CLINICO-PATHOLOGICAL STUDY OF SCHISTOSOMA MANSONI IN OLIVE BABOONS (PAPIO CYANOCEPHALUS ANUBIS) VACCINATED WITH S. MANSONI ANTI-OXIDANT ENZYMES AND FILAMIN ENCODING GENES

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (MEDICAL BIOCHEMISTRY) OF KENYATTA UNIVERSITY
DECLARATION

I, Robin C A Omedo declare that this thesis is my original work and has not been presented for a degree in any other University or any other ward.

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This work is dedicated to my wife Philo and children, Michelle, Pat, Marie, Bobo and Peace for their patience, prayers, continued support and encouragement.
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ABBREVIATIONS AND ACRONYMS

BSA  Bovine Serum Albumin
CDC  Center for Disease Control
CT-SOD  Cytosolic Superoxide Dismutase
DNA  Deoxy-ribonucleic acid
ELISA  Enzyme-Linked Immuno-Sorbent Assay
GPX  Glutathione Peroxidase
Hb  Haemoglobin
IgE  Immunoglobulin E
IgG  Immunoglobulin G
IPR  Institute of Primate Research.
MCH  Mean Cell Haemoglobin
MCHC  Mean Cell Haemoglobin Concentration
MCV  Mean Cell Volume
PAN  *Papio anubis*
PBS  Phosphate Buffered Saline
pc  Plasmid carrying
PCV  Packed Cell Volume
Pi  Post infection
ROS  Reactive Oxygen Species
S  *Schistosoma*
SEA  Soluble Egg Antigen
Sm  *Schistosoma mansoni*
SWAP  Soluble Worm Antigen Preparation
WHO  World Health Organization
XPG  Reverse of Glutathione Peroxidase (GPX)
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ABSTRACT

Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma*. It is second to malaria in importance and at present the most effective control method is chemotherapy with use of praziquantel as the drug of choice. With the rising cases of drug resistance and re-infection, the focus is on development of a vaccine that can significantly reduce the incidence of the disease. The ability of genes encoding *S. mansoni* Cu/Zn superoxide dismutase (CT-SOD) and glutathione peroxidase (GPX) to confer significant levels of protective immunity in a murine model of *S. mansoni* have been demonstrated. In addition to anti-oxidant enzymes, a gene encoding Sm-filamin has also been shown to consistently induce a significant level of protection against schistosomiasis in mice. In this study, therefore the aim was to determine whether the protection observed in murine models can be replicated in the olive baboons as no such work has been done in higher mammals. In the study, animals in three groups were inoculated with 500μg each of DNA vaccines containing genes encoding *S. mansoni* CT-SOD, GPX and Sm-filamin respectively. The baboons in the fourth group received 500μg each of a vaccine containing a gene encoding GPX cloned in the reverse orientation (XPG) and the animals in the fifth group were injected with normal saline (0.85% NaCl). The animals were boosted at week 4, 8 and 12, and then rested for 4 weeks. Thereafter, the animals were challenged with 800 *S. mansoni* cercariae per animal percutaneously. The DNA vaccines did not alter the blood parameters, indicating that they are safe for use in animal models. Genes encoding *S. mansoni* anti-oxidant enzymes (CT-SOD and GPX) and Sm-filamin induced production of specific IgG against SEA, and the response to SWAP was low, but this immune response was un-protective to baboons against *S. mansoni* infection. The egg out-put was low in SOD, GPX and XPG vaccinated baboons and highest in Sm-filamin vaccinated and normal saline injected baboons. Worm burdens, gross and histo-hepato-intestinal pathology including the mean hepatic egg-granuloma sizes in vaccinated and non-vaccinated controls were statistically insignificant. Although the baboons inoculated with Sm-pcGPX and Sm-pcXPG had a worm reduction of 7.47% and 2.63% respectively, this was insignificant and below the WHO requirement for vaccine. Those vaccinated with pcCT-SOD and Sm-filamin had also insignificant worm reductions as compared to the controls. However, all animals gained weight before inoculation, but lost weight after challenge infection, with those vaccinated with SOD and GPX loosing less weight than other groups and those vaccinated with XPG loosing more weight. Although, these vaccines were able to induce immune responses to some worm antigens, some of them (SOD and GPX) were able to cause less weight loss pi and also low egg out put, but worm reduction and pathology in both vaccinated and non-vaccinated were insignificant to indicate any protection. This study recommends several things; these vaccines should be used with suitable adjuvant in future studies, vaccines should also be combined into one polyvalent vaccine, less weight loss and anti-fecundity effects of SOD and GPX should also be further investigated. Genes encoding other schistosome antigens that have shown protection in murine models should also be tested in non-human primates as a prelude to human clinical trials.
CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

DNA vaccination is a recent and an effective vaccination technology that delivers DNA constructs encoding specific immunogens into the host cells, inducing both antigen-specific humoral and cellular immune responses (Bergquist, 2001). The first demonstration of protective immunity was against viral challenge induced by DNA encoding influenza A nucleoprotein. Since then many trails with various degrees of success have been achieved against viral and other parasitic pathogens (Ulmer et al., 1993; Guranathan et al., 1998; Doolan and Hoffman, 2001). The main methods of plasmid-DNA application are intramuscular injection and intra-dermal delivery into the skin by a gene gun (Smahel, 2002).

Recently many researchers have reported a number of findings highlighting the fact that vaccination can be used to deliberately alter the phenotype of the immune response that develop during subsequent S. mansoni infection, with the result that the immune-pathology associated with egg deposition in the tissues is minimised (Wynn, 1996). Vaccination with DNA has been shown to induce encoded Schistosoma antigen-specific immune responses in murine models (Capron et al., 1997; Dupre et al., 1997, 2001; Yang et al., 2000) and partial protection against challenge in mice, underlining the potential of this method of vaccine delivery for schistosomiasis (Mohamed et al., 1998; Hanem et al., 2006).
Internationally, efforts to develop a vaccine against schistosomiasis have been going on for many years, but until the recombinant DNA techniques were introduced, antigens production remained a major hindrance to the development of anti-schistosomiasis vaccine (Pearce, 2003). Many antigens continue to be identified as possible vaccine candidates, and promising results in animal models have been reported with a number of these antigens, although in an independent test none of them accomplished the pre-set target level of 40% protection (Bergquist and Colley, 1998).

Vaccination can achieve its effect both by preventing parasite entry and development, and by interfering with the production and delivery of eggs (Pearce, 2003). A reduction in worm burden is the gold standard for anti-schistosome vaccine development, but as *Schistosoma* eggs are responsible for both pathology and transmission, a vaccine targeting parasite fecundity and egg viability also appears to be relevant (Hanem et al., 2006). Vaccination with naked DNA encoding SM21.7 protein has been observed to induce a significant level of protection in mice (Hanem et al., 2006). Plasmid cDNA constructs encoding *S. mansoni* Cu/Zn cytosolic superoxide dismutase (CT-SOD), signal peptide containing superoxide dismutase (SP-SOD) and glutathione peroxidase (GPX) have also been used to induce protective immunity to *S. mansoni* infection in murine models (Shalaby et al., 2003).

### 1.2 Schistosomiasis

Schistosomiasis also known as bilharzia, bilharziosis or snail fever is a parasitic disease caused by several species of flukes of the genus *Schistosoma,*
which affects human beings, domestic livestock and wild animals including non-human primates (Warren, 1984; Stevens et al., 2002; Pearce, 2003). The disease is a major health hazard in tropical countries, especially in areas with water that is contaminated with freshwater snails which may carry the parasites. It is approximated to affect between 200-300 million people, particularly children who may acquire the disease by swimming or playing in infested water (Anon., 1993). More than 80% of those infected are found in sub-Saharan Africa, where it is a poverty related health problem and a major cause of morbidity (Chitsulo et al., 2000).

The parasites cause chronic morbidity in large numbers of infected individuals, with severe life threatening consequences in a small percentage of those affected. Although it has a low mortality rate, schistosomiasis is often a chronic illness that can damage internal organs and in children impair growth and cognitive development. Currently it is estimated that 280,000 people die from schistosomiasis in sub-Saharan Africa each year (van der Werf et al., 2003). Schistosomiasis is of major socio-economic and public health importance in endemic areas due to its adverse pathology, which ranks second to malaria (Pikes, 1987).

A number of Schistosoma species have been reported to affect humans and other mammals (Farah et al., 2000), but only three are of significant importance in human schistosomiasis. The three species are Schistosoma haematobium, S. mansoni and S. japonicum (Cheesbrough, 1981). S. haematobium causes urinary schistosomiasis and occurs in Africa, Asia and
Europe (Anon., 1993). *S. mansoni* which causes intestinal schistosomiasis occurs in Africa, South and Central America, while *S. japonicum* the other causative agent of intestinal schistosomiasis is restricted to the Far East countries such as Philippine, China, Thailand, Laos and Cambodia (Anon., 1993). Other species, such as *S. intercalatum* and *S. mekongi* also affect humans, but to a lesser extent. Species such as *S. bovis*, *S. curassoni*, *S. margrebowiei*, *S. matheei* and *S. rhodhiani* infect animals but may also infect human beings on rare occasions (Sturrock, 2001).

In Africa, East African countries have higher incidences of *S. mansoni* infections than West African countries (see Fig. 1). More than 50% of morbidity/pathology cases associated with *S. mansoni* are found in Tanzania, DR Congo, Nigeria and Kenya. Sudan and Somalia have very low endemicity for *S. mansoni* (van der Werf et al., 2003). *S. haematobium* infection seems to be less in Ethiopia, Uganda and Madagascar (prevalence ≤ 1%). Burundi, Rwanda, Equatorial Guinea and Eritrea also have very low endemicity for *S. haematobium* (van der Werf et al., 2003). In most West African countries the percentage of the total population with haematuria associated with *S. haematobium* infection is >15% (see Fig. 2).

In Kenya, where about 1.5 million persons are affected with schistosomiasis, the species are *Schistosoma haematobium* and *S. mansoni* (van der Werf et al., 2003). *Schistosoma haematobium* is found in the coastal districts of Kwale, Kilifi and Taita-Taveta. It is also found in most districts in eastern and western provinces (King et al.,
2003). While *S. mansoni* is mainly found in Central, Nyanza and Western provinces (Highton, 1974).

