THE EFFECTS OF IMMUNIZING OLIVE BABOON, *Papio anubis*, AGAINST *Schistosoma mansoni* WITH SNAIL SOLUBLE PROTEINS, AND THEIR IDENTIFICATION

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DECLARATION

This thesis is my original work and has not been presented in any other institution for degree award or other qualification

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DEDICATION

I dedicate this thesis to my departed brother Robert Kabundu Ataya, who inspired me to love and cherish education during my formative years. May his spirit rest in eternal peace.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell cytoxicity
Ag	Antigen(s)
ANOVA	Analysis of variance
Bo	Booster
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Cluster Designation
Ch	Challenge
DG1	Soluble proteins from digestive gland of intermediate host, *Biomphalaria pfeifferi*
DG	Baboons immunized with antigens from digestive gland of intermediate host
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosobernt Assay
GSTs	Glutathione-S-transferases.
Hr	Hour
IC	Infected control
IFN-γ	Interferon Gamma
Ig E	Immunoglobulin Episilon
IgD	Immunoglobin D
IgG	Immunoglobin Gamma
IgM	Immunoglobin Mu
IL	Interleukin
IM	Immunization
IPR	Institute of Primate Research
KDa	Kilo Dalton
KEMRI	Kenya Medical Research Institute
LT	Lymphotoxin
MAPs	Multiple antigenic peptides
Ml	Millilitre

NC	Naïve Control
NK	Natural Killer cells
Nm	Nannometer
0-3 hr	0-3 hr protein release
P	Perfusion
PC	Post challenge
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
RRR	Replacement, reduction and refinement
RT1	Soluble proteins from digestive gland of intermediate host, *Biomphalaria pfeifferi*
RT	Baboons immunized with antigens from the rest of the tissues of intermediate host
S*Bg*A	*Biomphalaria glabrata* antigen
S*Bg*A	Soluble *Biomphalaria glabrata* antigen
SEA	Soluble egg-antigens
SLAP	Lung Antigen Preparation
SWAP	Schistosome worm antigen preparation
TB	Tuberculosis
TBRI	Theodor Bilharzia Research Institute
Th1	T helper-1
Th2	T helper-2
TNF-α	Tumor necrosis factor alpha
TPI	Triose phosphate isomerase
T-test	Student t-test
UV	Ultraviolet
µg	Microgramme
µl	Microlitre
WHO	World Health Organisation

