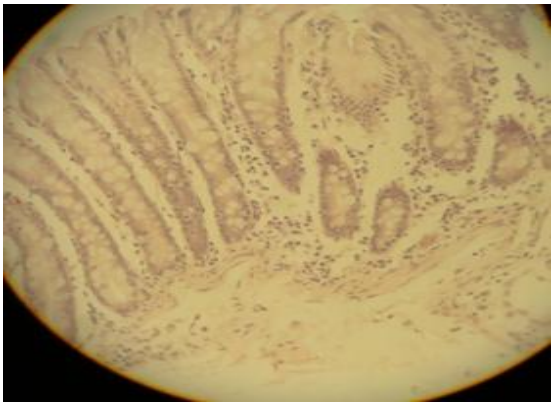
The mesenteric lymph nodes displayed varying pathology from mild to intensive proliferation of lymphocytes. Figure 4.4.3.4 is a section of lymph node tissue which presented intense lymphocytic proliferation which resulted to destruction of lymph node structure.

Table 4.4.3.1: Histopathological findings in the 4 *Cercopithecus aethiops* with Experimental *Cyclospora* infections studied **at IPR, Nairobi in 2009**

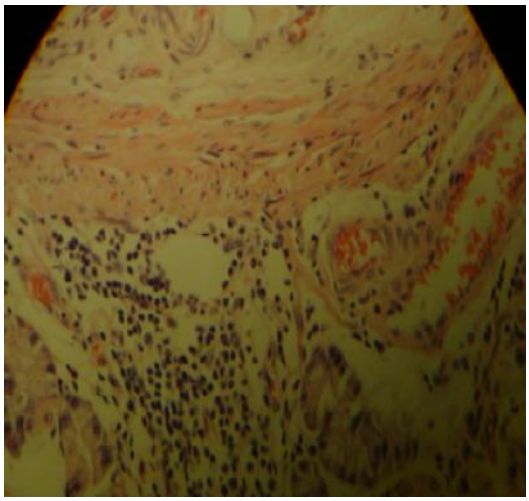
AnimID	Stomach	Intestines	Lymph nodes	Liver
VER 1974 F/J	Mucous membrane entire, capillaries filled with blood- congested blood vessels	Congestion of the blood vessels, moderate lymphocytic infiltration	Hyperplasia, loss of structure of lymph node, moderate proliferation of lymphocytes	Lymphocytic infiltration of the periportal area, no clear demarcation of hepatocytes and lobules, generalized lymphocytic infiltration
VER 2009 F/J	No observable defects	No observable defects	No observable defects, normal follicles	No observable defects, well demarcated liver lobules with central vein, bile duct, hepatic artery and vein and hepatocytes in radiating chains
VER 2065 M/A	Disruption of the mucosal layer with mild lymphocytic infiltration and many goblet cells	Bloated epithelial border at different loci, intense lymphocytic infiltration, parasites in enterocytes	Extensive hyperplasia, intense lymphocyte proliferation	Few granulomas with loss of hepatocytes and haemosidrin deposits
VER 2092 M/A	Disruption of mucosal layer	Intact intestinal borders, mild to moderate lymphocytic infiltration, parasites in the enterocytes	Mild lymphocyte proliferation	Granulomas, numerous and at different stages of development, loss of liver structure

Key: Animal Identification of four Experimental *Cyclospora* inoculated study animals comprising of 2 female juvenile male adult African green monkeys. VER African green monkey, F Female, M Male, A Adult and J Juvenile.





A B



C

Figure 4.4.3.1: Stomach wall of *Cercopithecus aethiops* with Experimental *Cyclospora* infection examined at IPR, Nairobi in 2009.

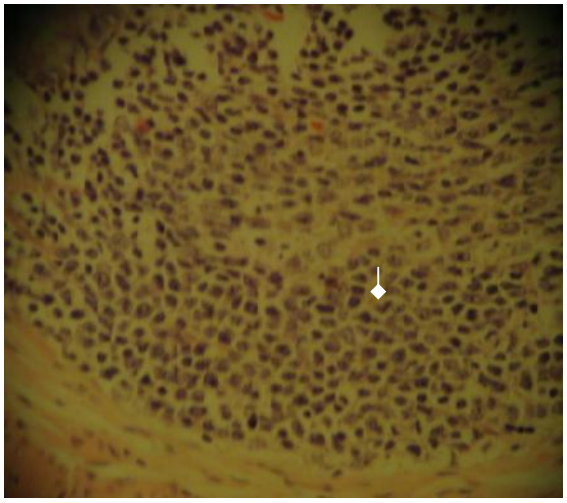
H&E Stain, X 400**.**

Key:

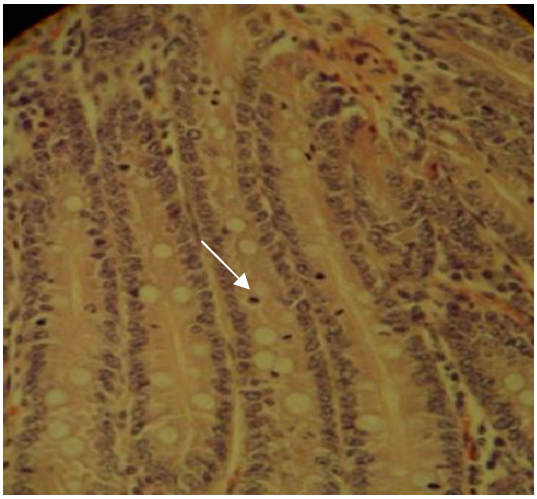
A- Normal stomach mucosa

B- Normal stomach wall with gastric pits and glands

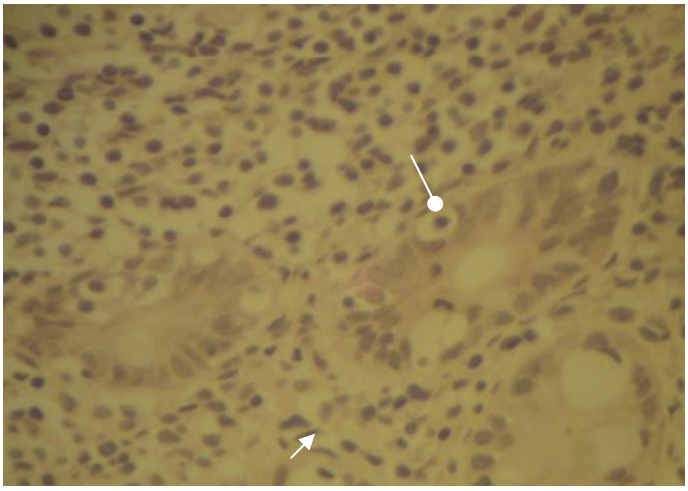
C- Stomach wall showing mild lymphocytic infiltration



A



B



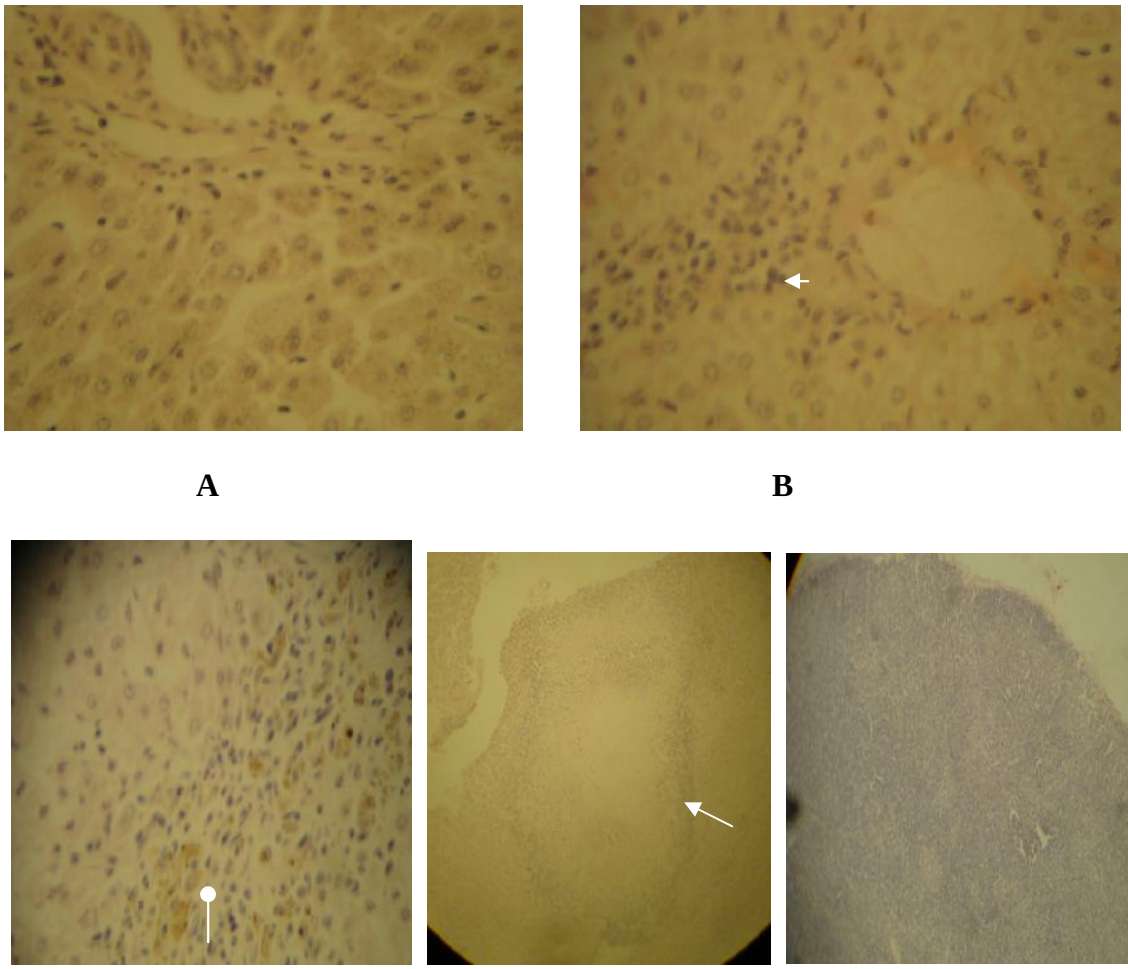
C

Figure 4.4.3.2: Intestinal wall of *Cercopithecus aethiops* with Experimental *Cyclospora* infection examined at IPR, Nairobi in 2009. H&E Stain, X 400.

Key: A **showing intensive lymphocytic infiltration**

B vacuoles in the enterocytes

C Parasite vacuoles in the enterocytes



A **B**

C **D** **E**

Figure 4.4.3.3: Liver sections of *Cercopithecus aethiops* with Experimental *Cyclospora* infection examined at IPR, Nairobi in 2009.

H&E Stain, X 400.

A- normal hepatic tissue **B-** showing mild periportal lymphocytic infiltration **, C-** lymphocytic infiltration, and haemosiderin deposits, D- well demarcated granuloma, E- mature granuloma formation

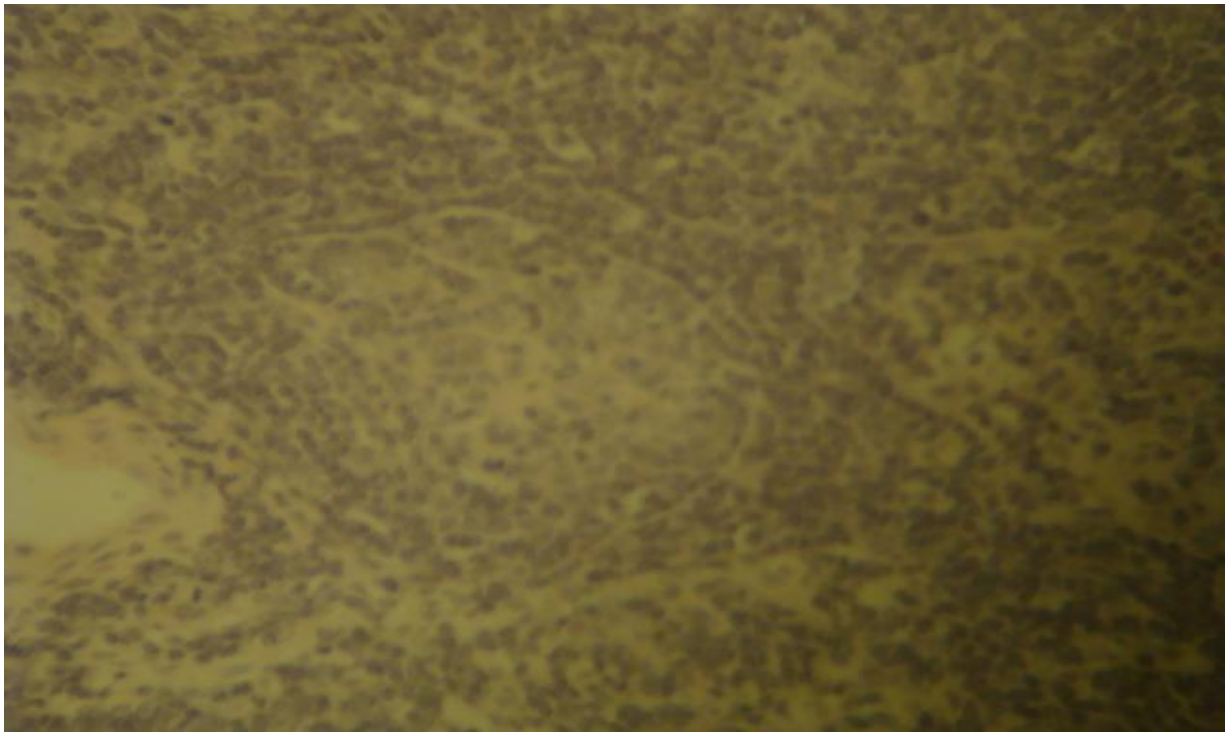


Figure 4.4.3.4: Lymph node section of *Cercopithecus aethiops* with Experimental *Cyclospora* infection showing lymphocytic infiltration with loss of lymphoid structure examined at IPR, Nairobi in 2009.