### 1.3 Diagnosis of schistosomiasis

Microscopic identification of eggs in stool or urine is the most practical method for diagnosis (Feldmeier and Poggensee, 1993). The stool examination is the more common of the two. Stool examination is performed when infection with *S. mansoni* or *S. japonicum* is suspected and urine examined when *S. haematobium* is suspected. Eggs can be present in the stool in infections with all *Schistosoma* species. The examination can be performed on a simple smear (1-2 mg of faecal material). Since eggs may be passed intermittently or in small amounts, their detection can be enhanced by repeated examinations and/or concentration procedures (Elliott, 1996). The egg output can also be quantified by using Kato-Katz technique (20-50 mg of faecal material) or the Ritchie technique. Tissue biopsy (rectal biopsy for all species and urinary bladder biopsy for *S. haematobium*) may demonstrate eggs when stool and urine examinations are negative. Antibody detection can also be used in both clinical management and for epidemiologic surveys (Bergquist, 1992).
Figure 1: A map of Africa indicating predicted proportion of the total population with hepatomegally from *S. mansoni* infection by country (van der Werf *et al.*, 2003)
Figure 2: A map of Africa indicating predicted proportion of the total population with haematuria from *S. haematobium* infection by country (van der Werf et al., 2003)
1.4 The morphology and life cycle of the parasites

The males of *S. mansoni* are creamish in colour, broader than the females, bear a gynaecophoric canal and measure 6-12mm long with a diameter of 1.1mm. The number of testes varies from 4-13 and these are anteriorly placed (Cheesbrough, 1981). The morphology of male *S. haematobium* is similar to that of *S. mansoni* except it is longer. *Schistosoma japonicum* also have similar morphology to *S. mansoni*, but like *S. haematobium* it is longer and narrower. The females of the three species are darker, slender and relatively longer than the males, measuring 10-20mm long by 0.6mm wide. The uterus is placed in the anterior half of the female body (Yole *et al.*, 1996).

The eggs of *S. haematobium* have a terminal spine, while those of *S. mansoni* and *S. japonicum* have lateral spine (Cheesbrough, 1981). The eggs of *S. mansoni* and *S. japonicum* are laid by the female adult worms in the mesenteric blood vessels from where they penetrate into the lumen of the intestines of the definitive hosts and are passed out with faeces (Cheever and Andrade, 1967). *S. haematobium* eggs are passed out in urine, rarely in faeces because they are laid in the walls of the urinary bladder (Cheesbrough, 1981).

Mature eggs once passed with faeces or urine in fresh water hatch into female and male miracidia, under condition of light and warmth (Kassim and Gilberton, 1976). Miracidium swims actively by means of cilia until it encounter an appropriate snail or die within 48 hours. Penetration into the
vector snail is through the soft parts of the body and is achieved by secretions from the anteriorly placed penetration glands of the parasite (Jourdane and Theron, 1987). Once inside the vector, miracidia lose their ciliated epidermal layer and develop into mother sporocysts (Cheesbrough, 1981). The mother sporocysts then become filled with germ balls and burst after 8 days releasing several germ balls. The germ balls migrate to the digestive glands and after a series of multiplications they develop into thin walled daughter sporocysts (Cheesbrough, 1981).

A further process of asexual multiplication takes place and the daughter sporocysts become filled with final larval stage, the cercariae, which emerge from the snail between 4-5 weeks after infection a process influenced by light (Yole et al., 1996). One snail can release about 1000-2000 cercariae daily, but these numbers decrease with time. The cercariae measure between 300-400μm in length and have a bi-forked tail (Cheesbrough, 1981). The cercariae once out of the snail may swim for about 12-14 hours, and when they come into contact with wet skin of the host, they penetrate between the hair follicles by means of anterior spines and capopystic secretion of the cephalic glands (Jourdane and Theron, 1987). The penetration process takes 5-15 minutes; cercariae lose their tails and become schistosomula (Cheesbrough, 1981).

The schistosomula enter the peripheral lymphatic or venous vessels and are carried to the lungs via the heart and appear in the lungs 4-7 days after penetration. From the lungs they move to the portal vessels via the heart, where they grow into male and female adult worms (Anon., 2003). The adult,
male and female then pair up, and remain in copula with the female lying in the gynaecophoric canal of the male. In this state, the worm pair migrates to the mesenteric venules of the bowel/rectum (*S. mansoni* and *S. japonicum*) and venous plexus of the urinary bladder (*S. haematobium*) where the female begin to lay eggs. In the case of *S. japonicum* and *S. mansoni*, a proportion of the eggs are carried to the lumen of the colon and passed with faeces, while others remain attached to the gut wall and die. But *S. haematobium* eggs are concentrated in urine (Cheever, 1969). The life cycle of three *Schistosoma* parasites is illustrated in Fig. 3. It continues when eggs come into contact with fresh water and hatch into miracidia (Lyons, 1978; Anon., 2003).

![Figure 3](image-url)

*Figure 3.* The life cycle of *Schistosoma* parasites: adapted from Center for Disease Control. Laboratory identification of parasites of public concern, 2nd edition, 2003.
1.5 Host preference and tissue localization of *Schistosoma mansoni*

Schistosomiasis due to *S. mansoni* is a chronic infectious disease of humans and other primates (Sturrock *et al.*, 1978). Many primates are naturally infected. Miller, (1960), observed that in endemic areas baboons, vervet monkeys and sykes are affected, suggesting presence of a true zoonosis. The parasites may also develop to maturity in a number of experimental animals, referred to as permissive hosts. These include mice, hamsters and Rhesus monkeys. Rodents such as rats are rarely infected (Kuntz *et al.*, 1971; Cheever *et al.*, 1974).

Snails of various groups act as intermediate hosts for different species of schistosomes (van der Werf *et al.*, 2003). The geographical distribution of the snails, therefore influences the endemicity of the parasite. *S. mansoni* is transmitted by snails of the species of the genus *Biomphalaria* and in East Africa the species *Biomphalaria pfeifferi* is the main vector (Lyons, 1978). The snail vectors of schistosomiasis live in well aerated water with vegetation. The vegetation serves as food and also supply leaf surface for egg deposition. Although snails can survive at higher temperatures, they thrive well at an optimum temperature range of 22-23°C (Malek, 1958). Most snail populations fall during the rainy season and increase in the drier and warmer months of the year. However, where water temperatures are more stable, some snail populations, show no seasonal trends. But droughts and floods reduce their populations (Malek, 1958).
In tropical lakes, the extraordinary growth of certain species of floating plants, particularly *Eichhornia crassipes* (water hyacinth), *Salvinia auriculata* (water fern) and *Pistia stratiotes* (water lettuce), provides rich support for multiplication of vector snails, especially *Bulinus* and *Biomphalaria* species, and of insects (Anon., 1993). Snail dispersal along water-courses is also assisted by floating islets of vegetation. The spread of water hyacinth is having a severe economic impact on Eastern African lakes. Submerged vegetation, of which *Ceratophyllum demersum*, *Polygonum senegalense* and *Utricularia inflexa* are examples, may support large snail colonies, especially when the aquatic plants are growing vigorously. As it sometimes occurred in Lake Nasser, bottom algae can support a snail population that ensures the transmission of schistosomiasis (Anon., 1993).

*Schistosoma mansoni* prefer the lower mesenteric veins of humans and other primates. Humans and baboons that get infected pass through an acute phase of the disease from 6-12 weeks post-exposure (Farah and Nyindo, 1996). In humans, severe and acute cases of *S. mansoni* occur usually in young individuals under 9 years (Smithers and Terry, 1969; Woolhouse *et al.*, 1991; Hagan, 1992). Infected older individuals are protected against re-infection by concomitant immunity probably mediated by IgE antibodies (Smithers and Terry, 1969; Hagan, 1992).
1.6 Pathology

The parasitological and pathological changes due to acute and chronic schistosomiasis in humans, non-human primates and mice have been documented (Warren, 1966; Sadun et al., 1966; Cheever and Andrade, 1967; Myers et al., 1970). Baboons infected with S. mansoni develop similar signs of acute human schistosomiasis, including eosinophilia and fever (Damian et al., 1992). The immune responses that develop following infection with schistosomiasis often proceed to cause pathological changes that are primary cause of the disease (Kariuki and Farah, 2005). The pathology of schistosomiasis may be divided into three main stages. These stages include; the invasion phase, the maturation of infection and the chronic phase of the disease. The invasion stage, include the actual penetration of the definitive host by the cercariae and the following 2-7 days during which the cercariae migrate from the skin to the lungs (Cheesbrough, 1981). The phase is marked by a moderate to intense skin reactions called the “Swimmer’s itch” at the site of penetration depending on individual’s sensitivity to the parasites (Capron and Capron, 1994). Individuals in endemic areas show little or no reaction, while visitors develop marked skin rashes and urticaria (Clarke et al., 1970).

The acute phase starts 6-12 weeks after infection, and coincides with peak egg deposition and the characteristic pathological manifestation in this stage, in moderate and heavy infections is an acute febrile reaction often referred to as the “Katayama syndrome” (Clarke et al., 1970). The symptoms includes; fever, fatigue, urticaria and eosinophilia. The eggs produced induce an inflammatory reaction in the liver and walls of the colon and rectum, with
formation of granulomas where giant and epitheliod cells infiltrate around the egg and are held together by an extra cellular matrix (Farah et al., 1997). The clinical signs at this stage are; abdominal pain and tenderness and intermittent dysentery or diarrhoea (King and Mohmoud, 1998). The intestines may have serosal nodular lesions called “Sandy patches” or in severe cases polyps leading to pseudo-tumours or bilharziomas (Cheever and Andrade, 1967). There is also a remarkable enlargement of the mesenteric lymph nodes (Clarke et al., 1970).

Histologically, the acute granuloma in the liver is seen to consist of a centrally placed *Schistosoma* ovum surrounded by concentric layers of inflammatory cells, predominantly eosinophils (Moore et al., 1976; Lenzi et al., 1987). Lymphocytes, macrophages, plasma cells and neutrophils are also present in the granulomas. The chronic phase which is a sequel to modulation of the granulomatous inflammation, is characterised by reduction in liver granuloma size and diminished cellular infiltration particularly eosinophils. This stage is largely sub-clinical, but it contributes to long term debility associated with chronic schistosomiasis (Mitchinson and Oliviera, 1986).

In some cases of schistosomiasis, the granulomas may fail to modulate resulting in the hepatosplenic disease (Wyler and Telebian, 1997). This is the main cause of schistosomiasis related mortality. The reaction to the eggs in the liver may eventually cause the peri-portal fibrotic reaction known as “Symmers clay pipe stem fibrosis” which may lead to formation of varicose around the rectum, stomach and oesophagus (Andrade, 1987). In rare cases the
eggs may be lodged in the brain, spinal cord, lungs and the kidneys. Neurological schistosomiasis is due to eggs being trapped in the brain or spinal cord (Pittella and Lana-Peixoto, 1981), and renal schistosomiasis manifest as nephropathy or glomerulo-nephritis (Andrade, 1987). Death due to *S. mansoni* infection is as a result of haemato-emesis, portal hypertension and occasionally oesophageal varices burst to cause haemorrhage (Dessein, 2003; Dune, 2003).

1.7 Immune responses to *Schistosoma mansoni* infection

All stages of *S. mansoni* induce a very pronounced immunological response, with the more predominant being directed against the eggs (Omer-Ali *et al.*, 1988). The adult worms are largely unaffected due to development of evasive mechanisms which enable the worms to survive in the hostile environment of the host (Dunne, 2003). These evasive mechanisms of the adult parasite include induction of anti-inflammatory molecules (Ramaswamy and Kumar, 2000), incapacitation of lymphoid cell function (Angeli *et al.*, 2001), coating with the host’s surface antigens (Loukas *et al.*, 2001) and production of anti-oxidant enzymes among many other mechanisms (LoVerde *et al.*, 2004).