ABSTRACT

Schistosomiasis infects about two hundred million people around the world. Currently, treatment for *Schistosoma mansoni* infection is by use of Praziquantel. However, a longer lasting solution would be a vaccine to enhance the use of drugs. Unfortunately, no human vaccine againt schistosomiasis is available. Snails that are the intermediate host of *S. mansoni* have been discovered to have common proteins with the schistosome worms. Research conducted in a mouse model showed that two candidate vaccines derived from *Biomphalaria pfeifferi*, RT1 (soluble proteins from the rest of snail tissue) and DG1 (soluble proteins from the digestive gland), were protective against *S. mansoni*. Both candidates met the World Health Organization criteria of over 40% protection. This study was undertaken to evaluate potentials these vaccines in *Papio anubis* as hosts. The baboons were divided in four groups, 3 groups comprising of three baboons: Two experimental groups, DG (Baboons immunized with DG and then challenged with *S. mansoni*), RT (Baboons immunized with RT and then challenged with *S. mansoni*), one control IC (Baboons only infected with *S. mansoni*) and the Naïve group comprising of 2 baboons. The study involved vaccination of baboons followed by challenge with *S. mansoni* cercariae. At the beginning of the experiment (minus week 9), DG and RT groups were vaccinated each with their specific protein. In minus week 6 and minus week 3, DG and RT groups were boosted with their specific protein. Three weeks after final vaccination (0 wk), baboons in the 3 groups were infected with 600 cercariae of *S. mansoni*. At Week six post challenge, perfusion to recover adult worms was carried out on the 3 groups of baboons. Chemical identification and toxicity test of the proteins was done. Results revealed that baboons immunized with snail soluble proteins were better protected than non-vaccinated baboons. The mean worm burden was higher in IC group than both DG and RT group of baboons. Worm reduction in DG was significantly higher ($p \leq 0.05$) at 11.44% while in RT it was 6.14%. In cellular responses, DG significantly stimulated increased IL-6 production and consequently resulted in Th2 protection in olive baboons. Percentage CD4, CD8 and CD25 counts in the spleen, lymph node and blood were similar in DG and RT ($p > 0.05$). In humoral responses, both DG and RT stimulated higher production of IgM in a similar manner at most sampling points than the IC, causing agglutination and cytolytic reactions suggesting both DG and RT antigens offered protection to the baboons. Both DG and RT stimulated higher production of IgG in a similar manner at most sampling points than IC, an indication that more worms were killed using antibody dependent cell-mediated cytotoxicity (ADCC). DG had least gross pathology and histopathology indicating that DG baboons were better protected than those from RT and IC. Brine shrimp lethality bioassay results for DG and RT snail soluble proteins at the three different concentrations showed that both proteins were non-toxic and are therefore safe vaccines. Chemical identification of DG and RT using the GC-MS spectrum established the presence of different chemical compounds with varied retention times. The results from the GC-MS were confirmed by UV spectrum and the FTIR results which confirmed the presence of proteins. This was evidenced by the amide (peptide) bands in both cases of the DG and RT soluble protein extracts. In conclusion therefore, although the two vaccine candidates were protective, they fell below the 40% protection threshold of the World Health Organization. Baboons in the DG exhibited greater worm reduction, greater cellular and humoral responses and minimum pathology, therefore making it a better vaccine candidate.

CHAPTER ONE:

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background Information

Schistosomiasis is an intravascular debilitating disease that is as a result of infection with parasitic Schistosomes which reside in the human bloodstream (Steinmann *et al.,* 2006). WHO (2014) approximated that 732 million individuals were vulnerable to infection globally in endemic areas. South America, Asia and Africa are estimated to have over 200 million cases of individuals that are infected with schistosomiasis. World tropical disease load caused by schistosome infections and geohelminthes with the exclusion of malaria is above 40% (WHO, 2014). In sub-Saharan Africa, Prevalence of schistosomiasis is still high. Treatment of people with schistosomiasis worldwide in 2008 stood at 17.5 million with sub-Saharan Africa accounting for 11.7 million people (WHO, 2014). In sub-Saharan Africa about 120 million people show symptoms associated with schistosomiasis where about 20 million individuals undergo hardship caused by chronic presentations of the disease (Chitsulo *et al.,* 2000). The main means of intervention of schistosomiasis is by use of drugs e.g. Praziquantel, such interventions have challenges of rapid re-infection, high costs and increased risk of resistance. The re-infection and the risk of resistance needs frequent re-treatment calling for long-term solutions. A vaccine has a long term effect, but at the moment, a vaccine for schistosomiasis meant for humans is unavailable. Snails have been found to have shared proteins with the Schistosoma mansoni parasite (Chacón *et al*., 2000). Enzyme Linked Immunosorbent Assay (ELISA) done using sera from individuals infected with schistosomes agglutinated

with soluble extracts of *Biomphalaria glabrata* antigen (S*Bg*A) with sensitivity of 100%. Further experiments by Chacon and others (2000) using Western-blot showed agglutination of sera derived from mice vaccinated with S*Bg*A with numerous homologous molecules of the snail.

A study was conducted using the mouse model (Kobia *et al*., 2011), in which two soluble proteins, DG (soluble proteins from digestive gland of the snail, *Biomphalaria pfeifferi*) and RT (soluble proteins from the rest of the tissues of the snail, *Biomphalaria pfeifferi*) were tested. A 60.5% reduction in worms was observed in RT and 43.3% in DG. This was above the 40% protection threshold of the World Health Organization (WHO, 1996). DG and RT stimulated cellular response as demonstrated by production of interferon gamma. The response was higher in RT. In addition, the two proteins also stimulated the humoral response as demonstrated by production of Interleukin-5 and IgG, the responses being higher in RT. They also reduced the pathology, again with RT having most reduced pathology.