H&E Stain, X 400.

4.5 Evaluation of Trimethoprim-Sulphamethoxazole Therapy for Treatment of Cyclosporiasis in *Cercopithecus aethiops*

Six African green monkeys with natural *Cyclospora* infection were treated with Trimethoprim 160 mg–Sulphamethoxazole 800 mg twice daily for seven days offered in ripe banana. Response to treatment was defined by laboratory criteria of enumerating the *Cyclospora* oocysts shed in faeces, evaluation of haematological values and *Cyclospora* specific antibody responses post treatment. Controls of 4 *Cyclospora* ositive and 2 *Cyclospora* negative AGM were included.

4.5.1 *Cyclospora* Oocysts Shedding Post-treatment with Trimethoprim-Sulphamethoxazole in *Cercopithecus aethiops*

Cyclospora oocyst shedding was monitored beginning seven days post-treatment on twice weekly basis for a period of about nine weeks. This was done by examination of faecal slide smears following standard FEA concentration technique and hot Safranin staining. The six TMP-SMX treated monkeys shed large numbers of *Cyclospora* oocysts between day 7 and day 14 and shedding ceased at different times for all the animals. Among the four positive control monkeys, the female adults shed moderate numbers of oocysts while the female juveniles shed low numbers for different number of days in the study period. No *Cyclospora* oocysts were observed in the 2 negative controls (Table 4.5.1, Figure 4.5.1).

Among the TMP-SMX treatment group, the female adults (TST-1 and TSt-2), initially shed low numbers at baseline with maximum shedding on day 7 and they continued to shed for period ≥ 32 days. TSt-1 shed few *Cyclospora* oocysts (38) at baseline with peak shedding (2355) on day 7 then decreased, and shedding ceased after day 35. TSt-2 generally shed low numbers of oocysts with maximum shedding of 315 on day 7 then shedding ceased after day 32. The female juveniles (TSt-3 and TSt-5), initially shed low numbers at baseline with maximum shedding at day 7 and day 14, then they continued to shed low numbers for short period and shedding ceased after day 28. The male juveniles (TSt-4 and TSt-6), initially shed low numbers at baseline with large numbers shed on day 7, day 10 and day 14, the numbers shed decreased and shedding ceased after day 28 (Table 4.5.1).

In the positive control group, the female adult PC-2 initially shed moderate numbers of oocysts on day 0 (100) and day 7 (120) then the number of oocysts shed decreased to between 15 and 30 and shedding ceased after day 32. PC-3 initially shed moderately high numbers of oocysts on day 0 (200) and day 7 (240) then decreased to between 10 and 40 and shedding ceased after day 25. The female juvenile PC-1 shed low numbers of oocysts of between 20 and 80 and shedding ceased after day 35 while PC-4 shed low numbers of between 40 and 100, and shedding ceased after day 18. No *Cyclospora* oocysts were shed by the two negative control monkeys (NC-1 and NC-2) (Table 4.5.1). Recurrent asymptomatic cyclosporiasis developed in two TMP-SMX treated female adult monkeys (TSt-1 and TSt-2) by day 60 post-treatment. On second TMP-SMX

treatment of these two monkeys, TSt-1-R shed maximum number of *Cyclospora* oocysts on day 7 (460) then decreased to between 50 and 60 then shedding ceased after day 18. TSt-2-R shed large numbers of *Cyclospora* oocysts with maximum shedding on day 7 (2800) with high numbers on day 14 (600) and day 28 (520). Tst-1 shed lower numbers of oocysts on second TMP-SMX treatment than in first tratment while TSt-2 shed higher numbers of oocysts on second treatment than on first treatment (Table 4.5.1).

Table 4.5.1: Number of *Cyclospora* oocysts shed by *Cercopithecus aethiops* post-treatment with Trimethoprim-Sulphamethoxazole studied at IPR, Nairobi in 2009

Group	Age/ Sex	D 0	D 7	D 14	D 21	D 28	D 35	D 38	D 42	D 49	D 60
TSt-1	F/A	38	2355	155	320	24	102	0	0	0	**60**
TSt-2	F/A	60	315	150	60	32	0	0	0	0	**100**
TSt-3	F/J	20	2600	600	250	50	0	0	0	0	**0**
TSt-5	F/J	60	1000	720	0	0	0	0	0	0	**0**
TSt-4	M/J	40	1400	1200	0	0	0	0	0	0	**0**
TSt-6	M/J	25	1960	240	100	350	0	0	0	0	**0**
PC-1	F/J	40	80	50	20	20	20	0	0	0	**0**
PC-2	F/A	100	120	30	15	0	0	0	0	0	**0**
PC-3	F/A	200	240	20	10	0	0	0	0	0	**0**
PC-4	F/J	50	60	40	0	0	0	0	0	0	**0**
NC-1	F/J	0	0	0	0	0	0	0	0	0	**0**
NC-2	F/J	0	0	0	0	0	0	0	0	0	**0**
		D 60	**D 67**	**D 74**	**D 81**	**D 87**	**D 94**	**D 101**	**D 108**	**D 115**	**D 120**
TSt-1-R	F/A	60	460	50	0	0	0	0	0	0	**0**
TSt-2-R	**F/A**	**100**	**2800**	**600**	**500**	**520**	**0**	**0**	**0**	**0**	**0**

Key:

TSt-1 to TSt-6 treated with Trimethoprim-Sulphamethoxazole; PC-1 to PC-4 were positive control, no treatment; NC-1 and NC-2 were negative control, no treatment; TSt-1 and TSt-2 were given second treatment with Trimethoprim-Sulphamethoxazole after relapse *Cyclospora* infection hence TSt-1-R and TSt-2-R. A Adult, J Juvenile, M Male, F Female and D Days

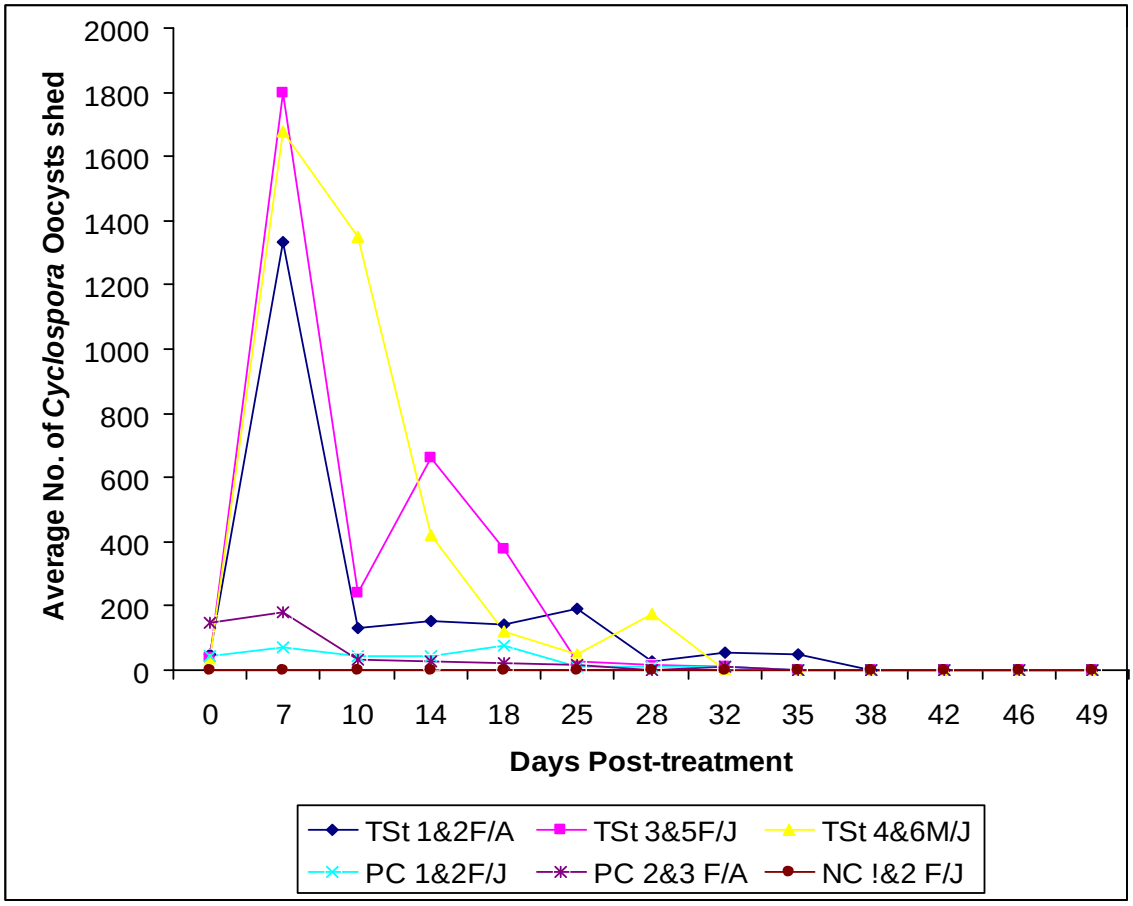


Figure 4.5.1: Pattern of *Cyclospora* oocysts shedding in *Cercopthecus aethiops* post-treatment with Trimethoprim-Sulphamethoxazole studied at IPR.

Six AGM treated with Trimethoprim-Sulphamethoxazole (TSt-1 and 2, TSt-3 and 5, TSt-4 and 6), four positive control no treatment (PC-1 and 4, PC-2 and 3), and two negative controls no treatment given (NC-1 and 2).

Key:

F Female, M Male, A Adult and J for Juvenile

4.5.2 Haematological analysis of AGM post-treatment with Trimethoprim-Sulphamethoxazole

On haematologic analyses, most of the erythrocyte values were decreased in all the six TMP-SMX treated monkeys within 42 days post-treatment. In the female adults TSt-1 and TSt-2, the PCV changed from a mean of 37.4% to 33.5%. Similarly the MCV decreased from a mean of 76.2fl at baseline to 70.0fl at 42 days post treatment. The red blood cell counts dropped marginally from a mean of 5.02 to $4.73x10^6/\mu l$. Haemoglobin values showed minor changes. However there was an increase in MCHC values in the TMP-SMX from a mean of 29.8 to 39.4 g/dl (Table 4.5.2).

In the female juveniles TSt-3 and TSt-5, the PCV changed from a mean of 41.5% which decreased to 31.0%. Similarly the MCV values decreased from a mean of 90.8fl at baseline to 70.2fl at 42 days post treatment. The red blood cell counts dropped marginally from a mean of 4.65 to $4.42x10^6/\mu l$. Haemoglobin values showed minor changes. However there was an increase in MCHC values in the TMP-SMX from a mean of 32.4 g/dl to 40.2 g/dl (Table 4.5.2).

In the male juveniles TSt-4 and TSt-6, the PCV changed from a mean of 41.2% to 34.0%. Similarly the MCV values decreased from a mean of 89.5fl at baseline to 73.0fl at 42 days post-treatment. The red blood cell counts dropped marginally from a mean of 4.79 to $4.58x10^6/\mu l$. Haemoglobin values showed minor changes. However there was an

increase in MCHC values in the TMP-SMX from a mean of 32.7 g/dl to 38.7 g/dl (Table 4.5.2).