Schistosomula are most susceptible to immune attack both in-vitro and in experimental murine models. The surface of schistosomule has glycoprotein antigens that share carbohydrate epitopes with large polysaccharide antigens in the parasite egg. These antigens induce IgE and IgG dependent cellular cytotoxicity reactions involving eosinophils, macrophages, platelets and

Infection with *Schistosoma mansoni* has been shown to elicit both the cellular and humoral arms of the immune response. Cellular immune response to *S. mansoni* infection in baboons is largely of mixed Th1\Th2 phenotype, similar to the situation in the human hosts (Mola *et al.*, 1999). Studies to determine the role of antibodies in the immune response in baboons have demonstrated IgM as the predominant antibody in the primary response, while the secondary response is characterised by production of other classes specifically IgG and IgE (Nyindo *et al.*, 1999).

Cytokine levels peak during acute *S. mansoni* infection, but decline with chronic infection and become almost undetectable after treatment (Mola *et al.*, 1999). Re-infection after treatment induces two to three fold increases in soluble egg antigen (SEA) specific interleukin-4(IL-4), IL-5, IL-10, IL-2 and transforming growth factor β (TGF-β) production. SEA-induced gamma interferon production does not increase with re-infection after treatment. SEA induced TGF-β remains elevated as the infection become chronic and correlates with diminished hepatic granuloma size, implying its participation in the down-modulation (Mola *et al.*, 1999). SEA in baboons also induces lymphocyte proliferation during the acute phase of the disease.
1.8 Control of schistosomiasis

Five main approaches have been applied in an attempt to control schistosomiasis. These include control of vector snails, chemotherapy, improved sanitation, prevention of water contact and vaccination (Pearce, 2003). Molluscicides have been widely used to control snails, but this approach has several limitations. First, the chemicals are expensive and their application must be undertaken frequently. Some molluscicides are harmful for example sodium chlorphenate causes skin irritation and hence is dangerous to the handlers. Molluscicides also kill some of non-target beneficial molluscs and other organisms. These drawbacks have limited the use of molluscicides (Klump and Chu, 1987).

Biological methods have also been tried. Birds and large snails such as Marisa Carmuarietus and Tarebia granifera that feed on other snails have been used to control the vector snails (Yole et al., 1996). The other methods of control involves proper disposal of faecal and urinary waste in order to avoid contamination of the environment with S. mansoni ova (CairnCross and Feachem, 1983). The building of latrines would if practised ensure that this is achieved. But in practice, not every homestead in endemic areas has a latrine and in some cases the local population has cultural beliefs that are opposed to the practice (CairnCross and Feachem, 1983).

Chemotherapy has been the only successful method in controlling schistosomiasis. The drugs currently available for treatment of schistosomiasis
are praziquantel, metrifonate, oxamnaquine and artemisinins (Richter, 2003). Praziquantel is the drug of choice and all species that cause pathology in humans and other animals are susceptible to it (Anon., 1998). Apart from the original product, generic praziquantel is also available and because of this, the price of the drug has gone down drastically (Richter, 2003). The efficacy of oxamnaquine and praziquantel is comparable, with a slightly better efficacy for praziquantel. Resistance to oxamniquine has however been observed in Brazil (Lambertucci et al., 2000; Saconato and Atallah, 2000).

Although schistosomiasis control can be approached with praziquantel alone, the possibility of developing resistance to praziquantel must be taken into account. This drug is effective against adult blood flukes only and under vigorous selection in the laboratory, it has been possible to demonstrate that some schistosomes possess the genes that carry a lesser susceptibility to praziquantel. Reduced cure rates have also been observed recently in Senegal due to very high re-infection rates with a relatively high number of developing immature parasite not susceptible to praziquantel (Stelma et al., 1995; Anon., 1998; Kussel and Hagan, 1999; Chitsulo et al., 2000). Another problem is that in spite of recent reduction in price of praziquantel, the drug is still far from being available in the endemic areas especially in Africa and treatment does not also prevent re-infection (Richter, 2003; Kariuki and Farah, 2005). Due to these reasons, it is clear that the most feasible long-term solution to schistosomiasis control is a protective vaccine (Pearce, 2003).
1.9 Vaccines for schistosomiasis

Although chemotherapy, especially with praziquantel has been successful in controlling schistosomiasis, it has not been able to prevent re-infection, which can be as often as once a year (Bergquist, 1998; Pearce, 2003). There is thus a need for a complementary tool with effect for the longer term and cost efficient, notably a vaccine. Vaccination can be targeted towards the prevention of infection or the reduction of parasite fecundity. A reduction in worm numbers is the “gold standard” for anti-schistosome vaccine development, but as eggs are responsible for both pathology and transmission, a vaccine targeted at parasite fecundity and egg viability also appears to be entirely relevant (Capron et al., 2002). The effective vaccine would prevent the initial infection and might reduce egg granuloma associated pathology (McManus et al., 1998).

Recently, a significant effort has been made to develop a protective vaccine against schistosome infections, and several vaccine candidates have been identified (Ahmed et al., 2001; Da’dara et al., 2001; Zhang et al., 2001; Dupre et al., 2001; Capron et al., 2002), but as the efficacy of any of these against schistosomiasis remains uncertain, the identification and characterization of new anti-schistosomiasis vaccine molecules remains a priority (Hanem et al., 2006). The development of vaccine remains to be an important long-term and a challenging goal in the control of schistosomiasis (Hafalla et al., 1999).
Vaccination with irradiated cercariae consistently produces high levels of protection in experimental animals, but delivery problems, the need for a standardised product and safety considerations rule out this approach for use in human beings (Damain et al., 1984; Kariuki and Farah, 2005). However, many \textit{S. mansoni} antigens continue to be tested as possible vaccines. These vaccine candidates may not be the final answer to controlling schistosomiasis, but may provide measurable protection and since morbidity rather than sterile immunity is the target, only partially protective vaccines are required (Bergquist, 1985). Because pathology in schistosomiasis is directly correlated to the number of \textit{Schistosoma mansoni} eggs in the host, a vaccine can achieve its effect both by offsetting parasite entry and development, and by interfering with the production and delivery of eggs (Bergquist, 1998; Pearce, 2003).

Several studies of immunity in schistosomiasis have been carried out; both in experimental animal models and human beings, but immune correlates of resistance remain undefined (LoVerde, 1998). This is because the various animal models and human studies have provided conflicting evidence regarding the immunological factors responsible for resistance (Coulson, 1997). Despite these conflicting evidences, two paradigms have emerged from the various animal models of schistosomiasis studied to date. One paradigm is that the larval stages (schistosomula) are the most susceptible and the target for immune elimination (Smither, 1982; LoVerde, 1998). The schistosomula elimination involves a cellular response that is potentiated by cytokines and/or antibodies. The cells involved include monocytes, macrophages, eosinophils and platelets (LoVerde, 1998).
The second paradigm is that the adult worms once they reach the portal circulation seem to evade the engendered immune response through the evolution of several defence mechanisms (Smithers and Terry, 1969; Maizels et al., 1993; LoVerde, 1998). The cells involved in cytotoxic response against the schistosomula are thought to produce reactive oxygen species (ROS) such as superoxide anion and hydroxyl radicals (LoVerde, 1998) (Fig 4). The adult worms are able to evade immune elimination by expressing anti-oxidant enzymes Cu/Zn superoxide dismutase (SOD), Glutathione peroxidase (GPX) among other mechanisms (Callahan et al., 1988; Mei and LoVerde, 1995). Hong et al., (1992) and Mei and LoVerde, (1997) demonstrated that expression of these anti-oxidant enzymes is developmentally regulated such that the lowest gene expression and enzyme specific activity are in the larval stages and highest in adult worms, the least susceptible to immune elimination (Mei et al., 1996). The anti-oxidant enzymes protect an organism from ROS derived damage (James, 1994; LoVerde, 1998) (see Fig.5). These enzymes have been immuno-localized to the tegument and gut epithelia of adult worms, but not to the larval stages of the parasites (Mei and LoVerde, 1997).

To provide direct evidence that antioxidant enzymes are important in immune evasion and thus viable candidate vaccines. DNA vaccination strategies have been used to evaluate the efficacy of DNA constructs containing genes encoding *Schistosoma mansoni* Cu/Zn cytosolic superoxide dismutase (Sm-CT-SOD), Signal peptide-containing *S. mansoni* SOD (Sm-SP-SOD) and *S. mansoni* glutathione peroxidase (Sm-GPX) in *S. mansoni* infection in murine models. After use of different doses of plasmid cDNA constructs containing genes that
encode these enzymes, mice exhibited a significant level of worm burden reduction when challenged with *S. mansoni* cercariae, with Sm CT-SOD showing 54%, and Sm-GPX 43.4% protection respectively (Shalaby et al., 2003).

**Figure 4.** An attack of schistosome tegument by host eosinophil resulting in release of reactive oxygen species (ROS) capable of attacking the parasite apical membrane initiating a lipid peroxidation that sets off a chain reaction that results in the death of the parasite (LoVerde et al., 2004)
Figure 5. The role of schistosome antioxidant enzymes in evasion from host cellular attack. Antioxidant enzymes such as CT-SOD, SP-SOD and GPX which localize to the host-parasite interface are postulated to protect the adult worm from the released reactive oxygen species (ROS) (LoVerde et al., 2004).
1.10 DNA vaccination

DNA vaccination is a vaccine methodology which is attractive and novel immunization strategy against a wide range of infectious diseases and tumours (Hanem et al., 2006). Injection of plasmid DNA as vaccine was first demonstrated to be effective using influenza as the model, where DNA construct containing a gene encoding nucleoprotein (NP) induced cytotoxic T-lymphocytes (CTLs) and cross-strain protection of mice (Ulmer et al., 1993). The effectiveness of DNA vaccines against viruses, parasites and cancer cells has been demonstrated in animal models (LoVerde, 1998; Tuteja, 1999; Seder and Guranathan, 1999). The DNA immunization induces both antigen-specific cellular and humoral immune response (Ramsay et al., 1999; Alarcon et al., 1999; Guranathan et al., 2000).

DNA vaccination strategies include the incorporation of immuno-stimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, utilization of localization/secretory signals and utilization of appropriate delivery system (Garmory et al., 2003). The vaccines usually consist of plasmid vectors that contain DNA coding for a specific component of a disease-causing organism. The heterologous genes (transgenes) are inserted under control of a eukaryotic promoter, allowing protein expression in the mammalian cells (Davis, 1997). To optimize the efficacy of DNA vaccines an appropriate choice of plasmid vector should be used. The basic requirements for the backbone of DNA vector are; a eukaryotic promoter, a cloning site, a polyadenylation sequence, a selectable marker and a bacterial origin of replication (Guranathan et al., 1998).
With DNA vaccination, the actual production of immunizing proteins takes place in the vaccinated host. This eliminates any risk of infection that has been observed with some live and attenuated virus vaccines. Other advantages of DNA vaccines are; production of long lived immune responses, vaccines for multiple diseases can all be given in a single inoculation, all DNA vaccines can be produced using similar techniques, DNA vaccines are extremely stable and candidate DNA vaccine can be recovered from diseased tissues (Guranathan et al., 2000). DNA vaccination against schistosomiasis has recently been investigated using a panel of plasmids encoding Schistosoma antigenic proteins such as sjc 26 GST, Sj79 (Waine et al., 1997; Zhou et al., 1999a; Zhang et al., 2001), Sm-CT-SOD and Sm-GPX (Shalaby et al., 2003), S. japonicum paramyosin (Yang et al., 1995) and Sm 23, 28 GST from Schistosoma mansoni (Dupre et al., 1997).