1.1.2 Schistosomes and their distribution

Schistosomes also known as blood flukes are digenetic trematodes belonging to the family Schistosomatidae. This genus belongs to the subclass Digenea, class Trematoda and phylum platyhelmelminthes (Sturrock, 2001). These are parasites in the blood – vascular system of vertebrates, endothermic birds and mammals. A characteristic feature of members in this family is that mature male worms are stouter than the female worms. The male worms have a groove on their ventral surface of their body where the female

worms are sheltered. Schistosomes utilize aquatic snails as intermediate hosts for the completion of their life cycles (Sturrock, 2001).

There are five main species responsible for causing human schistosomiasis. *S. haematobium, S. mansoni, S. japonicum, S. mekongi* and *S. intercalatum. S. haematobium* causes urinary schistosomiasis and is confined to the African continent and Middle East. *S. mansoni* occurs in South America, Africa, Caribbean region, Madagascar and Arabian Peninsula (Rollinson and Southgate, 1987). In Africa, the two most prevalent species of schitosomes are *S. haematobium* and *S. mansoni* (De Vlas *et al.*, 1997).

1.1.3 Prevalence of *Schistosoma mansoni* in some sub-Saharan Africa countries

In many different countries south of Sahara, schistosomiasis is the main root of anomalies for instance periportal fibrosis, splenomegaly and hepatomegaly. Data from a research study conducted in Alamata district in northern Ethiopia revealed a very high prevance of 73.9% of schistosomiasis, with 12.3% periportal fibrosis, 7.4% splenomegaly and 3.7% hepatomegaly presentation (Abebe *et al.,* 2014). In Machakos district in Kenya, a study that was done amongst school going children showed different intensities of infection with *S. mansoni* and its relationship with hepatomegaly. Children infected with schistosomiasis were found to have muscle wasting and stunted growth when compared with schistosome free children (Corbett *et al.,* 1992). Various studies have reported cases of appendicitis-related to infections with schistosomes. In endemic communities, *S. mansoni* infection causes schistosomal appendicitis, which has a prevalence of 0.02–6.3% prevalence (Elbaz *et al.,* 2013). In schistosome endemic areas, *S. mansoni* is the leading cause of pulmonary hypertension globably (Lapa *et al.,* 2009). A prevalence of

60.5% was found in a study involving school pupils in the age bracket of five to nineteen years around Lake Victoria and Mbita in Kenya (Odiere *et al.*, 2012).

1.1.4 Geographical distribution of *S. mansoni*

S. mansoni geographical distribution is shown in figure 1.1. *S. mansoni* parasite is found in South America (Brazil, Surinam and Venezuela), numerous countries in Africa, parts of the Middle East and Caribbean. These areas either have slow moving streams or large-scale water projects infested with intermediate snail hosts (WHO, 1987). In Kenya, it is found in Machakos, Kitui, Mwea irrigation scheme in Kirinyaga, Taita, Murang'a, Kiambu, Mwea, Embu, along the shores of L. Victoria, L. Kanyaboli and L. Jipe, Rusinga Island, Nandi hills, Kericho, Kajiado and Mwea (WHO, 1985).