The total white blood cell counts were increased in all of the TMP-SMX treated monkeys. In the female adults TSt-1 and TSt-2, the WBC increased from a mean of 8,690 at baseline to a mean of 11,490 at 42 days post-treatment. The neutrophil counts increased from mean of 47.5% to a mean of 55.5% while the lymphocyte counts were decreased from a mean of 51% to a mean of 44%. Few eosinophils were observed in one animal. The platelet counts decreased from a mean of $649x10^3$ at baseline to a mean of $460x10^3$ at the 42 days post-treatment (Table 4.5.2).

In the female juveniles TSt-3 and TSt-5, the WBC increased from a mean of 6,530 at baseline to a mean of 6,875 at 42 days post-treatment. The neutrophil counts increased marginally from a mean of 36.5% to a mean of 38.5% while the lymphocyte counts were increased from a mean of 54% to a mean of 60.5%. Few eosinophils were observed in one animal. The platelet counts decreased from a mean of $503x10^3$ at baseline to a mean of $448x10^3$ at the 42 days post-treatment (Table 4.5.2).

In the male juveniles TSt-4 and TSt-6, the WBC increased from a mean of 8,276 at baseline to a mean of 14,185 at 42 days post-treatment. The neutrophil counts decreased from a mean of 44% to a mean of 24.5% while the lymphocyte counts were increased from a mean of 49.5% to a mean of 72%. Few eosinophils were observed in one animal.

The platelet counts decreased from a mean of $599x10^3$ at baseline to a mean of $462x10^3$ at the 42 days post-treatment (Table 4.5.2).

In the positive control group, the female juveniles PC-1 and PC-4, the PCV changed from a mean of 28.6% which increased to 38.1%. Similarly the MCV values decreased from a mean of 87fl at baseline to 74fl at 42 days post-treatment. The red blood cell counts increased from a mean of $3.39x10^6$/ µl to a mean of $5.1439x10^6$/µl. Haemoglobin values increased from a mean of 8.7 g/dl to a mean of 12.3 g/dl. However there was a slight increase in MCHC values from a mean of 31.1 g/dl to 33.4 g/dl (Table 4.5.2). In the positive control group, the female adults PC-2 and PC-3, the PCV changed from a mean of 34.0% which to 28.6%. The MCV values increased from a mean of 77.2fl at baseline to 89.4fl at 42 days post treatment. The RBC, haemoglobin values and MCHC values had minor changes (Table 4.5.2).

The negative control animals, female juveniles NC-1 and NC-2, showed minimal changes in the erythrocyte values at the end of the 42 day study period. The PCV, haemoglobin and MCV) were decreased marginally while there was a slight rise in RBC and mean corpuscular hemoglobin MCHC values (Table 4.5.2).

In the female juveniles PC-1 and PC-4, WBC increased from a mean of 7,000 at baseline to a mean of 11,050 at 42 days post-treatment. The neutrophil counts increased from mean of 32% to a mean of 41% while the lymphocyte counts were decreased from

a mean of 67% to a mean of 58%. Few eosinophils were observed in one animal. The platelet counts decreased from a mean of $650x10^3$ at baseline to a mean of $521x10^3$ at the 42 days post-treatment (Table 4.5.2).

In the positive control group, the female adults PC-2 and PC-3, the WBC increased from a mean of 5,700 at baseline to a mean of 6,100 at 42 days post-treatment. The neutrophil counts increased from mean of 53% to 57% while the lymphocyte counts remained constant at 41%. Few eosinophils were observed in one animal. The platelet counts increased from a mean of $280x10^3$ at baseline to a mean of $290x10^3$ at the 42 days post-treatment (Table 4.5.2).

In the negative control group, female juveniles NC-1 and NC-2, the WBC decreased from a mean of 5,930 at baseline to a mean of 5,600 at 42 days post-treatment. The neutrophil counts decreased from mean of 39% to a mean of 32% while the lymphocyte counts were increased from a mean of 60% to a mean of 64%. Few eosinophils were observed in one animal. The platelet counts decreased from a mean of $488x10^3$ at baseline to a mean of $470x10^3$ at the 42 days post-treatment (Table 4.5.2).

Table 4.5.2: **Haematological values of *Cercopthecus aethiops* post-treatment with Trimethoprim-Sulphamethoxazole studied at IPR, Nairobi in 2009**

AnimID	Sample Time	PCV %	Hg	RBC	Plt	MCV	MCHC	WBC	Neu %	Ly %	Eo %
TSt-1, F/A	Baseline	39.17	11.1	5.11	683	79.6	28.3	9,050	35	64	**1**
	42 D PT	33.2	13.2	4.78	489	69.5	39.1	13,949	63	36	**0**
TSt-2 F/A	Baseline	35.7	11.2	4.92	615	72.7	31.3	8,330	60	38	**0**
	42 D PT	33.8	13.4	4.67	430	72.4	39.6	9,030	48	52	**0**
TSt-3, F/J	Baseline	44.8	14.7	4.01	400	110.5	33.3	6,660	35	54	**0**
	42 D PT	32.2	13.1	4.46	353	72.2	40.6	7,150	43	57	**0**
TSt-5, F/J	Baseline	38.1	12.1	5.28	605	73	31.5	6,400	38	54	**3**
	42 D PT	29.7	12.9	4.37	543	68.1	43.4	6,600	34	64	**0**
TSt-4, M/J	Baseline	40	12.9	5.51	697	73	32.1	9,152	34	54	**2**
	42 D PT	34	12.7	4.49	500	72.5	37.3	13,220	26	72	**0**
TSt-6, M/J	Baseline	43.1	14.3	4.06	500	105.9	33.3	7,400	54	45	**1**
	42 D PT	33.9	13.6	4.67	424	72.8	40.1	15,150	23	72	**0**
PC (1&4), F/J	Baseline	28.6	8.7	3.39	650	87	31.1	7,000	32	67	**0**
	42 D PT	38.09	12.3.	5.14	521	74	33.4	11,050	41	58	**0**
PC (2&3), F/A	Baseline	34.0	11.3	4.5	280	77.2	33.3	5,700	53	41	**1**
	42 D PT	28.6	11.9	3.98	290	89.4	33.5	6,100	57	41	**1**
NC (1&2), F/J	Baseline	47.3	13.2	5.75	488	82.4	27.9	5,930	39	60	**1**
	42 D PT	**45.5**	**13**	**6.32**	**470**	**80.3**	**29.2**	**5,600**	**32**	**64**	**0**

Red blood cells RBC, ($x10^6$/µl): Hemoglobin Hb, (g/dl): Hematocrit PCV: Mean corpuscular volume MCV, (fl): Mean corpuscular hemoglobin concentration MCHC, g/dl: White blood cells WBC, (/µl): Platelets Plt, (x 10^3/µl): Neutrophils Neu: Lymphocytes Ly: Eosinophils Eo: Animal Identification AnimID: TSt-1 to TSt-6 were TMP-SMX treated animals, PC-1 to PC-4 were positive control and NC-1 and 2 were the negative control.

Key: M Male, F Female, A Adults, J Juvenile, D Days, and PT Post-treatment.

4.5.3 *Cyclospora* Specific Responses in *Cercopithecus aethiops* Post-treatment with Trimethoprim-Sulpamethoxazole

Soluble proteins derived from *Cyclospora* parasites obtained from positive monkeys were used to probe the sera from TMP-SMX treated, positive and negative control monkeys. Varied responses were recognized at Day 30 and Day 42 post-treatment. Among the TMP-SMX treatment group, the female adults (TSt-1 and TSt-2), the *Cyclospora* specific responses denoted by the optical density values decreased at the Day 42 post-treatment with a mean drop of 0.075 units. Similar pattern was observed for the female juveniles (TSt -3 and TSt-5) and also for the male juveniles (TSt-4 and TSt-6) (Figure 4.5.3).

In the positive control group, the female juveniles (PC-1 and PC-4) showed slight increase in the *Cyclospora* specific responses while in the female adults (PC-2 and PC-3) there was a minor decrease in the values (Figure 4.10.3). In the negative control group, the female juveniles (NC-1 and NC-2) there was no change observed in the optical density values and the values remained lower than the determined value for *Cyclospora* specific responses of 0.500 (Figure 4.5.3). On second treatment of the two female adults TSt-1-R and TSt-2-R) that had relapse infection, the *Cyclospora* specific responses also dropped with TSt-2-R having a larger decrease in optical density value (0.252 units) than TSt-1-R (0.205 units) (Figure 4.5.3)

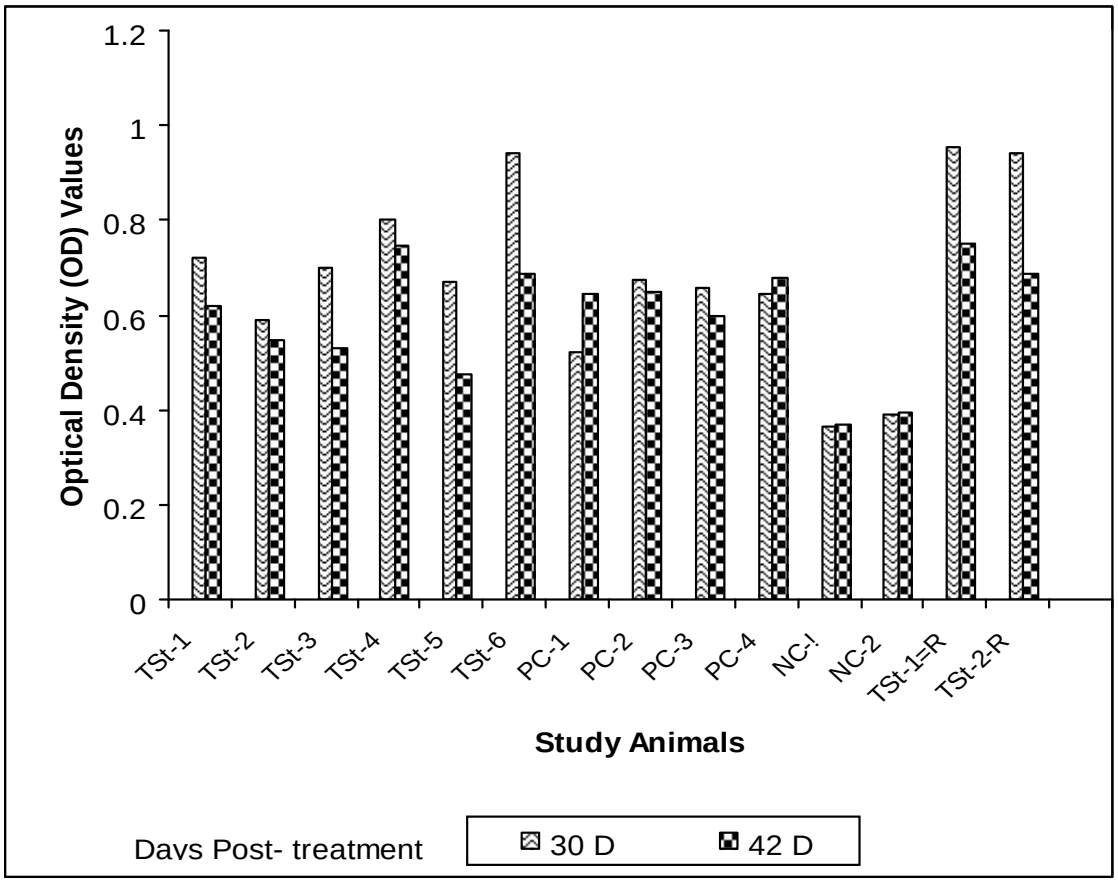


Figure 4.5.3: *Cyclospora* Specific Responses in *Cercopithecus aethiops* post-treatment with Trimethoprim-Sulpamethoxazole studied at IPR, Nairobi in 2009.

Key:

Animal Identification: Tst-1 to TSt-6 were TMP-SMX treated animals, PC-1 to PC-4 were positive control and NC-1 and 2 were the negative control.

4.6 Molecular Characterization of *Cyclospora* oocysts

Specific identification of *Cyclospora* was performed using conventional nested PCR amplifications as previouslydescribed. The expected size of the primary amplicon is 636 base pairs (bp) while the expected secondary amplicon is 294 base pairs.