1.11 Baboon as an ideal model in schistosomiasis studies

A number of animal species have been used as models to study the basic biology, immunology and pathogenesis of schistosomiasis (Farah et al., 2001). But the baboon is the most frequently used non-human primate in schistosomiasis research because of a multiplicity of qualities that make them more relevant models than rodents (Nyindo and Farah, 1999). Baboons are similar to humans in their anatomy, genetics and immunological responses (Villinger et al., 2001). They acquire natural infections and are highly susceptible to experimental infections (Farah et al., 1997). Baboons develop hepatic and intestinal pathology during the acute phase, modulate this pathology in chronic phase of the disease and acquire protective immunity as
do humans (Farah and Nyindo, 1996; Farah et al., 1997; Mola et al., 1999; Nyindo et al., 1999). In the wild, baboons show age-dependent prevalence of infection, with high rates of infection in juvenile baboons and young adults, a similar characteristic observed in humans (Fulford et al., 1999). Baboons are also abundant in Eastern Africa and are not an endangered species. They adapt readily in captivity and to changes in their environment, can give birth twice every 18 months and live up to 20 years and attain body weight of 20 kg (Farah et al., 2000). It is also easy to monitor the immune and disease progression in many different organs in individual baboons during the course of infection by performing some surgical manipulations (Mola et al., 1999).

Baboons acquire immunity on vaccination. For example, immunization with irradiated cercariae stimulates over 50% protection (Yole et al., 1996) which is associated with elevated serum levels of schistosome-specific IgG and IgE. In addition, vaccination with irradiated cercariae results in reduction of the intestinal pathology (Farah and Nyindo, 1996). Some protection to challenge infection following vaccination with recombinant antigens has also been reported in baboons (Soisson et al., 1993). The moderate size, possibility of repeated sampling, and ease of perfusion for recovery of adult worms make the baboon a good model for vaccine efficacy studies (Farah et al., 2000). Baboons can also sustain repeated schistosome infections without any untoward lesions such as portal-caval shunting (Sturrock et al., 1984) and with subsequent development of acquired immunity and increased resistance to re-infection. Such re-infection episodes are characterized by reduced adult worm burden and granulomatous inflammation (Mola et al., 1999) which, as in
humans, are associated with high serum levels of parasite specific IgE (Nyindo et al., 1999).

Baboons have also a few constraints limiting their use in schistosomiasis research, which include the high costs involved in trapping and maintaining them in captivity. There may also be variation in data from wild caught baboons due to their heterogeneous genetic background. Immunological reagents suitable for baboon work may not be currently available (Farah et al., 2001).

1.12 Statement of the problem

The severity of pathology in *S. mansoni* has direct correlation to the number of eggs in the host tissues. A vaccine may act by preventing parasite entry and development, or by interfering with the production and delivery of eggs (Hanem et al., 2006). The ability of *S. mansoni* Cu/Zn superoxide dismutase (Sm-CT-SOD) and glutathione peroxidase (Sm-GPX) as DNA vaccines to reduce worm numbers in murine models has been demonstrated (Shalaby et al., 2003; LoVerde et al., 2004). Whether this reduction in worm burden in murine models can also be replicated in humans and non-human primates has not been ascertained. The pathology of *S. mansoni* in animals vaccinated with genes encoding these antioxidant enzymes has also not been determined. Sm-filamin has also been demonstrated to induce protection against *S. mansoni* infection in murine model but not in primates such as baboons.
1.13 Justification of the study

The ability of DNA vaccines encoding *S. mansoni* anti-oxidant enzymes and filamin to consistently confer significant levels of protective immunity to *S. mansoni* infection in murine models have been demonstrated (LoVerde *et al*., 2004). But the parasite biology, pathology, immune response and immunity to *S. mansoni* infection in baboons differ significantly from the murine model of schistosomiasis and correlate better with the disease in humans. There is therefore a need to demonstrate whether the protection observed in mice can be reproduced in baboons as a prelude to human vaccine trials.

1.14 Research questions

i. Does vaccination with DNA constructs encoding *S. mansoni* anti-oxidant enzymes and filamin reduce adult worm numbers and hence eggs in stool?

ii. What is the clinico-pathology of *S. mansoni* infection in baboons vaccinated with these DNA vaccines?

1.15 Hypothesis

There is no difference in the clinico-pathology of *Schistosoma mansoni* infection in baboons vaccinated with genes encoding *S. mansoni* anti-oxidant enzymes and Sm-filamin and those not vaccinated.
1.16 Objectives of the study

1.16.1 Overall objective

To evaluate the effect of DNA vaccines containing constructs encoding *Schistosoma mansoni* antioxidant enzymes and Sm-filamin on the clinic-pathology in *S. mansoni* infected baboons (*Papio cynocephalus anubis*).

1.16.2 Specific objectives

i. To determine hemogram of baboons vaccinated with genes encoding antioxidant enzymes and filamin in baboons infected with *Schistosoma mansoni*.

ii. To determine the effects of these DNA vaccines on the worm burdens and number of eggs in stool.

iii. To determine the gross and histo-pathology of the liver and intestine of baboons vaccinated and then challenged with *S. mansoni* and compare the results with those from the control groups.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Experimental Animals

Twenty five (25) juvenile male and female olive baboons, *Papio cynocephalus anubis*, weighing 6-13 kgs captured from schistosome free area of Kenya were used in the study. The animals were quarantined for three months, during which period they were screened for common bacterial, viral diseases and intestinal helminths. In particular they were tested for tuberculin, according to the standard procedures at the Institute of Primate Research (IPR)-Karen, Kenya. Test for patent schistosomiasis infection was carried out by the Kato technique (Katz *et al.*, 1972) on samples of stool passed within a twenty four hour period. To further eliminate the possibility of prior exposure of animals to schistosomiasis, serum measurement of adult worm antigen-specific IgG was carried out as described for baboons (Nyindo *et al.*, 1999).

All the animals used in the study were Kato negative and had no prior exposure to schistosomiasis. The animals were then caged individually and fed on commercial monkey chow and supplemented with fresh fruits. Water was provided *ad-libitum*. All experimental procedures were reviewed and approved by the Institutional Scientific and Ethical Review committee at the Institute of Primate Research (IPR)-Karen, Kenya.
2.2 The parasites

*Schistosoma mansoni* eggs were obtained by homogenizing faecal matter of baboons chronically and experimentally infected with an isolate of *S. mansoni* recovered from the stool of an infected human patient. After a period of 30 minutes the supernatant was poured and water added to the homogenate. The settled debris was then put into a Petri-dish and exposed to light for the eggs to hatch into miracidia. By use of a dissecting microscope 4-6 miracidia were picked by a pipette and put into beakers each containing a single *Biomphalaria pfeifferi* snail for 30 minutes to facilitate infection. Five weeks after infecting the snails, they were able to shed cercariae when exposed to light for two hours. The cercariae shed by the infected snails were counted using a dissecting microscope and 800 cercariae were used to challenge each of the 25 olive baboons percutaneously, using the pouch method (Sturrock *et al.*, 1976). The baboons had been sedated by a mixture of ketamine/xylazine (Agrar Holland BV, Soest, The Netherlands) at a dose of 0.1mg/kg body weight. The infection rates were higher than 99% as determined by counting the remaining cercariae that did not penetrate.

2.3 Plasmid constructs

The DNA vaccines were obtained from the department of Microbiology and Immunology, School of Medicine and Biomedical Science, Witebsky Centre for microbial pathogenesis and immunology, State University of New York, USA. The vaccines were cDNA fragments containing the entire coding sequence of *Schistosoma mansoni* genes, cytosolic superoxide dismutase (CT-SOD), glutathione peroxidase (GPX) and filamin (Sm-filamin) cloned into the
expression vector pc DNA 1/AMP. A construct with a GPX cDNA cloned into the pc DNA/AMP vector in the reverse orientation (pc DNA/XPG) was used as placebo.

### 2.4 Vaccination of baboons

The baboons were divided randomly into five (5) groups of five animals each. Vaccination was performed in accordance with the Institute of Primate Research’s standard procedures. The animals were anaesthetised using ketamine and xylazine mixture prior to vaccination. The baboons in group one were given 500μg naked DNA vaccine pcDNA cytosolic superoxide dismutase (CT-SOD), group two animals received same amount of pcDNA glutathione peroxidase (GPX), third group was immunised with same amount of pcDNA filamin (Sm-filamin). The fourth group received also the same amount of pcDNA/XPG, and the fifth, the control group received normal saline (0.85% NaCl). The vaccines and normal saline were administered intramuscularly (IM) into the quadriceps using a 23 gauge needle and one ml syringe. The animals were then boosted at 4, 8 and 12 weeks with same amount of vaccine. Vaccination was carried out as depicted in Table 1.
Table 1: Vaccination schedule plan for the five experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vaccine</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; vaccination at week</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; vaccination at week</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; vaccination at week</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; vaccination at week</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CT-SOD</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>GPX</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Filamin</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>XPG</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Normal Saline</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

The animals were then rested for four weeks and challenged with 800 cercariae per animal percutaneously on the abdomen.

2.5 Haematological analysis

Blood was collected from each animal every two (2) weeks and thoroughly mixed with ethylene-diamine-tetra-acetic acid (EDTA) anticoagulant by repeated inversion of the containers. The blood was then diluted in Turks solution to a dilution of 1:20. All red blood cell (RBC) parameters; total red blood cells, haemoglobin (Hb) concentration, packed cell volume (PVC), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean capsular haemoglobin concentration (MCHC), and total white cell count were determined by use of Particle counter (PCE-170 ERMA Inc Tokyo, Japan). Differential count of white cells was done by preparing a thin blood film using
EDTA blood, fixed with absolute methanol for 5 minutes and then stained
with Giemsa stain. The film was air dried and the count done at 100 x
magnification. Relative white cell values were then expressed as percentages.
Total plasma protein was determined by use of a refractometer.