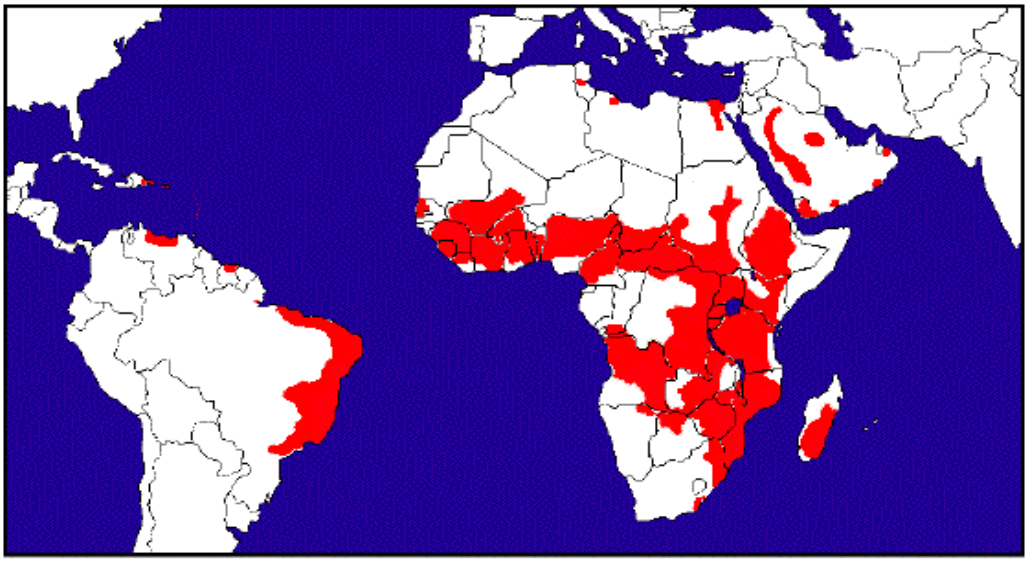

Figure 1.1 Geographical Distribution of Schistosoma mansoni (www.striepen.uga.edu/med para/ Schistosome.ppt) accessed on 14/07/2015

Key: ***Schistosoma mansoni* endemic areas**

1.1.5 Definitive hosts of *S.mansoni*

The most important definitive hosts of *S. mansoni* are humans, especially children. However, rodents and baboons are rare hosts (Cheesbrough, 1987).

1.1.6 Intermediate hosts of *S. mansoni* and their geographical distribution

S. mansoni is mainly transmitted by the intermediate snail hosts of the genus *Biomphalaria. S. mansoni* chiefly causes intestinal and hepatic schistosoma infection in areas such as South America, Africa and Arabian countries. Snails of the genus *Bulinus on* the other hand transmit *S. haematobium* which mainly causes urinary schistosomiasis in Arab world and virtually all countries in Africa (Gryseels *et al*., 2006). There are many species of the *Biomphalaria* snails. These include *B. pfeifferi, B. alexandrina, B. choanomphala*, and *B. sudanica.* The genus *Bulinus also* comprises of many species. These include *B. africanus, B. tropicus*, *B. forskalli*, *B. truncatus* and *B. globosus* (Ekpo *et al.*, 2012). Fresh water snails of the genus *Oncomelania* transmit *Schistosoma japonicum*, the main cause of hepatosplenic and intestinal schistosomiasis in China, Indonesia and Phillipines (Gryseels *et al.,* 2006).

1.1.7 Life Cycle of *S. mansoni*

An illustration of the life cycle of *S. mansoni* is shown by figure 1.2. Eggs of the schistosomes containing miracidia are passed out together with faeces. On contact with water, the miracidia hatches out of the egg. The hatching is stimulated by light, temperature and dilution of faeces with water. Miracidia then seeks for an appropriate intermediate host (*Biomphalaria straminea* , *B. glabrata* , *B. sudanica* or *B. tenagophila*) and then penetrates it (Gatlin *et al.,* 2009). In the intermediate host, the parasite

undergoes further development to form a larval stage known as cercariae that is infective to man (Gatlin *et al*., 2009).

During daylight, the cercaria emerges from the snail and actively seeks out for their definitive host. On recognition of the final host, they penetrate the skin. Upon penetration, the cercarial head changes in structure into a larval form known as the schistosomule. Schistosomules do not enter the general circulation immediately. They normally live for a few days in the skin depending on blood for nutrition. Entry into the general circulation is at the venules and dermal lymphatics (Olivera *et al*., 2000). 5-7 days after penetration, the schistosomule relocate to the lungs. From the lungs, they take less than fifteen days to move through the circulation to get to the left side of the heart and then to the hepato-portal circulation. Pairing of worms of the opposite sex takes place at the hepatic portal circulation leading to the development of the worms into sexually mature adults. Paired worms finally move to the mesenteric veins (Beltran & Boissier, 2008).