Total genomic DNA extracted from faecal samples of test animals by sodium dodecyl sulphate proteinase K treatments was visualized on agar rose gel On visualization of DNA product isolated from *Cyclospora* oocysts of positive *Cercopithecus aethiops,* the observations were as presented by Lane 1 to Lane 7 in Figure 4.6.1. Lanes 1, 3 and 7 a smear at the beginning where the extract was deposited and three bands (I strong and 2 light) were observed which indicated that the faecal samples from each of the animals tested were positive for *Cyclospora* DNA. On the other hand, in Lanes 2, 5 and 6 there was a smear but no band formation hence, in this case the faecal samples of the animals tested were negative for *Cyclospora* DNA. In Lane 4 there was neither smear no band formed. This was a negative control in which the DNA extraction chemical Urea was used.

The polymerase chain reaction products of *Cyclospora* oocysts isolated from faecal specimen from *Cercopithecus aethiops* (African green monkeys) studied were as presented by Lanes 1 to 7 in Figure 4.6.2. Lane 1 showed a single strong clear band and was an extract from positive control animal. Lane 5 was an extract from the *Cyclospora* oocysts used for animal inoculation tests and presented as strong clear bands. Lane 2 and

4 were extracts isolated from animals with natural *Cyclospora* infection and presented as single faint clear bands. Lane 3 and 6 were extracts isolated from animals with experimental *Cyclospora* infections and presented as clear single bands. All these Lanes (Lane1 to 6) were at the same location which indicates that the same protein was amplified. Lane 7 was PCR reaction mixture with no *Cyclospora* oocysts, no smear or bands were observed and this was the negative control with no amplification. As demonstrated in Figure 4.6.2, the same PCR product was isolated and visualized from faecal samples of AGM with natural and experimental *Cyclospora* infections. The same product was also amplified from the inoculum used for experimental *Cyclospora* infections.

The *Cyclospora* DNA protein was denaturated and amplification of short sequence of DNA by use of polymerase chain reaction (PCR) was done. Visualization of PCR product was on agarose gel stained with ethidium bromide. Molecular characterization techniques targeting single-nucleotide in the 18S rRNA gene using a nested polymerase chain reaction for *Cyclospora* specific species were used and a PCR product of 294 base pairs (bp) visualized which confirmed the parasite as *Cyclospora* (Figure 4.6.3).

The PCR product of the oocysts isolated from faecal samples of *Cyclospora* positive *Cercopithecus aethiops* (African green monkeys) studied was visualized as a clear band at 294 bp mark on the DNA ladder which is positive for *Cyclospora* species. Lane A was the 100 bp ladder, Lane B was a negative control (Figure 4.6.3).

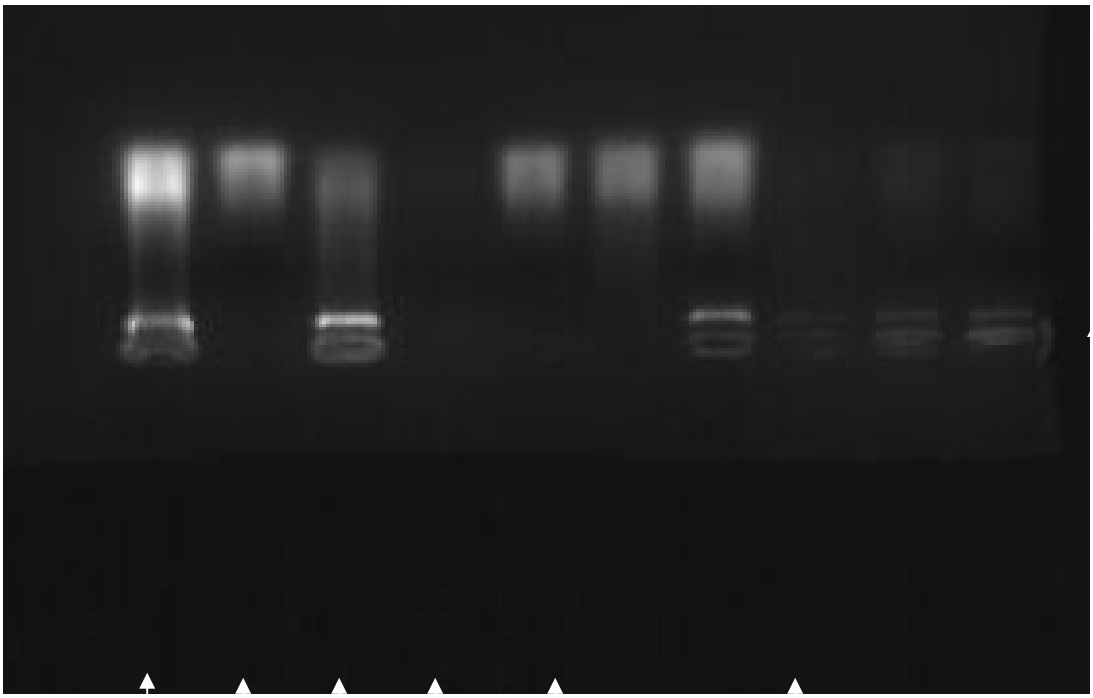


Figure 4.6.1: Visualization of DNA product isolated from *Cyclospora* oocysts of positive *Cercopithecus aethiops* studied at IPR, Nairobi in 2009.

Lane 1 to Lane 7; Lanes 1, 3 and 7 smear and bands were observed which indicated that the faecal samples of animals tested were positive for *Cyclospora* DNA while Lanes 2, 5 and 6 there was smear and no band formation hence, negative for *Cyclospora* DNA. Lane 4 neither smear no band formation was observed, extraction chemical urea had been used as negative control.

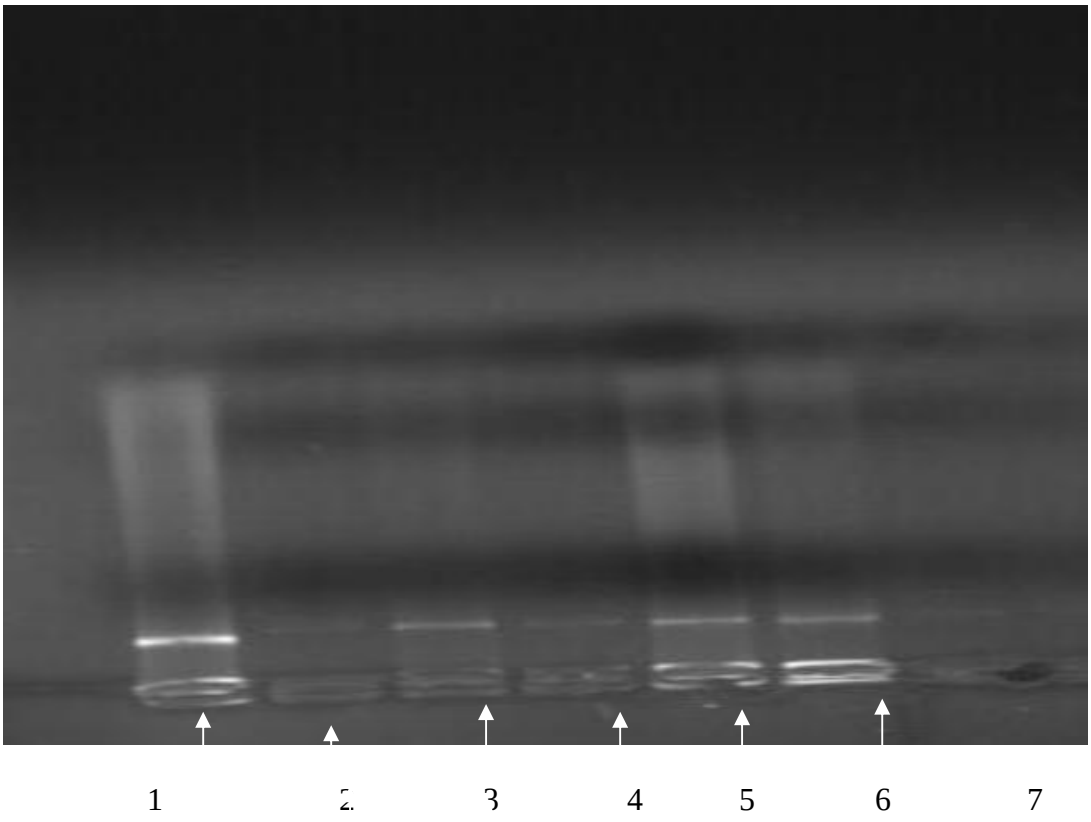


Figure 4.6.2: Visualization of Polymerase Chain Reaction (PCR) products of *Cyclospora* oocysts isolated from faecal specimen from *Cercopithecus aethiops* studied at IPR, Nairobi in 2009.

Lanes 1 to 7; Lane 7 was negative control, PCR reaction mixture with no oocysts - no amplification; Lane 1 was positive control animal with a strong band; Lane 5 was extract from the *Cyclospora* oocysts used for animal inoculation test; Lane 2 and 4 were extracts isolated from animals with natural *Cyclospora* infection while Lane 3 and 6 were extracts isolated from animals with experimental *Cyclospora* infections.

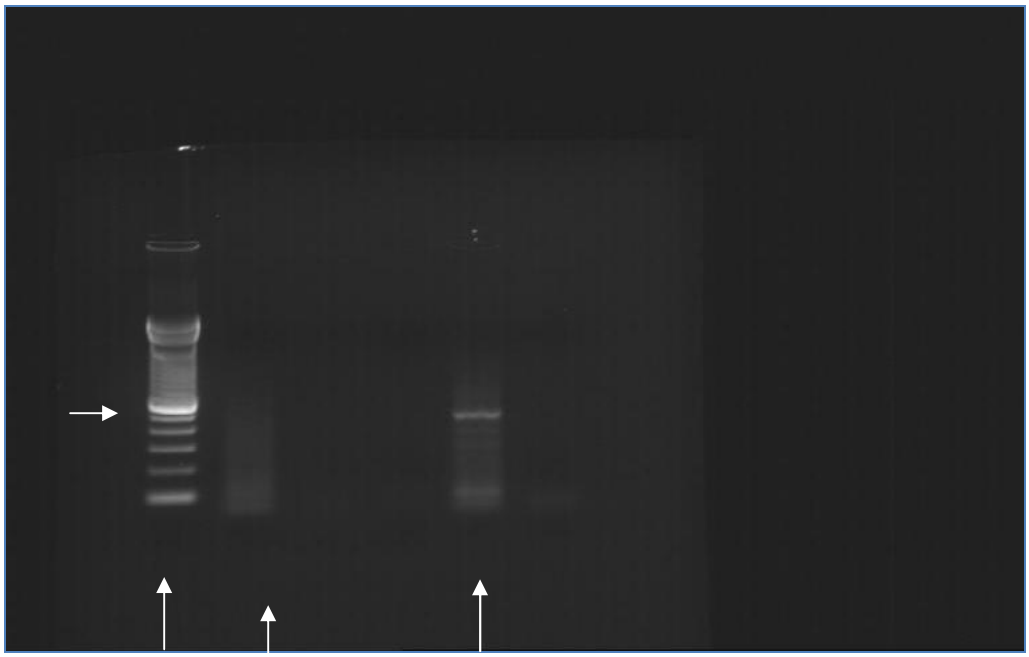


Figure 4.6.3 Visualization of PCR product of *Cyclospora* oocysts isolated from *Cyclospora* positive *Cercopithecus aethiops* studied at IPR, Nairobi in 2009.

Lane A is the 100 bp ladder, Lane B is a negative control sample and Lane C is *Cyclospora* positive sample with a clear band at the 294 bp mark.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 General Discussion

This study was conducted to develop a non-human primate model for cyclosporiasis. The study was able to elucidate the clinical epidemiology of *Cyclospora* infections in non-human primates, evaluate co-infections of *Cyclospora* with other gastrointestinal parasites and simian immunodeficiency virus infections in NHPs, to demonstrate the gross and histopathological lesions caused by *Cyclospora* infections and to evaluate trimethoprim-sulphamethoxazole therapy on *Cyclospora* species infections in African green monkeys. This study yielded useful and systematic information on methodology on harvesting, purifying and storage of *Cyclospora* oocysts from the African green monkey. The histopathological results clearly demonstrated *Cyclospora* as an intestinal parasite with extra-intestinal involvement particularly in the liver and mesenteric lymph nodes. The successful establishment of experimental *Cyclospora* in the *Cercopithecus aethiops* which was accomplished in the study, affords us an opportunity to study other aspects of *Cyclospora* and evaluate new or existing drugs for therapy. The fact that *Cyclospora*-SIV co-infections were observed in the *Cercopithecus aethiops* suggests that this model will be useful for further investigation to gain a better understanding of *Cyclospora*-HIV co-infections in humans.