2.6 IgG antibody linked immunosorbent assay

Blood samples were collected and incubated at 25°C for 1-2 hours to allow for
coaugulation and then centrifuged at 1500rpm for 10 minutes. The sera were
then aliquoted and stored at -20°C. Specific antibody (IgG) levels to soluble
egg antigen (SEA) and soluble adult worm preparation (SWAP) in individual
sera were determined by enzyme linked immunosorbent assay (ELISA). A 96-
well immunosorp ELISA plate (Dynex) was coated with 50µl of either SEA or
SWAP at concentration of 2µg and 5µg, respectively. The antigens were
diluted from a stock of 1mg/ml in phosphate buffered saline (PBS) at pH 7.2.
After over night incubation at 4°C, the non-specific binding sites were blocked
with 100µl of 3% bovine serum albumin (BSA) and then incubated for 1 hour
at 37°C. Serial dilutions of serum were made from a dilution of 1:200 to a
dilution of 1:25,600 in 0.5% tween and 50µl of these dilutions added into each
well and incubated overnight at 4°C. The plate was then washed six times
using an automatic ELISA washer (MR 5000; Dyntech) and incubated with
50µl of Horseradish Peroxidase conjugated to mouse ant-monkey IgG per well
at a dilution of 1:2000 for one and half hour at 37°C. The wells were then
washed six times and 50µl of TMB Micro well Peroxidase substrate added
into each well. Absorbance was then measured as optical density using a Maxi
Kinetic Micro plate Reader at 630nm after 20 minutes.
2.7 Challenge infection of baboons

Four (4) weeks after the last boost vaccination, all the 25 baboons were anaesthetised using ketamine/xylazine mixture. Each animal was then placed on the operating table lying on its back and the limbs tied on the table. Using the cloth pegs to hold loose skin, the skin around the inguinal region was folded to create a pouch. Then 800 cercariae were transferred from the beaker to the pouch that had been created. To ensure that all cercariae were transferred, the beaker was rinsed several times with distilled water and the water used pipetted and added to the pouch. The cercariae were allowed to penetrate for 30 minutes and then water was pipetted back from the pouch to the beaker again rinsing the pouch several times. The number of cercariae that had not penetrated was counted using a dissecting microscope. In all cases the penetration was above 99% as determined by counting the remaining cercariae.

2.8 Determination of *Schistosoma mansoni* eggs in stool

The Kato thick smear technique was used to quantify eggs in stool. A 24 hours stool passed by each animal was collected weekly from the 4th week post-challenge infection, then weighed and mixed well. 2 grams of faecal material was taken and then forced through a 125μm mesh to remove fibrous material. Using a spatula, the stool sample was recovered from the back of the mesh and filled completely into the hole of the template (perforated plate containing 50mg capacity well), which was then placed over a microscope slide. The template was removed carefully from the slide and the 50mg stool specimen on the slide covered with a cellophane cover slip which had been soaked in a
50% glycerine malachite green solution for 24 hours before use. The slide was then inverted against a smooth surface and pressed down gently until the sample was spread uniformly over the whole cellophane cover slip. The slide was left for 24 hours in the dark before observing and counting the eggs under the microscope. To obtain the number of eggs per gram of stool sample, the number of eggs counted per slide was multiplied by 20 and then the number of eggs in a 24 hours faecal sample was obtained by multiplying the number of eggs per gram with the weight of 24 hours stool. This was to obtain the 24 hours egg output.

2.9 Perfusions of baboons

Perfusions were carried out using a modified Smithers and Terry (1969) method at ten weeks after challenge. The animals were sedated with a mixture of ketamine and xylazine as earlier described and placed on their backs on operating table. A blood sample was taken from the femoral vein. Using the same needle, 5000 units of heparin was injected through the same vein. This was followed by a final injection of 5-10ml pentobarbitone sodium, concentration 200mg/ml (Euthatal-May and Baker Ltd, England) at dose rate of 1ml/2kg body weight to kill the animal. Then the four limbs were tied to the operating table to make dissection easier.

A mid-line incision was made from the neck to the groin and the skin undermined to expose the ribs and abdominal muscles. The abdomen was opened and the rib cage cut open at the costo-chondral junctions leaving the
diaphragm intact. The inter-costal muscles were cut to allow for clamping of the aorta and the posterior vena cava. The abdominal aorta was clamped just before the bifurcation into iliac arteries and the posterior vena cava was clamped just before entering the heart. A small incision was made into the abdominal aorta just after it leaves the heart and a cannula which was connected to the perfusion equipment put in. Another incision was made into the hepatic portal vein. The perfusion fluid made up of 0.85% sodium chloride and 1.5% sodium citrate was introduced with the help of a perfusion pump into the abdominal aorta and collected at the hepatic portal vein having circulated through the mesenteric veins and the venous plexus of the urinary bladder. The worms present in these locations were collected in the fluid.

The fluid was sucked into a bell jar using a standard vacuum pump and then perfusion fluid container kept at 4°C until the foam had settled. The fluid was then gently strained through a 105µm mesh sieve and the container was rinsed with phosphate buffered saline (PBS) to ensure that no worms were left. Using a squirt bottle containing PBS, the worms were concentrated in one area of the sieve and transferred into urine glasses which were then kept on ice. With a tweezer, hair and fat tissue were removed and worms washed three times. Then using a dissecting microscope worms from each animal were counted.
2.10 Determination of gross and histohepato-intestinal pathology

After the animals were euthanized, the viscera of each animal, was exposed and the gross pathology of the liver, intestine and mesenteric lymph nodes evaluated. The liver and intestinal samples were taken and fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethyl-alcohol, cleared in xylene, impregnated and embedded in paraffin wax. The paraffin blocks of tissues were then sectioned and stained with haematoxylin and eosin. Paraffin blocks of liver were sectioned in perpendicular orientations to ensure adequate sampling. Serial sections of each liver were made to visualize as many granulomas as possible. All sections were searched completely and only granulomas containing an ovum at its centre were measured at 100× magnification using a calibrated ocular micrometer as previously described (Farah et al., 1997). Horizontal and vertical diameters of a granuloma were determined and the mean of the two taken as the granuloma size. A total of 10 granulomas were measured for each animal.

2.11 Data management and analysis

Data generated from the study was entered into Excel software and then exported to SPSS program version 11.0 for statistical analysis. Data were presented as Tables and Figures. Results were expressed as Means ± Standard deviation (SD) because most of the measured parameters were normally distributed. Differences between investigated groups (CT-SOD, GPX, Filamin, XPG and Normal saline) were assessed by ANOVA and post-ANOVA. Results were considered significant at P < 0.05.
CHAPTER THREE
RESULTS

The results obtained were categorised under the following headings; immunological findings, clinical, haematological, parasitological and pathological findings.

3.1 Immunological responses

This study evaluated whether vaccination of olive baboons with DNA vaccines was able to induce specific IgG antibody responses. Sera from the vaccinated baboons were analysed for specificity to soluble egg antigens (SEA) and soluble worm antigen preparation (SWAP) and for intensity of the specific response by ELISA. Generally, all vaccinated animals (pc-SOD, pc-GPX and pc-Filamin) had significantly higher total IgG responses ($P<0.05$) to SEA than the placebo (XPG) and saline control animals. However, there was no statistically significant difference within the vaccinated groups. In the case of SWAP, all animals showed little response initially, but later there was slightly higher increase in the vaccinated animals than the controls. This shows that the animals which were vaccinated responded and the response was increased by boost vaccination with DNA vaccines.

3.2 Clinical signs

All the animals in the study were clinically examined throughout the experimental period. The baboons in each group had good appetite before and after challenge infection with *S. mansoni* cercariae. Three (3) out of the 5 of
those vaccinated with pc-SOD (group 1) developed diarrhoea six weeks post infection (pi), with two baboons having bloody diarrhoea. Four of the baboons vaccinated with pc-GPX (group 2) also developed diarrhoea post-infection; two of them developed diarrhoea 6 weeks pi and the other two, 8 weeks post-challenge. Two baboons in this group had alopecic lesions on the skin.

Of those vaccinated with pcSm-filamin (group 3), four of them also had bloody diarrhoea post-infection, and one baboon in the group started to diarrhoea 6 weeks pi and three, 8 weeks pi. One animal in this group had also alopecic skin lesions. The same results were observed in the group vaccinated with the placebo (pc-XPG) (group 4), with two baboons in the group having diarrhoea from the 6th week pi and two others from the 8th week pi. One animal in the group had no significant clinical symptoms throughout the experimental period.

All baboons in the control group (normal saline injected) (group 5) had bloody diarrhoea post-infection. Four of them started having diarrhoea from the 6th week pi and one from the 8th week pi. The number of animals that developed diarrhoea post-infection in each group is shown in Figure 6. The temperatures and other clinical parameters for all experimental animals were within the normal range for baboons. The results on the mean temperatures of animals in each group are presented in Figure 7.
Figure 6: The number of baboons that developed diarrhoea in each group after vaccination with DNA vaccines and then challenged with *S. mansoni* cercariae.

Figure 7: The temperatures of baboons vaccinated and non-vaccinated at different sampling times before and after challenge with *S. mansoni*.

The weight of each animal in all groups was determined every two weeks. In all groups animals gained weight from the 1st up to the 17th week. The mean weight gain for each group was as follow; Group 1(CT-SOD); 0.78 kg, group
2 (GPX); 0.82 kg, group 3 (Filamin); 0.98 kg, group 4 (XPG); 0.76 kg and group 5 (normal saline); 1.036 kg. The mean gain in weight was calculated by subtracting the mean weight of the group at week zero from the mean weight at time of challenge infection. Group 5 had the highest weight gain before challenge infection and group 4 with the lowest weight gain. However, the difference in weight gain between the groups was not statistically significant by one-way ANOVA test (P>0.05). All groups showed reduction in mean weight after challenge infection (from the 17th week). The mean weight reduction for each group was as follow; Group 1(0.01 kg), group 2(0.15 kg), group 3 (0.27 kg), group 4(0.42 kg) and group 5(0.24 kg). The weight reduction was calculated by subtracting the mean weight at time of inoculation (challenge infection) from the weight at the last sampling point. Weight reduction among groups was statistically significant (P< 0.05). Group 1 had the least weight reduction, and group 4 with the greatest weight loss. Figure 8 shows the mean weight for each group at different sampling points.

![Figure 8: The mean weights of baboons at different sampling times.](image-url)
3.3 Haematological parameters

3.3.1 Leukocyte counts

Total white blood cells (WBC) and differential leukocyte counts were determined at two weeks interval from the first week up to the time of perfusion. Figure 9 shows the white blood cell values for each group. Generally, the total white blood cell counts for all groups were within the normal range for baboons (see Appendix 1). The total WBC values were highest at sampling point 6 for pc-SOD and pc-GPX, which was 2 weeks after the second boost vaccination, with pc-GPX having the highest WBC count at this sampling point. White blood cell values were generally high for baboons vaccinated with DNA vaccine pc-GPX throughout the experimental period. WBC counts for all groups declined at sampling point 9, which was 2 weeks pi and then increased at point 11, six weeks post challenge (pi). The change in WBC counts within and between groups, however was not statistically significant (P>0.05) before and after challenge infection.