In the male, there is the ventral groove that forms a gynaecophoric canal where the mature female schistosome worm is sheltered. Schistosome male worm carries the female within the gynaecophoric canal and are able to move in the opposite direction to that of the blood flow to their final resting place; mesenteric circulation. In less than thirty two days, they start egg laying. The greatest number of eggs are passed into the lumen of the intestines of the host and passed out in the faeces (Loverde & Chen, 1991). Other eggs are carried by circulation and deposited in tissues.

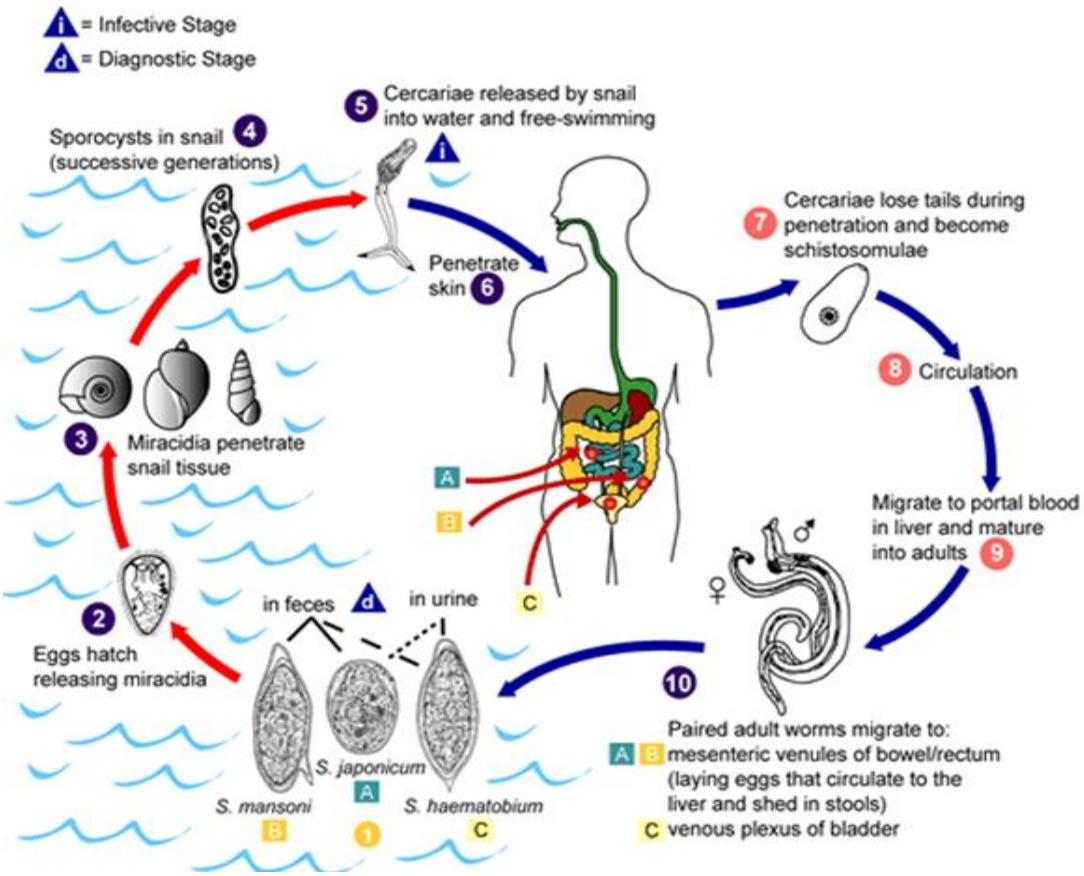

Figure 1.2: Life cycle of mammalian Schistosomes (CDC, 2009)

1.1.8 Control of schistosomiasis

Control of schistosomiasis is undertaken in various ways. These include: chemotherapy, sanitation, health education and intermediate host control.