5.1.2 Gastrointestinal parasites in non-human primates

The general climatic conditions in the areas where the study animals were wild-trapped were wet and warm throughout the year of capture. These conditions most likely favoured parasite transmission, and may partly explain the rich fauna of parasite species, present in *C. aethiops*, comprising 5 helminth and 6 protozoan species. The parasites found present in the Olive baboons was very similar to that reported elsewhere, in non-human primates (Muller-Graf *et al*., 1996; Stoner, 1996; Munene *et al*., 1998; Muriuki *et al*., 1998; Ocaido *et al.*, 2003; Gillespie, 2004; Okanga *et al*., 2006; Levecke *et al*., 2007).

The prevalence of protozoan parasites was higher than that observed for helminth infections, and this observation was similar studies, elsewhere (Appletown and Brain, 1995; Munene *et al*., 1998). The fact that the parasite assemblage in the baboons consisted largely of protozoans could possibly be explained by the fact that protozoan reproductive rate is higher than helminthes, have a higher survival rate as they form cysts and have a direct mode of transmission. The prevalence of both helminth and protozoan parasites was higher among the juvenile baboons than in the adult animals, suggesting that even in non-human primates, juveniles are more susceptible to parasite infections than adults, and probably that immunity is developed as the animals grow older. Multiple parasites species infections were most common in adult (55.9 %) compared with the prevalence in juvenile baboons (20.4 %), and this confirms the vulnerability of adult baboons to parasite infections.

Intensity of helminth infections in both the juvenile and adult baboons was generally low, and this could be possibly attributed to a long dry spell experienced in the location where the baboons were wild-trapped prior to capture. This finding is in agreement with the observation of Appletown and Brain (1995) in which the gastrointestinal parasites of baboons living in the Namib Desert were predominantly protozoa.

Similarly faecal examination of *C. aethiops* revealed natural infection with *Trichuris, Oesophagostomum, Strongyloides*, *Trichostrongylus* and *Enterobius vermicularis* for helminths while the protozoa were *E. coli, Cyclospora*, *E. histolytica/ dispar* and *Cryptosporidium, Isospora* and *Paramecium* species. Statistically significant higher prevalence was observed in females than in males for *Strongyloides*, *E. coli*, *Cyclospora* and *Enterobius* Majority of the helminth infections were categorized as light with egg counts ≤ 200 epg. Prevalence of protozoan infections was significantly higher in the male adult monkeys. Multiple parasite species infections were common, and 2-4 parasite species were present per individual animal in combinations of helmith-helminth, helminth-protozoa or protozoa-protozoa infections. Similar observations have been reported elsewhere in non-human primates in Sub-Saharan Afica (Muller-Graf *et al.*, 1996; Stoner, 1996; Munene *et al.*, 1998; Muriuki *et al*., 1998; Gillespie, 2006; Okanga *et al*., 2006).

Earlier studies in Kenya by Munene *et al*. (1998) reported a low prevalence of waterborne or soil-borne parasite infections in arboreal Sykes monkeys (*Cercopithecus*

mitis). The low intensities could be explained by the monkey's way of life. The Sykes monkeys spend most of their time on tree branches. Similarly, the current study, observed a higher prevalence of helminth parasites in the Olive baboons than in the African green monkeys. Free ranging baboons scavenge for food which may favour acquisition of parasite infections (Eley *et al.,* 1989), and in the current study the baboons were wild-trapped fron localities close to human habitation (at the Aberdare Ark hotel) where they had opportunity to scavenge in the rubbish dumps. *Entamoeba coli*, *Cyclospora* and *Strongyloides*, parasites of public health significance present in the baboons, these results are similar those by Muriuki *et al*. (1998).

The protozoan parasites *E. histolytica/ dispar*, *Cyclospora* and *Cryptosporidium* were observed in both vervets and baboons. These parasites are considered to be easily transmissible from animals to man through contaminated food or water (Ortega *et al.,* 1997; Muriuki *et al.,* 1998). These parasites are also known to be the most common causative agents of debilitating diarrhoea in both immunocompetent and in immunocompromised individuals (Pape *et al.,* 1994). In Kenya, *Cryptosporidium* infections have been reported with a prevalence of 4% prevalence in children 0-5 years of age (Gatei *et al.,* 2006). Infections with *E. histolytica/ dispar* are also common in Sub-Saharan Africa (Beaver *et al*., 1988; Alakpa and Fagbenro-Beyioku, 2002; Nguhiu *et al.,* 2009).

In the African green monkeys, *Cyclospora* infections were observed in all age groups of the study animals and infections were asymptomatic, at the time of the time of faecal sample collection.Other workers investigating *Cyclospora* infections in non-human primates have also previously made similar observations (Smith *et al*., 1996; Eberhard *et al.,* 2001; Legesse and Erko, 2004). The *Cyclospora* oocysts present in both monkeys and baboons were spherical in shape measuring 9.96µ in diameter, and the parasites in the vervets aand baboons were indistinguishable. The oocysts stained variably in Ziehl Neelsen but were uniformly stained with hot Safranin as previously described and were identified as *Cyclospora* (Eberhard *et al*., 1997; Visvesvara *et al.,* 1997). The prevalence of *Cyclospora* infections in Olive baboons was low (6.2%) compared with that in African green monkey (64.1%). Similar observation was made previously in Kenya by Eberhard *et al*. (2001). These results suggest that the monkeys are more susceptible to infection with *Cyclospora* than baboons although other explanations cannot be ruled out. This high prevalence of *Cyclospora* in African green monkeys was the basis of selecting these monkeys for further investigations with the aim of developing a laboratory model for human cyclosporiasis. The non-human primates however, were asymptomatic at the time of faecal and blood sampling**.**

Although *Cyclospora* infections have not been reported in humans in Kenya, inspite of their presence in from East African monkeys, *Cyclospora cercopitheci* in vervets, *C. colobi* in colobus monkeys and *C. papioni* in baboons (Eberhard *et al.*, 1999b; Eberhard *et al.*, 2001), human cases of cyclosporiasis have been reported from Tanzania, Nigeria

and Egypt (Cieqelski *et al*., 1999, Alakpa *et al.,* 2002; Nassef *et al.* 1998). There is a likelihood that this parasite could easily be transmitted from non-human primates in to humans in in Kenya especially in areas where contact between humans and non-human primates is high as was the case in the two localities of Leimuria and Nguruman where the primates used in the present study were captured, and where the humans, non-human primates and other animals shared the same water sources.

The low intensities of helminth parasites in both monkeys and baboons observed in this study could be attributed to the low rainfall reported from the localities where the study animals were wild-trapped. It has previously been reported that non-human primates in dry areas harboured fewer parasite species with low intensities, than in primates captured from relatively wet areas (Appleton and Brain, 1995). Again, majority of the parasite species present in the non-human primates were protozoan and this observation was similar to those made in previous studies (Munene *et al.,* 1998; Muriuki *et al*., 1998).

5.1.3 Simian Immunodeficiency Virus and *Cyclospora*-SIV Co-Infections

In the present study, only the adult monkeys tested positive for SIV infection, with no clinical signs of the disease. SIV infection has previously been detected in monkeys in Kenya and elsewhere (Otsyula *et al.*, 1996; Joy *et al*., 2001). The sero-prevalence for SIV infection observed in the present study closely compared with that made in aprvious study in Kenya (Otsyula *et al.*, 1996). SIV infection in the juveniles AGM was also low in the previous study (Otsyula *et al*., 1996). The current study reported a sero-prevalence

of 30% for SIV among the adult Olive baboon but no infection was detected in the juvenile baboons. This was the first time baboons are being reported to have SIV in Kenya. Failure to detect SIV infection in African green monkey and Olive baboons is peculiar, but it does suggest that juvenile non-human primates are not susceptible to SIV infections. Further studies will need to determine the factors contributing to this low insusceptibility to SIV in juvenile animals. The present and a previous study (Otsyula *et al*., 1996) have observed that the SIV seropositive primates had no clinical symptoms at all. It is difficult to explain lack of symptoms without further studies. However, SIV infections occur naturally in indigenous primates and the animals remain asymptomatic, while SIV strains induce profound immunodeficiency when administered to Asian macaque species such as *Macaca mulatta* (Joy *et al*., 2001; Jones, 2004). Consequently, high viral loads may be maintained in indigenous primates and may be a source of fatal disease to any exotic primates that they may interact with. In our current study, SIV infections occurred naturally in the adult population of both Olive baboons and African green monkeys.

SIVagm strain is closely related to HIV-2 strains which cause AIDS in humans. The potential of monkeys to harbour simian retroviruses presents a potential zoonotic risk to animal handlers and researchers, since transmission of these viruses to humans is possible (Joy *et al*., 2001). A study done by Locher *et al*. (2003) showed increased viral replication and virulence after serial passage of human immunodeficiency virus (HIV) in baboons. This finding is significant in that HIV from baboons if transmitted to humans

is more likely to produce a virulent disease. Cyclospora -SIV co-infections were detected in both Olive baboons (prevalence of 10%) and in the African green monkey (prevalence of 30%) in the present study. Although *Cyclospor*a and SIV have been previously reported from non-human primates (Eberhard *et al*., 1999 b, Eberhard *et al.*, 2001; Otsyula *et al*., 1996, Joy *et al*., 2001) this is the first time Cyclospora-SIV co-infections are being documented in non-human primates. This finding is significant and and may explain the reason for protracted disease in immunocompromised human individuals. In the context of HIV, co-infections with coccidian parasites, such as *Cryptosporidium*, *Isospora* and *Cyclospora* may lead to severe diarrhoeal disease in humans (Cranendonk *et al*., 2003; Karp and Auwaerter, 2007).

5.1.4 *Cyclospora* infections in African green monkeys

The cohort study of *Cyclospora* in African green monkeys was designed to characterize the course of infection, immune response to infection, and determine the effect of infection on haematological parameters. Majority of the animals remained positive for *Cyclospora* for at least 6 months but by 9 months in captivity the animals had lost infection. Whereas no record to establish the source and course of *Cyclospora* in non-human primates was available, Soave (1996) reported that the disease in humans occurs within 2-11 days after consumption of contaminated food or water and run for a course of one or more months with few or no clinical signs and this was the general course observed in our study. Furthermore the colony-bred juvenile AGM remained *Cyclospora* negative throughout the study period which indicates that the breeding colony at IPR is *Cyclospora* clean and that the *Cyclopsora* infections observed in the current study were

acquired in the wild. The relapse infections observed in 25% of the study animals are in general agreement with work done by other researchers who have reported that some human patients would experience a relapse infection after a month (Pape *et al*., 1994; Madico *et al*., 1997). In accordance with current practice in humans, the therapy for *Cyclospora* infections in AGM should be followed by prophylactic treatment to avoid relapse infections.

The sporulation of the *Cyclospora* oocysts in potassium dichromate cultures maintained at ambient temperatures was observed from day 4 of incubation and completed within two weeks were in agreement with sporulation tests done by others from *Cyclospora* positive baboon faecal samples (Smith *et al*., 1997). The sporulation conditions were relatively easy to establish and yielded sporulated *Cyclospora* oocysts which were used as inoculums in establishing experimental infections.

The trends of the hematology values of the *Cyclospora* positive AGM were a general decline in the red blood cell values observed in all sex-age categories while the white blood cell counts were increased in female adults and male juveniles but decreased in the sex-age categories. The decline in red blood cells which may have been caused by blood loss as haemorrhages were observed in the stomach and intestinal mucosa leading to a depletion of existing cells or low production of cells may be an effect of *Cyclospora* infection. Consequently, the hemoglobin values were increased marginally in all the study animals.

The total white blood cell counts (WBC) in the *Cyclospora* infected AGM were higher in adult females than in other animals. The increase in WBC was closely associated with an increase in neutrophil proportion which would indicate body's positive response to *Cyclospora* infection. A decline in lymphocyte percent was observed in all the *Cyclospora* positive AGM. This decline in lymphocyte percent would indicate depletion in this cell population by the mild to intense lymphocytic infiltration observed in the intestinal mucosa and liver even though the mesenteric lymph nodes had mild lymphocytic proliferation. This cellular effect was a major finding in the animals that were positive for *Cyclospora* infection. The lymphocytes which are chief immunological cells, and being depleted in the current study indicate that *Cyclospora* adversely affects the immune system and may explain the protracted disease observed among Acquired Immunodeficiency syndrome (AIDS) patients in humans (Pape *et al*., 1994 Sifuentes-Osornio *et al.,* 1995; Peterson, 1998).