Figure 9: The total WBC counts in baboons vaccinated with DNA vaccines at different time post vaccination and post challenge.
Leukocyte differential counts were also performed and the following results obtained. Neutrophil counts for pcCT-SOD, GPX, filamin, pc-XPG and normal saline are presented in figure 10. All the baboons in the groups had their neutrophil values within the normal range for baboons at all sampling points (Appendix 1). However, the neutrophil counts were averagely high in baboons vaccinated with the placebo (pc-XPG) than the rest. Baboons vaccinated with pc-filamin had on average the lowest neutrophil counts throughout the sampling period. The neutrophil counts for all groups declined at sampling point 6, which was 2 weeks after the second boost vaccination and also at point 9, two weeks post challenge. The neutrophil values within the group and between groups were found not to be statistically significant by one-way ANOVA test (P>0.05).

![Graph of neutrophil counts](image)

**Figure 10:** The relative neutrophil counts in DNA vaccinated baboons and non-vaccinated ones at different sampling times after vaccination and post challenge.

Figure 11 presents eosinophil counts for all experimental groups. Values of eosinophil counts were within the normal range (1-4%) before challenge,
though pcCT-SOD vaccinated baboons had high eosinophil counts than other animals at this stage (before challenge). After challenge infection with *S. mansoni*, all animals showed a consistent increase in eosinophil counts reaching peak at different sampling points post-infection for different groups and then declining to normal values at the last sampling point. The post-infection increase in eosinophil counts was however not statistically significant between groups (*P* > 0.05). Baboons that were vaccinated with pc-Sm-filamin had the greatest variation with values ranging from 1-8% and the highest count being observed at sampling point 11, which was the sixth week post-infection. Animals vaccinated with pcCT-SOD also had high eosinophil counts, with peak at 6% at 12th sampling point (8th week pi).

![Figure 11: The relative eosinophil counts in baboons after vaccination and post challenge at various sampling times. There was eosinophilia post challenge (pi).](image)

Lymphocyte counts were also determined, and the counts are presented in Figure 12. Baboons vaccinated with pc-GPX had consistently high levels of lymphocytes compared to the other animals except at sampling point 8, when the
value dropped to below the baseline level. Baboons given pc-XPG had the lowest counts of lymphocytes with values falling below the baseline value at some sampling points. Lymphocyte values for those vaccinated with pcCT-SOD, Sm-filamin and those in the control group were basically within the normal range (Appendix 1) and above the baseline value throughout the study period. Analysis of the lymphocyte counts between groups by one-way ANOVA test showed that they were not statistically significant (P>0.05).

![Graph showing relative lymphocyte counts in baboons vaccinated with DNA vaccines and non-vaccinated ones at different sampling times during the study.](image)

**Figure 12:** The relative lymphocyte counts in baboons vaccinated with DNA vaccines and non-vaccinated ones at different sampling times during the study.

### 3.3.2 Red blood cell parameters

*Schistosoma mansoni* infection causes anaemia and due to this fact, it was necessary to determine the following red blood cell parameters in the experimental animals. The total red blood cell counts, haemoglobin concentration, packed cell volume, mean cell haemoglobin concentration and mean cell volume were determined at all sampling points. Results for mean total RBC counts, haemoglobin (Hb), packed cell volume (PVC) and mean cell volume (MCV) for vaccinated baboons and control are presented in
Figures 13, 14, 15 and 16. Total RBC counts were consistent throughout the study period for all animals. The values increased two weeks after the last boost vaccination and declined from the second to fourth week after challenge infection, but increased again at the next two sampling points and dropped at the last sampling point. The mean total RBC counts were within the normal range for olive baboons for all experimental groups at all sampling points (Appendix 1). Other red blood cell parameters, HB, PCV and MCV were also within the normal ranges throughout the experimental period (see Appendix 1). The mean differences in red cell parameters among all the groups were found to be statistically not significant when subjected to one-way ANOVA test (P>0.05).

Figure 13: The total red blood cell counts in baboons at different sampling times post vaccination and challenge.
Figure 14: The haemoglobin levels in baboons vaccinated with DNA vaccines and saline at different sampling times after vaccination and infection.

Figure 15: The PCV values for baboons vaccinated with DNA vaccines and saline at different sampling times post-vaccination and after challenge.
3.3.3 Total plasma protein concentration

Mean total protein concentrations for the experimental groups were also determined and are presented in Figure 17. Total plasma protein concentration values were also consistent throughout at all sampling points for all animals in all groups and were within the normal range for olive baboons (see Appendix 1). The mean differences in plasma protein concentration among groups were also found to be statistically not significant (P>0.05) at all sampling times.
3.4 Parasitological findings

To determine the efficacy of DNA vaccination with constructs containing CT-SOD, GPX, Sm-filamin and XPG, vaccinated and the control baboons were challenged with 800 *S. mansoni* cercariae each and the number of adult worms recovered at perfusion (which was 10 weeks after challenge infection) determined. The number of eggs in a 24 hour stool was also determined for each animal from the 4th week post-challenge at weekly interval up to the 9th week, which was one week before perfusion. At perfusion, male and female adult worms recovered were counted and the results expressed as mean male, female and total (male and female) worms for each group. The results are presented in Figure 18 below.

Figure 17: The total plasma protein in baboons vaccinated with DNA vaccines and those injected with normal saline at different sampling times post vaccination and post challenge.
Figure 18: The mean adult worm burden of *S. mansoni* recovered at perfusion from baboons vaccinated with pcCT-SOD, pcGPX, Sm-filamin, XPG and normal saline

At perfusion, the mean worm burden of *S. mansoni* recovered from group CT-SOD was 283, GPX, 211, filamin, 251, XPG, 222 and saline, 228. The male and female worm number for each group was as follows: CT-SOD had 153 males and 130 females, GPX, 116 males and 95 females, Sm-filamin, 126 males and 125 females, pc-XPG, 115 males and 107 females, and saline (control) had equal number of males and females, which was 114 each. The CT-SOD group had more worm numbers than the other groups including the control groups. The mean worm burden for GPX was the lowest. However, the difference in mean worm counts between each of the vaccinated groups (CT-SOD, GPX, filamin and XPG) and that of the control (normal saline) was statistically not significant as shown by one-way ANOVA test (P>0.05).

The number of eggs in stool in each group at all sampling points post-infection is presented in Figure 19. Four weeks post-challenge there were no eggs in
stool. The eggs started appearing in stool from the fifth week after challenge infection and increased steadily in all groups except in the control (normal saline) group, where the eggs declined at 5th sampling point, which was 8 weeks pi but then shot to the highest count at the last sampling point. Groups 1, 2 and 4 had fewer eggs in stool, but group 3 had generally the highest numbers of eggs in stool. Group 5 had the highest mean egg counts at the last sampling point. The difference was found to be statistically significant (P<0.05).

Figure 19: The mean number of eggs collected at different sampling times from DNA vaccinated and normal saline injected baboons.
3.5 Pathological findings

The pathological findings in this study were divided into gross and histopathology.

3.5.1 Gross pathology

At perfusion, gross pathological examination of the liver, small and large intestines and mesenteric lymph nodes was done for all animals in the five groups. The pathology was categorised as few, moderate, numerous and severe granulomas on the surface of liver and large intestine. In group A (vaccinated with pcCT-SOD), two baboons had livers with numerous granulomas, the other three had moderate liver granulomas. The same animals in the group had severe large intestinal granulomas. Two with moderate and one had few large intestine granulomas. Granulomas appeared as raised pinhead sized foci distributed over the surface of the liver lobes and intestine. The livers from all groups were friable, oedematous, with rounded edges, indicating acute inflammation. All animals in the groups had no significant lesions in the small intestine. Mesenteric lymph nodes were enlarged in four baboons in the group CT-SOD and one baboon in the group had moderately enlarged mesenteric lymph nodes.

Group 2 animals (vaccinated with pc-GPX), four of them showed moderate liver granulomas and one with numerous granulomas. One with numerous and one other with moderate liver granulomas had large intestine with few granulomas, the remaining three baboons in the group had moderate granulomas on the large intestines. The small intestine of one baboon was
congested with red blood cells, but the rest had no significant lesions in the small intestine. One had normal mesenteric lymph nodes; three others had enlarged mesenteric lymph nodes (MLN) and one with moderately enlarged MLNs.

Group 3 which was vaccinated with Sm-filamin, had three baboons with moderate granulomas distributed over the surface of all the liver lobes, one with severe liver granulomas and one had numerous raised pinhead-sized foci on the surface of the liver. Two baboons had large intestine with few granulomas, two others had severe large intestine granulomas and one had moderate granulomas over the surface of large intestine. For the small intestine, one animal had enteritis but the rest had no lesions. All baboons in the group had enlarged mesenteric lymph nodes (MLNs).

In group 4 (those vaccinated with pc-XPG), three baboons in the group had numerous granulomas on the surface of the liver and two animals with severe liver granulomas. Two baboons had few calcified granulomas on the large intestine, one baboon in the group had few scattered granulomas, one with severe granulomas over the large intestine and one other had numerous granulomas. The small intestines of all baboons had no significant lesions. MLNs were moderately enlarged in two animals in the group; two others had enlarged mesenteric lymph nodes and one with much enlarged lymph nodes.

The control group 5 had three baboons with numerous granulomas over the surface of their liver lobes. One had severe granulomas and one other with
moderate granulomas on the liver lobes. Large intestine of two baboons in the group had severe granulomas. Two other baboons in the group had numerous granulomas and one with moderate granulomas on the large intestine. One baboon, had enteritis in the small intestine and the rest had no lesions in the small intestine. The mesenteric lymph nodes (MLNs) were very enlarged in one, enlarged in two animals, and moderately enlarged in other two. The gross pathology in the livers and large intestines was almost similar in all groups, with baboons in groups, CT-SOD, pc-XPG and normal saline having slightly severe gross pathology.

3.5.2 Histo-pathology of the livers and intestines

The egg granuloma size in the liver of each baboon was determined by calculating the mean granuloma diameters by taking the average measurement of horizontal and vertical diameters. A total of 10 granulomas were measured for each baboon. The mean granuloma sizes for all the groups were similar (Table 2).

Table 2: The mean liver granuloma sizes in μm.

<table>
<thead>
<tr>
<th>VACCINE</th>
<th>GRANULOMA SIZE (μm)</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-SOD</td>
<td>268.24±37</td>
<td></td>
</tr>
<tr>
<td>GPX</td>
<td>254.54±29</td>
<td></td>
</tr>
<tr>
<td>FILAMIN</td>
<td>254.80±43</td>
<td></td>
</tr>
<tr>
<td>XPG</td>
<td>268.50±52</td>
<td></td>
</tr>
<tr>
<td>NORMAL SALINE</td>
<td>315.40±68</td>
<td></td>
</tr>
</tbody>
</table>
Though the mean granuloma size for the control baboons was higher than vaccinated animals, One-way ANOVA test showed that the difference in mean granuloma size among the groups was not statistically significant (P>0.05).

Histological examination of the livers of the baboons showed one animal in group 1 (CT-SOD) had few granulomas; the rest of the baboons in the group had many granulomas. Sm-GPX and pcXPG groups all had many granulomas in the liver. Groups 3 and 5 (Sm-filamin and the control group (normal saline)) had two animals each with few granulomas, but the other baboons in both groups had many egg- granulomas in the liver.