1.1.8.1 Chemotherapy of Schistosomiasis

In schistosomiasis endemic regions, the main goal of controlling the disease burden is to limit morbidity mainly by use of praziquantel (WHO, 1985). Other drugs used in schistosomiasis treatment are metrifonate, oxamniquine and derivatives of artemisinin. Metrifonate is active against *S. haematobium* only but it is no longer in the market due to administrative cost (Genovese *et al.*, 2000). Oxamniquine is the only alternative drug

against *S. mansoni* but fewer people prefer to use it due to adverse side effects (Cioli *et al.,* 2000). Derivatives of artemisinin renown for their action against malaria have shown some activity against schistosomiasis (Utzinger *et al*., 2003; Xiao *et al.,* 2013).

1.1.8.2 Sanitation and health education

Control and elimination of schistosomes can be achieved by embracing high standards of hygiene and provision of clean water (Rollinson *et al.,* 2013). The prevalence and intensity of schistosomiasis can be reduced significantly by provision of enough sanitation facilities and clean adequate water supplies. Provision of health education is mainly targeted at preventing indiscriminate urination and defecation which contaminate water sources with schistosome eggs and improving water use practices (WHO, 1985).

1.1.8.3 Intermediate host control

There are 3 main methods involved in intermediate host control; chemical control, environmental control and biological control. In chemical control, incorporation of molluscicides in intergrated control of the intermediate host has been a key supportive measure. Snails, being the intermediate host, play an integral role in transmission. The use of molluscicides has always been considered to be a major supportive procedure in integrated control approach. Although copper sulfate and sodium pentachlorophenate were in use in the 1950s, Niclosamide is still the molluscide of choice. It is capable of acting upon all the life phases of the intermediate and also on larval stages of the schistosome worms. However, Niclosomide is lethal to fish and is expensive. Moreover, it does not hinder recolonization of the places by the snail remnants, which would result into selection of molluscide resistant populations (Larden & Dissous, 1998).

In a comparative study conducted in Central Morocco, the researchers used three varied ways of environmental control of snails; removal of egg masses, removal of aquatic plants and removal of silt and snails (Laamrani & Boelee, 2001). They observed that persistent washing of hydraulic parts resulted to a short period decline in population of *B. truncatus* although this control measure was not sustainable by the peasants in the locality. It was noted that the snails re-occupied the areas rapidly (Laamrani *et al.,* 2000). Removal of the aquatic plants resulted in the elimination of *B. truncatus* in the Rahad irrigation scheme in Sudan was noted by in the Madsen and Christensen (1992). Poor results in terms of snail reduction were noted from the Gezira irrigation which had only engaged mechanical removal of the weeds. The snails rapidly re-colonized the canals (Hilali *et al.,* 1985).

There are different biological control agents used to control the snail population. Predators such as small fish are successfully used in limiting the snail populations in islands such as Saint Lucia and Puerto Rico (Pontier *et al.,* 1992). Snail pathogens such as *Echinostomatidae* which sterilizes the snails are utilized in controlling snail population in China, Japan, and Brazil (WHO, 2002). Introduction of snail competitors to take over the targeted intermediate host populations (Madsen, 1990) or to compete for parasites by possible decoy effect has been studied (Combes, *et al.,* 1987). *Marisa cornuarietis* and *Melanoides tuberculata* have been successful in displacing *Biomphalaria glabrata* and *Biomphalaria stramineae* in Martinique Island. A major setback however is the potential of colonizing snails becoming susceptible to local schistosomes (Sturrock, 1995).