The general trends of the hematology values of the *Cyclospora* negative AGM were that the erythrocyte values were higher in the male adults and lower in the female juveniles while the white blood cell counts varied among the different study animals with the highest values recorded among the male adults. The normal hematological values recorded in this study for red and white blood cells are in general agreement with published data which reports marked variation in hematological values among AGM (Schalm *et al*., 1975; Kagira *et al.,* 2007). The study provides additional data on differential cell counts for eosinophils, basophils and total plasma proteins and plasma

fibrinogens in Kenyan AGM which complements previously reported haematological findings in captive *Cercopithecus aethiops* by Kagira *et al*. (2007) and which are in agreement with published data by Schalm *et al*. (1975). Furthermore, age- and sex-related hematology values have been described in vervet monkeys by Sato *et al*. (2005), who reported that male vervets tended to have higher erythrocyte and haematocrit values than females which is in concurrence with the current study. The higher values in adult males can be attributed to production of male sex hormone and higher muscular mass. The hematological changes in vervet monkeys during eight months adaptation to captivity with most hematological parameters becoming stable after 4 months have been described (Kagira *et al*., 2007). However, published data shows wide animal variations in hematological parameters in vervet monkeys (Schalm *et al.*, 1975) hence the necessity of establishing baseline hematological data in the current study.

Cyclospora specific antibody responses with high optical density values were observed in the *Cyclospora* positive animals. These observations were significant in that the antibody responses were an indication of an active immune system that was mounting humoral defense to invading micro-organisms. Low *Cyclospora* antibody responses below baseline were observed in the negative controls. These responses were as anticipated and display the body's immune response to infection. *Cyclospora* specific antibody responses were recorded in the animals with natural *Cyclospora* infections with optical density (OD) values being higher in males than females although the differences in observations were not statistically significant. The high OD values remained fairly

constant in male adult animals whereas in the juveniles, the OD values were initially low levels and increased steadily throughout the study period. This pattern observed indicated that the initial exposure to *Cyclospora* infections was probably in the juveniles, ilicitated a response of antibody production and by the time the animals were adults a maximum peak and further exposure maintained this level. This current study was the first documentation of *Cyclospora* antiboby responses in AGM.

5.1.5 Experimental *Cyclospora* infection in African green monkeys

Experimental *Cyclospora* infections were successfully established in four AGM by oral inoculation of 1000 *Cyclospora* oocysts using a catheter. The dose range was adopted from previous studies reported by Eberhard *et al*. (2000), where attempts were made to establish experimental *Cyclospora cayetanensis* infection in laboratory animals. The successful establishment of *Cyclospora* infection was confirmed by identification of *Cyclospora* oocysts from faecal samples of infected AGM. The experimental infections revealed *Cyclospora* oocysts that were indistinguishable from those of naturally infected AGM. The experimental animals started shedding from day 14 post- inoculation and shed oocysts for varying periods. The male adult had high numbers of *Cyclospora* oocysts initially with peak shedding on day 22 post-inoculation followed by a sharp decline and then shedding ceased by day 35 while the female juveniles continued to shed low numbers of oocysts until they were sacrificed at day 49. The pattern of oocyst shedding would indicate an incubation period of less than 14 days and a disease course greater than one month and the cessation of shedding would corresponded to clearing of infection in the animals.

The successful establishment of experimental *Cyclospora i*nfections in AGM was significant as it offers an animal model that may be used in studying various aspects of cyclosporiasis as previous attempts to establish infections with *Cyclospora cayetanensis* in various domestic animals and human volunteers have been unsuccessful (Eberhard *et al.,* 2000; Alfano-Sobsey *et al,* 2004). Studies in humans after consumption of *Cyclospora* infected food and water have reported incubation periods of 2-11 days (Soave, 1996). Hence the finding in the current study of an incubation period of 14 days when the animals started shedding oocysts compares well with human studies.

This result of asymptomatic *Cyclospora* infection in AGM with shedding of oocysts in stools has been reported by other authors (Eberhard *et al*., 2001; Legesse and Erko, 2004). This finding is significant in that the animals can silently harbour infections transmissible to humans, hence, pose potential risk to public health. Human cyclosporiasis is a self- limiting disease which persists for one month or more.

Cyclospora specific antibody responses with high optical density values were observed in the *Cyclospora* positive animals. These observations were significant in that the antibody responses were an indication of an active immune system that was mounting defense to invading micro-organisms. Low *Cyclospora* antibody responses were observed in the negative controls. These responses were as anticipated and display the body's immune response to infection.

5.1.6 Pathological findings in African green monkeys with *Cyclospora* infections

On gross post mortem evaluation of AGM with experimental *Cyclospora* infection, only few changes were observed in juveniles with adults displaying more pathology. These were interesting findings which may suggest that the juvenile non-human primates were more resistant to *Cyclospora* infection even though the infectious dose was the same i.e. 1000 *Cyclospora* oocysts administered orally. There are no reports on this phenomenon in *Cyclospora* infections in non-human primates were available, hence this finding was a first report. The gross changes observed in the juvenile AGM were mild enlargement of mesenteric lymph nodes while the adult AGM showed more severe lesions to include haemorrhages in the stomach and intestinal mucosa, mildly enlarged mesenteric lymph nodes, moderate pitting and necrotic foci on the livers. Again these differences in gross lesion indicate differences in susceptibility to *Cyclospora* infections among the different age groups of AGM. To the best of my knowledge, this phenomenon has not been previously reported. Though gross pathological lesions in humans with *Cyclospora* infections were scanty, Conner *et al*., (1993) reported moderate erythema of the duodenum in human patients with *Cyclospora* infection which is in agreement with the findings of this study of haemorrhages in the intestinal mucosa.

The significant histopathological findings were observed in the stomach, intestines, liver and lymph nodes of both juvenile and adult AGM included congested blood vessels, lymphocytic infiltrations, lymphocytic proliferation and granulomas. *Cyclospora* parasite species were observed in the enterocytes on microscopy. Distal duodenal and

jejunal aspirates of human patients with *Cyclospora* infections had similar histopathological findings, namely: acute and chronic inflammation, reactive hyperemia with vascular dilatation and villious capillary congestion, parasitophorous vacuoles that contain both asexual and sexual forms, crypt hyperplasia, epithelial disarray, and partial villous atrophy (Nhieu *et al.*, 1996; Ortega *et al*., 1997; Shoff, 2007). On electron micrographs, intracellular particles similar to sporozoites have been demonstrated (Ortega *et al*., 1997).

In the current study, the histopathological changes of the liver were only observed in the adult AGM which male indicate that the adults are more susceptible to *Cyclospora* infections and acquire extra-intestinal complications. Extra-intestinal complications following *Cyclospora cayetanensis* infections in humans have been reported mainly involving the bile ducts (Sifuentes-Osornio *et al.,* 1995). The granulomatous lesions observed may indicate an active immune system trying to wall off offensive parasites present. The lymphocytic proliferation observed in this study indicates a positive immune response.

5.1.7 Evaluation of Trimethoprim-Sulphamethoxazole therapy for *Cyclospora* infections in African green monkeys

Among the *Cyclospora* positive AGM treated with trimethoprim-sulphamethoxazole (TMP-SMX), on faecal sample examination, the samples were *Cyclospora* negative in all animals within 35 days post-treatment. Although no treatment for *Cyclospora* has been documented in non-human primates, this response to treatment was in agreement

with studies by other researchers in human studies with *Cyclospora cayetanensis* (Pape *et al*., 1994; Madico *et al*., 1997). In a study in Haiti, Pape and others reported that diarrhoea ceased and stool samples were *Cyclospora* negative within a mean period of 2.5 days post-treatment in infected humans which was in general agreement with the observations of the current study. The work of other researchers established that the treatment of human cases with trimethoprim-sulphamethoxazole was effective and had a relatively shorter post-treatment period than was reported in our study using AGM (Pape *et al*., 1994).

The pattern of oocyst shedding by the positive control animals over prolonged period and ceasing is characteristic of *Cyclospora* infections which is a protracted disease but self limiting (Pape *et al.,* 1994). The total number of oocysts shed was low as compared to the TMP-SMX treatment group which shed large numbers and this phenomenon further emphasizes the role of TMP-SMX in expunging *Cyclospora*. The two negative control animals did not shed any oocysts which indicate that they were *Cyclospora* naïve. These two animals were colony-bred and demonstrating that the colony is not contaminated with *Cyclospora* parasite species.

Although the source of infection was not established, recurrent asymptomatic cyclosporiasis developed in two (33%) of the female adult monkeys after two months and the animals started shedding *Cyclospora* oocysts. The two monkeys responded to second course of treatment with TMP-SMX and shedding ceased by day 28 post-second

TMP-SMX treatment. This finding was in agreement with the Haitian study in which recurrent symptomatic infections occurred in 43% of study population in 1-3 months and they responded to second treatment (Pape *et al*., 1994).

The general trends of the haematology values were indicative of positive response to TMP-SMX in all the treated animals. The white blood cell counts increased in all the TMP-SMX treated monkeys with the lymphocyte percentages showing a marked rise while the mean neutrophil percent had a slight decrease. The increase in the white blood cells and particularly the lymphocyte series is an indication that the animal has overcome the infection and showing signs of recovery. The red blood cells counts decreased marginally in all the treatment animals. The choice of using female juveniles in the study was to avoid age and sex-related haematology variations among the study animals as has been previously described (Kagira *et al*., 2007; Schalm *et al.,* 1975).
Among the positive control animals, most of the haematological parameters decreased except the WBC counts and in particular the neutrophil series where *Cyclospora* infection continued to have significant negative effects on the animals whereas the haematological parameters among the negative control remained fairly constant hence the observed results among the TMP-SMX treatment group may be attributed to the positive effects of the treatment.

On *Cyclospora* specific antibody ELISA, the optical density values decreased in the six TMP-SMX treated monkeys indicating positive response to treatment while the positive

control monkeys had increased antibody responses indicative of continuous challenge. In addition, no *Cyclospora* antibody responses were observed in the negative controls. These responses were as anticipated and demonstrate that the AGM were mounting an immune response to both the infection and treatment.

5.1.8 Molecular chacterization of *Cyclospora* parasites detected from African green monkeys

For the DNA analysis of *Cyclospora* oocysts isolated from *Cercopithecus aethiops* with natural and experimental *Cyclospora* infections as well as the oocysts used as inoculums a nested polymerize chain reaction (PCR) was carried out and yielded a PCR product of 294 base pairs. The results of the current study were consistent with studies by other authors who have carried out molecular studies to identify *Cyclospora* (Relman *et al.*, 1996; Eberhard *et al.*, 1999). The molecular analysis in the current study confirmed that the organism isolated from both natural and experimental infections and the inoculum used to be *Cyclospora* with a PCR product of 294 bp although the isolates from natural had a weaker band speculative of increased virulence following passage.

5.2 CONCLUSIONS

The results of this study led to the following conclusions:

5.2.1. Though general climatic conditions in localities where the study animals were wild-trapped were favourable for intestinal parasite multiplication and transmission but the low parasite intensities recorded were due to much drier conditions experienced in the localities prior to capture.

5.2.2. In the survey of gastrointestinal parasites in *Pabio anubis* and *Cercopithecus aethiops* both helminth and protozoa parasite infections were observed. Majority of the infections were predominantly protozoan parasites.

5.2.3. Many of the parasites reported in this study namely: *Trichuris Oesophagostomum*, *Strongyloides*, *Enterobius*, *Entamoeba histolytica/ dispar*, *Cyclospora*, *Cryptosporidium* and *Isospora* have public health significance and may pose potential risk to humans who may come in contact with the animals particularly animal handlers and researchers as these animals were in a Quarantine Facility in the Research Institute.

5.2.4. *Cyclospora* infections which were the parasites of interest in the current study were recorded in all age groups of the study animals. The prevalence of *Cyclospora* infections was significantly higher in AGM than in Olive baboons with higher prevalence observed in male adult AGM. Although all these infections were all asymptomatic it was significant that the study animals could silently harbour parasites species of public health significance. The *Cyclospora* infections among the AGM were self-limiting and animals had lost the infection by the 9- month sampling time since introduction to captivity.

5.2.5. The IPR colony-bred juvenile AGM were naïve and free from *Cyclospora* infections.

5.2.6. The Simian Immunodeficiency Virus infections were wide spread in *Pabio anubis* and *Cercopithecus aethiops* with high prevalence observed in adult male AGM while the juvenile AGM were negative for SIV. Cyclospora-SIV co-infections were common in *Pabio anubis* and *Cercopithecus aethiops* with high anti-SIV antibody responses

recorded among the monkeys with co-infections which indicates that the co-infections produced a much greater antibody response.