The egg-granulomas in all animals were mostly acute and contained mostly eosinophils. Other cells, lymphocytes, neutrophils, macrophages and few plasma cells were also present. The livers also had modulating and resolving granulomas that consisted of a degenerated schistosome ovum surrounded by multinucleated Langerhan’s giant cells, macrophages, lymphocytes, epithelial cells and a few eosinophils. Granulomas observed had minimal amount of fibrosis surrounding them. The acute liver egg- granulomas of vaccinated and control baboons are shown in Figures 20a, 21a, 22a, 23a and 24a.

The histo-pathology of the colon of baboons was as follow; all animals except one baboon in group 3 (Sm-filamin) had smooth muscular layer in the colon thickened, atrophied villous, hyperplasia of crypts, and lamina propria infiltrated with lymphocytes and polymorph-nuclear cells. All baboons in group 1, (pc-SOD) had many ova/granulomas in the large intestinal wall. One
baboon had ulcerated mucosa of the colon. Group 2 (pc-GPX) had similar pathology in the colon except for one baboon in the group, which had few granulomas in the colon. One baboon also had ulcerated mucosa.

Group 3 (Sm-filamin) baboons also showed almost similar histo-pathology in the colon to groups 1 and 2, except one baboon that had very mild pathological changes with no ova/granulomas in the wall of its colon. Two baboons in the group had ulcerated mucosa. Group 4 (pc-XPG), three baboons had same pathology as group 1 and 2, but two animals had mild histo-pathology, with few granulomas/ova in the colon. One animal also had ulcerated mucosa. In group 5 (normal saline), three baboons had ulcerated mucosa in the colon in addition to having the similar pathology as that of groups 1 and 2.

Histo-pathology of the colon from various experimental animals is shown in Figures 20b, 21b, 22b, 23b and 24b. The histo-pathological changes in small intestine in all experimental baboons were not significant.
Figure 20: Liver and colon of a baboon vaccinated with CT-SOD and challenged.

A: Liver showing egg-granuloma formed around remnant of an ovum, with mononuclear inflammatory cells and eosinophils, neutrophils and histiocytes.

B: The colon showing infiltration with inflammatory cells and presence of ova. (Haematoxylin and Eosin stain, 100 x magnification).
Figure 21: The liver and colon of a baboon vaccinated with GPX. 
A: Section of liver showing a granuloma with an ovum surrounded by large number of inflammatory cells. Stained with H/E
B: The colon showing *Schistosoma* eggs and inflammatory cells. (Haematoxylin and Eosin stain, 100 x magnification)
Figure 22: Liver and colon of baboon vaccinated with Sm-filamin. 
A: Section of liver showing a granuloma with inflammatory cells. 
B: The colon with ova and infiltrated with inflammatory cells. 
(Haematoxylin and Eosin stain, 100 x magnification)
Figure 23: The liver and colon of baboon vaccinated with pc-XPG.
A: Liver section showing a granuloma surrounded with inflammatory cells.
B: The colon showing an ovum and infiltrated with inflammatory cells.
(Haematoxylin and Eosin stain, 100 x magnification)
Figure 24: Liver and colon of baboon injected with normal saline (0.85% NaCl).  
**A:** Section of liver showing a large granuloma with inflammatory cells.  
**B:** Section of colon with *S. mansoni* eggs and infiltrated with inflammatory cells.  
(Haematoxylin and Eosin stain, 100x magnification)
CHAPTER FOUR
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

In this study, the aim was to determine if the protection observed in mice can be reproduced in baboons as a prelude to human vaccine trials. The study evaluated antibody response, haematological values, egg output, worm burdens and the pathology in the liver and intestines of baboons vaccinated with DNA vaccines encoding \textit{S. mansoni} antioxidant enzymes and Sm-filamin, and challenged with \textit{S. mansoni} cercariae, and then compared with the control non-vaccinated but Sm-cercariae challenged baboons. The results obtained from this study were discussed at five levels, immunological, clinical, haematological, parasitological and pathological.

The first thing in this study was to ascertain whether the olive baboons (\textit{Papio anubis}) responded to injected DNA vaccines and also to determine the level of that response. For these reasons, sera from vaccinated baboons were analysed for specificity to soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) and for intensity of the response by ELISA pre- and post-challenge. This study hypothesized that IgG levels would be elevated in vaccinated baboons after vaccination and challenge infection. The serum IgG was determined at different sampling points after initial vaccination and during the boost vaccinations, and post-challenge. Results showed a highly significant and consistent increase in IgG response to SEA but mild to SWAP in vaccinated baboons (pc-SOD, pc-GPX and pc-Sm-filamin) than in the control and placebo (pc-XPG) baboons. This proved that the animals
responded to the DNA vaccines and the level of response increased with boost immunization and challenge infection. The results support the previous studies which have shown that eosinophil mediated killing occurs in the presence of IgG antibodies and also play a role in secondary responses to *S. mansoni* infection.

Data obtained demonstrated that vaccination with DNA vaccines (pc-SOD, pc-GPX and pc-filamin) elevates IgG levels in vaccinated olive baboons with an increase in IgG levels specific to SEA and SWAP, and being enhanced following challenge, suggesting a boost response, which follow the introduction of the parasites. The boosting response has also been observed after challenge infection in murine models immunized with DNA encoding Sm 28 GST, Sjc 26 GST and pcDNA-Sm 27.7 (Dupre *et al.*, 1997; Mohamed *et al.*, 1998; Ahmed *et al.*, 2001; Hanem *et al.*, 2006). The IgG response to SEA was higher than the one elicited by SWAP in all vaccinated groups, and this was because the samples assayed were from acute phase of the infection. This was consistent with available data that show that response in acute phase is mainly directed against the eggs (Farah *et al.*, 1997).

The present study examined the effects of different DNA vaccines on clinical outcomes in baboons (*Papio cynocephalus anubis*) before and after infection. In this study it was assumed that vaccination with genes encoding *S. mansoni* anti-oxidant enzymes and filamin cause no adverse effects in baboons and protect the animals against *S. mansoni* infection resulting in less or mild clinical disease. Diarrhoea was observed in animals in all experimental groups,
it's a common finding in moderate and heavy *S. mansoni* infections. It is caused by the pathology that occurs in the intestine due to immunological response to worm eggs in the intestinal wall. Because diarrhoea occurred in all groups, the vaccinated and non-vaccinated ones, this shows that the DNA vaccines containing genes encoding *S. mansoni* anti-oxidant enzymes and filamin did not prevent intestinal pathology due to *S. mansoni* infection, hence they were not protective. Those animals that did not come down with diarrhoea must have been protected by other mechanisms not induced by vaccination or the disease was still in acute phase and much of the large intestinal pathology had not developed.

All animals gained weight during vaccination period and their growth and development was normal. This is an indication that the vaccines had no adverse effects in the animals. The results are similar to others obtained by other researchers. DNA vaccines have been found to be safe in animal models and human beings (Garmory *et al.*, 2003; LoVerde *et al.*, 2004). But after challenge infection there were reduction in weight of animals in all groups. The reductions were statistically significant (P<0.05). Those vaccinated with SOD and GPX had least reduction in weight, but XPG group had the greatest weight loss. Less weight loss in SOD and GPX vaccinated animals pi may have been caused by the vaccines inducing production of IL-4, which have been shown to prevent development of a severe, lethal inflammatory condition in which the infected animal become cachectic prior to death (Brunet *et al.*, 1997; Fallon *et al.*, 2000) or the vaccines were able to reduce the stress caused by the infection. This observation indicates that these two DNA vaccines
induced some protection, but need to be investigated further to ascertain if it is true and also to be able to explain the mechanism behind it.

Appetite was good in all groups throughout the experimental period. The body temperatures of the baboons were also within the normal range for baboons (normal range 36-39°C) after initial and during boost vaccination and also after challenge infection with *S. mansoni* cercariae. These results differ from other reports which have documented acute disease characterised by anorexia and hyperpyrexia in baboons infected with *S. mansoni* (Sturrock *et al.*, 1976; Sturrock *et al.*, 1984; Damian *et al.*, 1992; Farah *et al.*, 1997). The possible explanation to these observations in this study may be that the dose of *S. mansoni* cercariae used for challenge infection was low to cause anorexia and hyperpyrexia. Anorexia and hyperpyrexia were observed in cases of heavy infestations with *S. mansoni* in children in Uganda, those that had mild infection had no fever nor were they anorexic (Koukounari *et al.*, 2006).

It was also assumed that vaccination with genes encoding anti-oxidant enzymes, Sm-CT-SOD and Sm-GPX, and Sm-filamin, and infection with *S. mansoni* would bring about changes in haematological values. All red blood cell indices such as red blood cell counts, packed cell volume (PCV), haemoglobin (Hb), mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC) were within the normal ranges for baboons. Total plasma protein, total leukocyte counts and differential leukocyte counts were also within the normal ranges (see Appendix 1).
The difference in red blood cell parameters among different groups at various sampling times pre- and post-challenge was not statistically significant (P>0.05). The red cell values were generally unaltered compared to the baseline values. These findings indicated that vaccination with genes encoding anti-oxidant \textit{S. mansoni} enzymes and Sm-filamin and (pc-XPG), and challenge infection with \textit{S. mansoni} did not change these parameters. Anaemia has been found with \textit{S. mansoni} infection when there are associated complications, such as hepatosplenomegally (Woodruff, 1966; Anon., 1990).

The results in this study are in agreement with other studies, where it has been found that in uncomplicated schistosomiasis, anaemia is not a feature and is only associated with the chronic stage of the disease and hypergammaglobulinemia (Mousa, 1967; Nagi \textit{et al.}, 1999). The other explanation to this observation in this study may be that the challenge infection was not heavy. In the same study in Uganda referred to above, anaemia was observed in children with heavy \textit{S. mansoni} infections (Koukounari \textit{et al.}, 2006). The potential mechanisms underlying a relationship between schistosomiasis and anaemia are unclear. But there are four possible mechanisms that might mediate this relationship, based on animal and human models. These include, iron deficiency caused by extra-corporal loss of iron, splenomegally leading to red blood cell sequestration, autoimmune haemolysis and anaemia of inflammation and chronic disease (Jennifer \textit{et al.}, 2005). Therefore, from the results obtained it can be stated categorically that the DNA vaccines used in this study did not prevent the anaemia that was not observed in the vaccinated and \textit{S. mansoni} challenged olive baboons.
In this study the eosinophil values were within the normal range (1-4%) in all groups before challenge infection, although pcCT-SOD vaccinated animals had generally high values than the other groups. However, after challenge infection, all experimental groups showed a consistent increase in eosinophil counts reaching peak at different sampling points post-challenge for different groups and then declining to normal values at the last sampling point. These findings are in agreement with observations by other researchers (Rahma and El-Sheikh, 1985; Nagi et al., 1999; Pearce and MacDonald, 2002). Eosinophilia is a common haematological finding in schistosomiasis, with marked leukocytosis and absolute eosinophilia being a feature of acute schistosomiasis (Rahma and El-Sheikh, 1985). The results on eosinophils demonstrated their role as effectors cells in *S. mansoni* infection.