1.2 LITERATURE REVIEW

1.2.1 Cellular immune responses

1.2.1.1 Cellular immune response in the mouse model

In the first 3–5 weeks following infection of mice, the exposure of the host to the migrating schistosomules induces an immune activation that is accompanied with a remarkable type 1 immune response. There is an elevated Th1 cells and secretion of interferon (IFN)-γ and IL-12. During the initial weeks of *S. mansoni* infection, the immune response is intensely type 1-mediated and mainly targets the antigens of the worm. During the same period, type 2- mediated responses are primed too. After five to six weeks, the responses changes in a remarkable manner as the parasites undergo maturity, then mate, and begin egg production. This leads to a declined type 1 response and simultaneous rise in a strong type 2-mediated reaction (Pearce and MacDonald, 2002). Mature female *S. mansoni* worms start egg laying from five to six weeks post infection. This causes the immune response to switch from type Th1 to type 2-mediated responses. Naive mice injected with *S. mansoni* eggs produce potent type 2 responses (Vella *et al*., 1992). Marked type 2 responses are also induced by soluble egg-antigens (SEA). Egg-antigen stimulated type Th2 responses cause cell enlargement, secretion of IL-4, IL-5, and IL-13 together with circulating eosinophils and upregulation in immunoglobulin (Ig) E levels. Maximal cell response that is channeled against the egg antigens leads to a peak response. The extent of granulomatous inflammation around the egg is closely related to this Th2 response. The chronic period of schistosome infection occurs at 12 weeks. This phase is marked with a declined Th2 response that is followed by a state of hypo-responsiveness. There is a dynamic relationship amongst Th1, T

regulatory cells besides Th17 in controlling the disease severity in schistosomiasis. This is related to the potential for Th2 response that results to regulated chronic schistosomiasis (Turner *et al*., 2011).

1.2.1.2 Cellular immune response in the Primates

It is unknown in the baboon model how primary immune reactions to schistosomes that prime the adaptive and innate arms of the immune response to induce Th1 and/or Th2 responses occur. It is assumed that the events are similar to what is known in the mouse model. In the mouse model, at the point where the parasites penetrate the skin, sentinel cells comprising of neutrophils, dendritic cells and macrophages in the upper layer of the skin; epidermis and the dermis layer sense them (Mountford & Trottein, 2004). In the baboon model, events that are clear are downstream of innate responses. As the infection with schistosomiasis becomes chronic in mouse model, the response progressively switches from a Th1 to Th2 phenotype. In baboons, the adaptive immune responses are governed by a mixed Th1/Th2 phenotype as observed in man (Morales-Montor *et al*., 2004).

Feeble PBMC reactions to the parasite larval antigens are prominent in the first four weeks of infection (Kariuki *et al.,* 2004). This phenomenon is accompanied by dramatic Th2 soluble egg antigen responses (Kariuki *et al.,* 2004). The advancement of acute schistosomiasis and increase in granuloma establishment is brought about by anti-inflammatory Th2 cytokines that include interleukin-4 (IL-4), IL-5 and IL-10 (Farah *et al.,* 2000; Chou *et al.,* 2000). IFN-γ, a Th1 cytokine is also produced during this period. Cytokine levels decline and this is coincident with onset of immunomodulation (Morales-Montor *et al.,* 2004). Studies carried out in hepatic tissue to evaluate cytokine mRNA

expression revealed that soluble egg antigens induced cytokines such as IL-6, IL-1β, macrophage inhibitory factor (MIF) and tumour necrosis factor (TNF)-α (Morales-Montor *et al.,* 2004). Intense IgM, IgG and IgE antibody response accompany the cytokine responses (Kariuki *et al.,* 2004, Nyindo *et al.,* 1999). The profound Th2 immune responses emergence is coincident with a progressive down-regulation of CD4+ T cell role and pathology, an occurrence called immunomodulation (Andrade, 1987). Immuno-modulation in baboons is accompanied by enlargement and recruitment of specific receptor cells for instance lymphocytes, eosinophils, basophils and mast cells in granulomas (Farah *et al.,* 1997).