5.2.7. Experimental *Cyclospora* infections were successfully established in two juvenile and two male adult AGM by oral inoculation of *Cyclospora* oocysts. The procedure for harvesting and preservation of viable *Cyclospora o*ocysts was perfected during the study.

5.2.8. *Cyclospora* species were found to be enteric parasites with extra-intestinal involvement with many of the histopathological changes being similar to findings described in humans with cyclosporiasis.

5.2.9. TMP-SMX therapy was effective for treatment of cyclosporiasis in African green monkeys and the AGM presents a model that may be used to evaluate other drugs used in therapy and to explain the relapse infections commonly observed among HIV patients given TMP-SMX treatment.

5.2.10. The African green monkey offers an appropriate model to study cyclosporiasis

5.3 RECOMMENDATIONS

The following recommendations are made from the study:

5.3.1. The colony manager at the Quarantine Facility in IPR, Karen should consider treatment for coccidian protozoa with trimethoprim-sulphamethoxazole as a complementary to routine deworming programme as a measure for controlling gastrointestinal parasite infections in wild-trapped non-human primates.

5.3.2. The research sc ientists and animal handlers should exercise care and take

necessary precautions when handling the non-human primates to avoid acquisition of zoonotic diseases.

5.3.3. For *Cyclospora* studies

a. The African green monkey would be the preferred species for any further work on *Cyclospora*
b. The African green monkey is the preffered age group for further work.
c. The colony-bred AGM were *Cyclospora* naive and due care should be taken to maintain a *Cyclospora* clean colony at IPR
d. Modified salt floatation technique yield sufficient quantities of *Cyclospora* oocysts from faecal samples
e. *Cyclospora* oocysts preserved in potassium dichromate remain viable for periods greater than six months at ambient temperatures
f. For successful establishment of *Cyclospora* infection, sporulated *Cyclospora* oocysts preserved in potassium dichromate need to be introduced by catheter into the stomach.

5.3.4 .SIV and *Cyclospora*-SIV Co-infections

a. The infections were only detected in adult Olive baboons and AGM, hence further work on this may benefit from using this age class.
b. *Cyclospora* infections should be controlled in the adult Olive baboons and AGM

5.3.5: Trimethoprim-Sulphamethoxazole Therapy

a. TMP-SMX is effective for the treatment of *Cyclospora* infection in AGM

b. A second course of TMP-SMX therapy be administered to treated monkeys to avoid relapse infections.

5.4 FURTHER WORK

a. The testing of other anti-*Cyclospora* drugs maybe done using the AGM
b. The AGM model be used for HIV-*Cyclospora* studies.
c. Water and soil from the locations where the non-human primates were wild-trapped be tested to establish source of *Cyclospora* infection.
d. Testing for *Cyclospora cayetanensis* among human populations around the locations where the non-human primates were wild-trapped
e. In the context of one health, and the significance of *Cyclospora* infections resulting in opportunistic infections with extraintestinal involvement, among immunocompromised patients further work may need to be done to gain better understanding of this parasite

CHAPTER SIX

6.0 REFERENCES

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CHAPTER SEVEN

APPENDICES

7.1 Appendix 1 Publications arising from this PhD research work

7.1.1. Papers published in Peer reviewed Journals

1. PN Nguhiu[1], CN Wamae[2], JK Magambo[3], DS Yole[4]. (2011). Prevalence and Intensity of Gastrointestinal Parasites in Wild -trapped *Pabio anubis* and *Cercopithecus aethiops* in Kenya**.** *The Kenya Veterinarian,* **36:** 35-44.

ABSTRACT

Gastrointestinal parasite infections by multiple parasite species involving helminths and protozoans occur in non-human primates. The objective of the study was to screen for gastrointestinal parasite infections from sixty five wild-trapped *Pabio anubis* (olive baboons) and sixty four *Cercopithecus aethiops* (African green monkeys) from various locations in Kenya and at the Institute of Primate Research (IPR), Nairobi, in March 2008 to June 2009 to obtain preliminary data as baseline for proposed further study on coccidian parasites. Four faecal samples per individual animal were collected on Mondays and Thursdays for two consecutive weeks and processed for parasitological examination for gastrointestinal parasites. The animals were asymptomatic at the time of faecal sampling. Twelve taxa of intestinal parasites, 5 helminth and 7 protozoan parasites were identified. The helminth genera observed were *Oesophagostomum, Trichuris, Strongyloides, Trichostrongylus* and *Enterobius* with species prevalence of

33.6%, 30.4%, 26.2%, 8.8%, 0.8%, 0.4%, and 10.0%, 47.6%, 16.0%, 3.2%, 0.4% for olive baboons and African green monkeys, respectively. The intensity of infections ranged from 0-2350 eggs per gram (epg). Most individuals (56.2%) had mild infections of 1-300 epg and only 8% had egg counts greater or equal to 400. The protozoan parasites identified in this study were *Entamoeba coli, Entamoeba histolytica*, *Cyclospora* spp, *Cryptosporidium* spp, *Isospora* spp, *Iodamoeba butschilii* and *Paramecium* spp with prevalence of 77.3%, 23.1%, 3.8%, 2.7%, 2.2%, 1.2%, 0%, and 76.8%, 23.6%, 63.6%, 6.2%, 0.4%, 0%, 0.4% for olive baboons and African green monkeys, respectively. Majority (63.1%) of the animals had 2-3 parasite infections. Though the infections were asymptomatic in these non-human primates, they harbour important parasites that pose potential danger to public health, livestock and wildlife animals**.**

2. Nguhiu PN, Wamae CN, Magambo JK, Mbuthia PG, Chai DC, Yole DS. (2012). Gross and histopathological findings in *Cercopithecus aethiops* with experimental *Cyclospora* infection in Kenya. *Pathology and Laboratory Medicine International*, **4:** 13-20.

ABSTRACT

In 2009, experimental *Cyclospora* infections were established in two juvenile female and two adult male *Cercopithecus aethiops* (African green monkeys) at Nairobi's Institute of Primate Research (IPR). The study animals were humanely sacrificed, and gross and histopathological evaluation was done at seven weeks post-infection. On gross

examination, the juveniles had no abnormalities except for a slight enlargement of the mesenteric lymph nodes, while the adults displayed more pathology of enlarged lymph nodes, hemorrhagic gastrointestinal tracts, widespread necrotic foci of the liver, and enlarged spleens. Significant histopathological findings were observed in both the juveniles and adults, which ranged from mild inflammatory reactions in the stomach and intestines to intense cellular infiltrations with mitotic activity and lymphocytic infiltrations around the periportal area of the livers. The lymph nodes had extensive hyperplasia with many mitotic cells.

3. Nguhiu P N , Wamae C N, Magambo J K, Yole D S. (2012). The African Green Monkey (*Cercopithecus Aethiops*) As A Non-Human Primate Model for Infections with *Cyclospora cayetanensis. The Kenya Veterinarian*, (In the press*)*

ABSTRACT

Cyclospora cayetanensis is a protozoan parasite causing gastroenteritis in immunocompetent as well as immunocompromised humans worldwide. Clinical epidemiology and pathogenesis of *Cyclospora* species was studied in 64 wild-trapped *Cercopithecus aethiops* at the Institute of Primate Research, Nairobi, Kenya. The monkeys were screened for *Cyclospora* parasites using conventional microscopy, examination of hot safranin stained faecal smears, *Cyclospora* specific antibody responses and molecular characterization of DNA following nested Polymerase Chain Reaction (PCR) amplification. Efficacy of Trimethoprim-Sulphamethoxazole therapy for *Cyclospora* positive monkeys was evaluated. Experimental *Cyclospora* infections were established by oral inoculation. Oocyst shedding was used to confirm

establishment and duration of infection. Gross and histopathological characteristics were described. The animals remained asymptomatic. *Cyclospora* parasites were identified in 41 (64%) of animals with infections mostly in male adults. Incubation period was 14-17 days, the duration of infection one to two months. A PCR product of 294 base pair was visualized, confirming *Cyclospora* species in the natural and experimental infections. *Cyclospora* specific antibody responses were recorded in positive monkeys. Response to treatment was evaluated by cessation *Cyclospora* oocyst shedding by day 17 post-treatment and decreased *Cyclospora* specific antibody levels. Relapse infections occurred in 33% of the treated animals but responded to second TMP-SMX therapy. Pathological findings recorded were moderate haemorrhagic enteritis, parasites vacuoles in the enterocytes, mild lymph node enlargement, lymphocytic infiltrations, focal necrosis and granulomas in the liver. In conclusion, the study demonstrated that AGM may be offer a suitable model for study of cyclosporiasis.

7.1.2 Presentations at Scientific Conferences and Meetings

1.Nguhiu P.N[1]., Wamae C.N[2]., Magambo J.K[3]., Kariuki T[5].,5 Kithome K[5]. , Yole D.S[5]. (2008). Prevalence and Intensities of Intestinal Parasites in 65 Wild-trapped Olive Baboons (*Pabio anubis)* in Kenya**.** 6th Biennial Conference, Faculty of Veterinary Medicine, University of Nairobi.

ABSTRACT

Gastrointestinal parasite infections and often multiple parasite species involving both helminths and protozoans may occur in non-human primates. Sixty five wild caught olive baboons from Aberdare, Lemuria forest reserve and transported to the IPR, from

March 2008 to May 2008 were screened for gastrointestinal parasites. Multiple feacal samples per individual animal were collected and processed for parasitological examination. The animals were asymptomatic at the time of faecal sampling. Twelve taxa of intestinal parasites, five helminth and seven protozoan parasites were identified. The helminthes occurring in this study were *Oesophagostomum, Strongyloides, Trichiuris, Trichostrongylous* and *Enterobius* with prevalence of 50.8%. 36.7%, 32.2%, 12.3% and 1.5%, respectively. The infections occurred in both adult and juvenile baboons. The intensity of infections ranged from 0-2200 epg, most of the infections were mild with 100-300epg. The protozoan parasites identified in this study were *Entamoeba coli. Entamoeba histolytica*, *Cyclospora* spp, *Cryptosporidium* spp, *Isospora* spp, *Iodamoeba butschilii* and *Paramecium* spp. Most of the gastointestinal parasites indentified in Olive Baboons been documented in humans, livestock and wildlife. Though the infections were asymptomatic in baboons, they harbour important parasites that pose potential danger to public health, livestock and wildlife.

2.Nguhiu PN, CN Wamae, JK Magambo, I Mulei, T Kariuki, K Kithome, Yole DS. (2008). Prevalence of acid fast staining protozoa in wild-trapped Olive baboons (*Pabio anubis*) from Aberdares forest. Kenya. Proceedings of the 4th JKUAT Scientific and Technological and Industrialization Conference pp 250-256.AICAD, JKUAT, Kenya.

ABSTRACT

The Institute of Primate Research (IPR) is a center for biomedical research that utilizes non-human primates (NHP) to address human health challenges. Wild-trapped NHP are transported to the center and held in quarantine for 90 days during which health

monitoring and teswts are carried out to ensure provision of clean animals for research. The objective of the current study was to determine the prevalence of acid-fast intestinal protozoan parasite species in 65 Olive baboons (*Pabio anubis*) captured from Aberdare Forest, Kenya. The baboons comprised of 24 female adults, 22 male and 19 juveniles. In the period from March to May 2008, four non-consecutive faecal samples were collected from each individual animal. The 260 baboon faecal samples were processed by formal ether concentration technique and smears made from the sediment. The slide smears were stained with Modified Ziehl Neelsen and examined microscopically for acid-fast staining cysts. Three genera of protozoan parasites were observed in this population; 11 (16.9%) were positive for *Cyclospora,* 8 (12.2%) harboured Isospora and 7 (10.85%) had *Cryptosporidium* parasites. The distribution of the infections according to sex age categories was 17.4% in adult females, 31.8% in ale juveniles and 44.4% in female juveniles. Polyparasitism was observed in 9.2% of the study population, and these infections were in the juveniles with no sex differences in prevalence. Four (6.2%) harboured *Cyclospora* and *Isospora,* 2 (3.1%) had *Cryptosporidium* and *Isospora* while 1 (1.5%) individual harboured all the three protozoan parasite species. The parasites observed may be zoonotic, and have recently gained significance as they result in disseminating infections in immunocompromised humans, therefore chemophylaxis against them would improve the health of the non-human primates and also safeguard the safety of the animal handlers and researchers.