Eosinophils are involved in killing of schistosomula and adult worms. The mediated killing of the parasites by eosinophils occurs in the presence of IgG antibodies and/ or cytokines (Pearce and MacDonald, 2002). The antibodies are specific to antigens present on the worm surface. Immune complexes formed between IgGs and antigens on the surface of the worm render eosinophils cytotoxic (Dupre *et al.*, 1997). After adherence to the surface of the worm, eosinophils flatten out to make intimate contact with the worm. The secretion granules of eosinophils accumulate adjacent to the point of contact and fuse to form vacuoles. Eventually the vacuoles fuse with the cell membrane and their contents are released into the worm. The secretory granules contain a variety of factors including phospholipase B and reactive oxygen species (Capron *et al.*, 1979).
As part of the cell cytotoxic response against the schistosomula, it is thought that reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals are released by the host eosinophils (LoVerde., 1998; LoVerde et al., 2004). The released reactive oxygen species (ROS) are capable of attacking the parasite apical membrane and initiating a lipid per-oxidation that sets off a chain of reactions resulting in death of the parasite (see Fig. 4). However, the adult worms are able to protect themselves against oxidant damage by producing anti-oxidant enzymes (Cu/Zn superoxide dismutase, CT-SOD and glutathione peroxidase, GPX) (see Fig. 5). The anti-oxidant enzymes are at the lowest levels in schistosomula, the stage which is most susceptible to immune killing, and highest in adult worms, the least susceptible (Hong et al., 1992; Maizels et al., 1993; Mei and LoVerde, 1995, 1997; LoVerde, 1998). Anti-oxidant enzymes are strategically located at the host-parasite interface (worm tegument) so as to offer optimal protection of adult worms against the reactive oxygen species produced by the eosinophils (LoVerde et al., 2004).

The pathogenesis due to schistosomiasis is majorly as a result of eggs produced by adult worm pairs that inhabit the portal circulation (Kariuki and Farah, 2005). The severity of the disease is a function of the worm burden (number of egg laying adult worms) and consequently, the inflammatory response to deposited eggs (Butterworth et al., 1992). A vaccine can either be targeted towards the preventing of infection or to the reduction of parasite fecundity. A reduction in worm numbers is the gold standard for anti-schistosome vaccine development, but as schistosome eggs are responsible for
both pathology and transmission, a vaccine targeted at parasite fecundity and egg viability also appears to be particularly relevant (Hanem et al., 2006).

In this study, the egg output and worm counts were determined in baboons vaccinated with genes encoding *S. mansoni* anti-oxidant enzymes and Sm-filamin and in the controls (XPG and normal saline injected baboons). This was the most direct way of determining the efficacy of these anti-schistosome vaccines. The difference in mean egg output between the groups was statistically significant (P< 0.05). The groups vaccinated with SOD, GPX and XPG had fewer eggs in stool than those vaccinated with Sm-filamin and normal saline. This implies that vaccination of olive baboons with genes encoding *S. mansoni* anti-oxidant enzymes, cytosolic superoxide dismutase (CT-SOD) and glutathione peroxidase (GPX) was able to reduce the egg output. These vaccines (SOD and GPX) may have an effect on female parasite fecundity (anti-fecundity effects). The results are similar to others obtained from other studies using other vaccine candidates. *Sm*-Glutathione-S-transferase (SmGST) and *Sh*-Glutathione-S-transferase (ShGST) have shown to induce immune responses that have anti-fecundity effects against female schistosome (Capron et al., 2001). The results on worm burdens were not statistically significant among groups (P>0.05). These results differ from the ones obtained in mice by Shalaby et al., (2003), where there was reduction of worm burden by CT-SOD and Sm-GPX of 54% and 43.4% respectively. These observations may suggest or indicate that the immunity to *S. mansoni* infection in murine models is different from that of primates. Whereas these
DNA vaccines induce protective immunity in murine models, the same did not occur in baboons (*Papio cynocephalus anubis*).

The gross pathology in the liver and large intestine of those olive baboons vaccinated and non-vaccinated and then challenged with *S. mansoni* cercariae was almost similar. These results, further show that the vaccines used in this study were unable to protect the animals against *S. mansoni* infection, because the gross pathology in vaccinated and the control animals was the same.

In schistosomiasis, morbidity is caused by the granulomatous reaction due to *Schistosoma* eggs lodged in the liver parenchyma and in the intestines (Farah *et al.*, 1997). The *Schistosoma* eggs release soluble proteins and glyco-proteins that are thought to be responsible for granuloma formation and other egg induced reactions (Boros, 1989). By surrounding the egg, the granuloma essentially segregates the egg from the hepatic tissue and allows continuing liver function. Mice lacking CD4 cells have been shown to be incapable of making granulomas and die due to the toxic effects on hepatocytes of certain egg proteins (Dunne and Doenhoff, 1983; Amiri *et al.*, 1992). The granuloma size is an indicator of morbidity of schistosomiasis. Larger lesions (granulomas) are detrimental and small ones ideal. Small lesions are due to compromise between egg sequestration and tissue pathology (Brunet, 1998).

The formation of granulomas around the schistosome eggs in the liver and in the intestine is the major cause of the pathology in schistosome infections. The
live miracidium within each egg secrete soluble antigenic materials through ultra microscopic pores in the eggshell (Boros and Warren, 1970). These antigens induce granuloma formation and this response has been extensively studied in murine models of schistosomiasis and initially show a coordinated influx of lymphocytes, epithelioid giant cells, macrophages, neutrophils, mast cells, fibroblasts and numerous eosinophils around eggs trapped in tissue of infected animals (Moore et al., 1976). Granulomas are thought to offer protection to the host, as they wall-off toxic egg products, such as hepatotoxic antigen Omega-1, which would otherwise kill the host and consequently the parasites too (Dunne et al., 1992). *Schistosoma mansoni* egg deposition begins in baboons at about 4 to 5 weeks post-infection with the first detectable granulomas present by about 6 weeks post-infection (Njenga et al., 1998). In this study, eggs were first detected in faecal samples from the 5th week post-challenge in all animals (vaccinated and non-vaccinated). An effective vaccine would prevent the initial infection and may reduce egg granuloma associated pathology (McManus et al., 1998). In this study, hepatic granulomas sizes were measured after perfusion. Although the mean hepatic granuloma diameter for control group was higher than the vaccinated and placebo group (pcXPG), one way ANOVA test showed that the difference in mean granuloma size among the groups was not statistically significant (P>0.05). The numbers of hepatic granulomas in all experimental animals were also not different. The granulomas were mostly acute with high infiltration of eosinophils. Few modulating and resolving granulomas were also present. The results show that the vaccines did not prevent or minimize
the liver pathology or protect the animals against the *S. mansoni* infection, because if they were protective, then the liver granulomas in vaccinated baboons would have been smaller and fewer than the ones in the control animals. These results differ from the results obtained in Egypt, in mice that were vaccinated with gene encoding SM 21.7 protein. This gene was able to protect the mice against *S. mansoni* infection as analysed parasitologically and pathologically (Hanem et al., 2006).

In general, the large intestine histopathology was also similar in both vaccinated and non-vaccinated baboons. This implies further that vaccination with genes encoding antioxidant enzymes and Sm-filamin did not prevent or minimize hepatic and intestinal pathology due to *S. mansoni* infection in olive baboons and therefore, did not protect these animals against *S. mansoni* infection.

### 4.2 Conclusions

1. Vaccination of baboons with genes encoding *S. mansoni* antioxidant enzymes, CT-SOD and GPX, and filamin induced production of specific IgG against soluble egg antigen (SEA), but the response to soluble worm antigen preparation (SWAP) was mild.

2. These DNA vaccines did not alter haematological parameters or cause adverse clinical effects in the experimental animals. Hence indicating that these vaccines are safe for use in animal models.
3. All baboons in all groups gained weight before challenge infection with *S. mansoni* cercariae. They reduced in weight post-infection, but those vaccinated with SOD and GPX had the least weight reduction. May be these vaccines were able to induce production of IL-4, which prevent vaccinated animals from becoming cachectic.

4. The schistosome egg output was highest in normal saline injected and Sm-filamin vaccinated baboons, but lowest in SOD, GPX and XPG vaccinated baboons.

5. Worm burdens in vaccinated and non-vaccinated baboons were not significantly different in this study.

6. The gross and histohepato-intestinal pathology of *S. mansoni* infection in vaccinated and non-vaccinated baboons was also not significantly different.

7. The results of this study are consistent with the hypothesis that there is no difference in the clinico-pathology of *S. mansoni* infection in baboons vaccinated with genes encoding *S. mansoni* anti-oxidant enzymes and Sm-filamin and the controls. Hence accept the hypothesis.

### 4.3 Recommendations

1. Due to the fact that, use of adjuvant IL-12 with radiation-attenuated cercariae in some cases has produced complete resistance to challenge infection (Wynn *et al.*, 1996). It is suggested that in future studies, IL-12 or any other molecule could be useful adjuvant for use with these DNA vaccines.
2. It is also being suggested that in future studies, these DNA vaccines should be combined into one polyvalent vaccine and applied with an adjuvant, and the phenotype of schistosome egg-specific immune-pathology in the liver and the intestine in vaccinated and non-vaccinated baboons compared for protection.

3. The cause of less weight loss in SOD and GPX vaccinated baboons post-infection should also be investigated in future studies.

4. In future studies, the effect of SOD and GPX on the fecundity of female schistosomes and egg viability should be further investigated.

5. The *Schistosoma mansoni* eggs in tissues of baboons vaccinated with these DNA vaccines should also be determined.

6. Genes encoding other *Schistosoma mansoni* antigens that have been shown to protect murine models against *S. mansoni* infection also need to be tested in non-human primates such as baboons as a prelude to human clinical trials.


### Appendix 1: Normal *Papio cynocephalus anubis* Haematological values.

(Source: Institute of Primate Research (IPR), Kenya)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (10 x 10⁶/ml)</td>
<td>5.48±0.47</td>
</tr>
<tr>
<td>Haemoglobin, g/dl</td>
<td>14.3±1.1</td>
</tr>
<tr>
<td>Packed cell volume (PCV) (%)</td>
<td>14.3±1.1</td>
</tr>
<tr>
<td>Mean cell volume (MCV) FIr</td>
<td>81±6</td>
</tr>
<tr>
<td>Mean cell haemoglobin concentration (MCHC) (%)</td>
<td>32.2±1.7</td>
</tr>
<tr>
<td>Mean cell haemoglobin MCH(pg)</td>
<td>25.8±3.1</td>
</tr>
<tr>
<td>White blood cells, WBC x 1000/ml</td>
<td>12.7±3.3</td>
</tr>
<tr>
<td>PMN cells (%)</td>
<td>51.1±12.3</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>0.9±1.3</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.0±1.1</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.1±0.2</td>
</tr>
<tr>
<td>Total plasma protein (g/100ml)</td>
<td>7.9±0.8</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>46.9±12.1</td>
</tr>
</tbody>
</table>