3.PN Nguhiu CN Wamae, JK Magambo, Yole DS. (2010). Association of *Cyclospora* infections with Simian Immunodeficiency Virus infections in wild-trapped Olive baboons (*Pabio anubis*) and African green monkeys (*Cercopithecus aethiops*) in Kenya. ITROMID 2nd Annual Health Science Conference. 27th and 28th May 2010. Jomo Kenyatta University of Agriculture and Technology, Kenya

ABSTRACT

Background: *Cyclospora* infection is an emerging protozoan disease of humans and non-human primates. In immunocompetent individuals, the disease is self limiting but in immunocompromised patients, the disease leads to a protracted life threatening diarrhea. In NHP, both *Cyclospora* and SIV are asymptomatic, but an evaluation of their co-infections has not been documented.

Objective: To determine the prevalence and co-occurrence *Cyclospora* infections and Simian Immunodeficiency virus (SIV) infections in wild trapped non-human primates (NHP) at Institute of Primate Research (IPR), Nairobi.

Design: Cross sectional study.

Setting: Wild trapped non human primates in quarantine at IPR, Karen.

Subjects: Ten adult female wild trapped olive baboons (*Pabio anubis*) and thirty three vervet monkeys (*Cercopithecus aethiops*) comprising of ten male and seven female adults, three male and thirteen female juveniles

Methodology: *Cyclospora* species were identified from faecal samples by conventional formalin ethyl acetate, microscopic examination of direct smear, modified acid-fast and

hot Safranin stained smears. Antibody titers were determined using a commercial SIV specific kit according to manufacturer's instructions.

Results: Out of the ten baboons tested, 3 (30%) were positive for SIV and only 1 (10%) was positive for *Cyclospora* infections. Of the 33 vervet monkeys tested, 10 (30%) were positive for SIV, 23 (70%) were positive for *Cyclospora* infections while 5 (18%) harboured both infections. The *Cyclospora* infections were found in all age groups of vervet monkeys, while the SIV infections were only observed in the adult populations. The co-infections were found in the adult population with no gender related differences.

Conclusion*: Cyclospora*-SIV co-infections are widespread in wild trapped non-human primates with infections predominantly observed in the adults.

4.Nguhiu P.N[1]., Wamae C.N[2]., Magambo J.K[3]., Chai D[4]., Yole D.S[4]. (2010). Evaluation of Trimethoprim-Sulfamethoxazole efficacy against *Cyclospora* infections in African green monkeys. Institute of Primate Research 18th International Scientific Conference. 6th -8th. July 2010. Kenya Institute of Education, Nairobi-Kenya.

ABSTRACT

Cyclospora cayetanensis causes gastroenteritis with immunocompromised individuals having protracted disease. The biology of this parasite has been unclear, with no known animal reservoirs and no laboratory methods to propagate the protozoa. *Cyclospora* species identified in non-human primates, accord us an opportunity to study aspects of this disease using an animal model. The efficacy of trimethoprim- sulfamethoxazole therapy for cyclosporiasis in African green monkeys was studied. Oocyst shedding

ceased 4 weeks post treatment in all the animals, recurrent asymptomatic disease observed in 33% of animals, but responded on second treatment.

5.Nguhiu P N [1], Wamae C N[2], Magambo J K[3], Yole D S[4]. (2012). The African Green Monkey (*Cercopithecus aethiops*) As A Non-Human Primate Model for Infections with *Cyclospora cayetanensis,* Faculty of Veterinary Medicine 8th Biennial Scientific Conference and the 46th KenyaVeterinary Association Annual Scientific Conference, 25th to 27th April, 2012**.** Safari Park Hotel, Nairobi-Kenya.

ABSTRACT

Cyclospora cayetanensis is a protozoan parasite causing gastroenteritis in immunocompetent as well as immunocompromised humans worldwide. Clinical epidemiology and pathogenesis of *Cyclospora* species was studied in 64 wild-trapped *Cercopithecus aethiops* at Institute of Primate Research, Nairobi. The monkeys were screened for *Cyclospora* parasites by conventional microscopy, examination of hot safranin stained faecal slide smears, *Cyclospora* specific antibody responses and molecular characterization of DNA following nested PCR amplification. Efficacy of trimethoprim-sulphamethoxazole therapy for *Cyclospora* positive monkeys was evaluated. Experimental *Cyclospora* infections were established by oral inoculations. Oocyst shedding was used to confirm establishment and duration of infection. Gross and histopathological characteristics were described. The animals remained asymptomatic, *Cyclospora* parasites were identified in 41 (64%) of animals with infections mostly in male adults. Incubation period was 14-17 days, the duration of infection one to two months. A PCR product of 294 base pair was visualized, confirming *Cyclospora* species

in the natural and experimental infections. *Cyclospora* specific antibody responses were recorded in positive monkeys. Response to treatment was evaluated by cessation *Cyclospora* oocyst shedding by D17 post-treatment and decreased *Cyclospora* specific antibody levels. Relapse infections occurred in 33% of the treated animals but responded to second TMP-SMX therapy. Pathological findings recorded were moderate haemorrhagic enteritis, parasites vacuoles in the enterocytes, mild lymph node enlargement, lymphocytic infiltrations, focal necrosis and granulomas in the liver. In conclusion, the study demonstrated that AGM may be offer a suitable model for study of cyclosporiasis.

6.Purity N. Nguhiu, Claire N.Wamae, Japheth K. Magambo, and Dorcas S. Yole. (**2012).** *Cyclospora*-Simian Immunodeficiency Virus Co-infections in *Pabio anubis* (Olive baboons) and *Cercopithecus aethiops* (African green monkeys): Implications for *Cyclospora*-Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome Patients. 1st Kenya Society for Immunology Symposium, 24th - 25th May 2012, KEMRI-Training Centre, Nairobi.

ABSTRACT

Introduction: *Cyclospora* infection is an emerging protozoan disease of humans and non-human primates (NHP). In immunocompetent individuals, the disease is self limiting but in immunocompromised patients, the disease leads to protracted life threatening diarrhoea.

Objectives: A study to determine the prevalence and co-occurrence of *Cyclospora* and Simian Immunodeficiency virus (SIV) infections in wild-trapped non-human primates (NHP) at Institute of Primate Research (IPR), Nairobi.

Materials and Methods: Ten female adult Olive baboons and thirty three African green monkeys, AGM (comprising of 10 male adults, 7 female adults, 3 male juveniles and 13 female juveniles) were screened for *Cyclospora* infections by microscopic examination of formal ethyl acetate processed faecal samples. Simian Immunodeficiency Virus (SIV) infection in these animals was screened using a SIV specific ELISA for antibody titres. The *Cyclospora* –SIV co-infections were evaluated from March 2008 to July 2009 at the Institute of Primate Research (IPR) Quarantine Facility.

Results: On microscopic evaluation of faecal smears, *Cyclospora* oocysts were identified in 10% of the female adult baboons, 80% of male adult AGM, 57% of female adult AGM, 67% of male juvenile and 69% of the female juvenile AGM of male adult AGM, 72% of female AGM. The juveniles were negative for anti-SIV antibody responses. The co-infections were observed in the 10% adult female Olive baboons and 30% in adult male and female AGM. All the study animals were asymptomatic at the time of sampling.

Conclusions: *Cyclospora* species infections occurred in both Olive baboons and AGM with higher prevalence observed in the AGM. SIV infections were only recorded among the adult study animals. The potential of these study animals to harbor SIVagm, a strain closely related to HIV-2 strains which cause AIDS in humans and remain asymptomatic presents a potential zoonotic risk to animal handlers and researchers. The finding of

Cyclospora- SIV co-infections in the baboons and AGM with high anti- SIV antibody responses may be significant in explaining the reason for protracted cyclosporiasis in immunocompromised individuals.

Key words: *Cyclospora,* SIV, Co-infections, opportunist infections, non-human primates.

7.1.3. Papers submitted to Journals for consideration

1.Purity N. Nguhiu, Claire N. Wamae, Japheth K. Magambo, Daniel C. Chai, Paul G. Mbuthia, Dorcas S. Yole. (2012). Establishment of Experimental *Cyclospora* Infections in *Cercopithecus aethiops* (African Green Monkeys) in Kenya. *Journal of Experimental Parasitology.* MS No. 155-2012.

ABSTRACT

Cyclospora cayetanensis is a coccidian parasite that causes gastroenteritis in humans worldwide. Attempts to establish experimental in different species of animals and humans have failed. However, *Cyclospora* infections are widespread in non-human primates. Experimental *Cyclospora* infections were established in two female juvenile and two male adult *Cercopithecus aethiops* (African green monkeys, AGM) to characterize infectivity. *Cyclospora* oocysts harvested from faecal samples of AGM with natural infection were used as inoculums. Each experimental animal was anaesthetized and 1000 *Cyclospora* oocysts were introduced into the stomach using a catheter. Study outcome measures were shedding of *Cyclospora* oocys in faecal samples and histopathological characteristics with parasite vacuoles in the enterocytes. Faecal samples were collected twice weekly for a 7 week period. *Cyclospora* oocyst shedding

was observed from day 17 post-inoculation and continued up to end of study period. On histopathological examination, the stomachs and intestines revealed acute and chronic inflammation, reactive hyperemia with vascular dilatation and villous capillary congestion, parasites in enterocytes, crypt hyperplasia, villous atrophy of the stomach and intestines. The successful experimental establishment of *Cyclospora* infection in *Cercopithecus aethiops* (African green monkeys) offers an opportunity to study cyclosporiasis.

2.Purity N. Nguhiu, C. Njeri Wamae, Japheth.K. Magambo & Dorcas. S. Yole. (2011) Haematological and Serological Findings in *Cercopithecus aethiops* (African green monkeys) with *Cyclospora* Infections in Kenya. *International Journal of Professional Practice*

ABSTRACT

Cyclospora, is a common cause of gastroenteritis in humans resulting in protracted life threatening diarrhoea in immunocompromised patients worldwide. *Cyclospora* infections have been reported in African green monkeys. The objective of the study was to determine the haematological and serological parameters in African green monkeys with *Cyclospora* infections. A cross-sectional laboratory based study was done at Institute of Primate Research, Nairobi, from March 2008 to June 2009. Thirty three African green monkeys comprising of 10 male adults, 7 female adults, 3 male juveniles and 13 female juveniles were analysed for blood cell counts using a hematological analyzer and screened for *Cyclospora* by ELISA. The red blood cell counts decreased in all study animals with mean decline of 1.2-1.5 (x 10^6/µl). The white blood cell counts

varied among the study animals with highest increase among female adults from 4.3-7.1 to 5.4-8.8 (x 10^3 /µl) and a decrease among the male adults from 3.9-8.7 to 1.5-5.3 (x 10^3/µl). Positive antibody responses to *Cyclospora* were observed in 20 of the study animals with highest mean optical density (OD) values of 0.816 ± 0.100 observed among the female juveniles and lowest among the male juveniles at mean OD value of 0.646 ± 0.055.

In conclusion, *Cyclospora* infections triggered both cellular and humoral responses in the African green monkeys.

7.2 Appendix II: Institutional Scientific and Ethical Review (IPR)

INSTITUTE OF PRIMATE RESEARCH
NATIONAL MUSEUMS OF KENYA
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INSTITUTIONAL SCIENTIFIC AND ETHICAL REVIEW COMMITTEE (ISERC)

FINAL PROPOSAL APPROVAL FORM

Dear **Dr. Dorcas Yole and Purity Nguhiu**

It is my pleasure to inform you that your proposal entitled "*Clinical-Epidemiology of Cyclospora Spp in Kenyan nonhuman primates: A laboratory model for cyclosporiasis*", in collaboration with *Dr. Njeri Wamae, KEMRI/CMR, Box 19464-0202, Nairobi*, has been reviewed by the Institutional Scientific and Review Committee (ISERC). The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed ..... Chairman ISERC: DR. PETER G. MWETHERA

Signed ..... Secretary ISERC: Dr. Hastings Ozwara

Date: 20th May 2008

7.3 Appendix III: KEMRI Scientific Steering Committee Review and Approval

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ESACIPAC/SSC/2754 30th June, 2008

Purity N. Nyambura

Thro'
Director, CMR
NAIROBI

REF: SSC No. 1417(Revised) – Clinical-epidemiology of Cyclospora SPP in Kenyan non-human primates: A laboratory model for Cyclosporiasis

I am pleased to inform you that the above mentioned proposal in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 147th meeting held on 3rd June 2008 and has since been approved for implementation by the SSC.

The proposal has been forwarded to the ERC and we advise that work on this project can only start when ERC approval is received.

C. Mwandawiro, PhD
SECRETARY, SSC