Matthias *et al.,* (2001) revealed from their study in chimpanzees that were vaccinated by a radiation-attenuated vaccine that the vaccine stimulated early interferon gamma production and a strong in vitro proliferative response. They further revealed that Th2 responses were dominant by the time of challenge. Weidong *et al.,* (2010) observed higher levels of Th1 response cytokine IFN-γ and IL-2 than Th2 response cytokines IL-4 and IL-10) in a study carried out in the baboons immunized with Sm-p80 vaccine.

1.2.1.3 Roles of CD4, CD8 AND CD25 T CELLS

Native T lymphocytes differentiates into either CD4+ Th1 cells that produce IL-2, IFN-γ and lymphotoxin which promotes cell-mediated immunity, or into Th2 cells that produce IL-4, IL-5, IL-6, IL-10 and IL-13, which promote antibody production and humoral immunity. These T cell subsets reciprocally regulate one another since one of the Th1 products, IFN-γ hinder the proliferation and functions of Th2 cells, while the Th2 products, IL-4 and IL-10, suppress cytokine production by Th1 (Kasukura, 1998).

CD8 T cells also known as cytoxic T Lymphocytes are responsible for defense against intracellular pathogens and for tumor surveillance. They are able to kill cells expressing class 1 MHC. They do this through cytokines, granule exocytosis and FAS mediated cytotoxicity. When CD8 T cells recognize antigenic peptides presented by MHC class 1 molecule, they are activated. They may secrete cytokines such as TNF and IFN. These have both anti-tumor and anti-microbial effects.

Granules exocytosis involves release of cytoxic granules that contain perforins and serine proteases. These activate apoptosis in the target cells. The other mechanism is through the FAS mediated cytotoxicity. In the FAS pathway, the FAS ligand on the activated CD8 T cell cross links FAS on the target cells all activating the FAS death pathway which leads to apoptosis of the target cells (Abbas & Litcham, 1991).

Human CD25 expressing B cells display a highly mature and activated phenotype and belong to memory B cell subset. CD25 expressing B cells play a major role not restricted to the physiology of the immune system but also play a role in the pathogenesis of autoimmunity. CD25 expressing B cells exhibit a mature and class switched phenotype, with higher expression of membrane bound IgA and IgG, and low expression of IgD and IgM when compared to exhausted CD25 B cells (Sylvie, 2008).

1.2.2 Humoral immune responses

1.2.2.1 Humoral immune response in the mouse model

IgG takes part in antibody-dependant cell-mediated cytotoxicity (ADCC) with neutrophils, macrophages and eusinophils which are damaging to the schistosomulea stage (Hagan *et al.,* 1998). Different antibody isotopes may have different effects on *S.*

mansoni infection. IgG2 and IgG4 block or compete with IgE which is associated with resistance to infection. IgG, IgE and IgA are important in protection, acting at several stages to prevent invasion, migration, development and egg production (Hagan *et al*., 1998).

The significance of B cell and antibody reactions to schistosomiasis related pathology was advanced from studies of *S. mansoni* infection in mice lacking B cells (Jankovic *et al.,* 1998, Fairfax *et al.,* 2012). Involvement of antibodies in protective immunity was shown earlier on by passively transferring resistance to naïve mice (Perlowagra, 1964). Only sera obtained from multiply vaccinated mice exhibited protective capacity (Mangold, 1986). IgG isotype, in particular IgG1 seems to offer protection and IgM enhances its function (Jwo & Loverde, 1989).

Two weeks after vaccination, antibodies specific to schistosome parasites are detected. Their levels peak at week five and six, and then gradually decline. Repetitive vaccinations using irradiated cercariae or after challenge infection using non-attenuated cercariae enhances titers (Lewis *et al*., 1987) showing a typical anamnestic response. There is no correlation between antibody titers and level of resistance although the presence of antibodies is important. However, antibody specificity is essential in protective immunity against schistosomiasis.

1.2.2.2 Humoral immune response in the baboon model

The significance of antibodies in immunity against schistosomiasis has been demonstrated in the baboon model. Antibodies assist in clearing the parasites and controlling the disease. Mechanisms that are involved in killing of schistosome parasites

in baboons are still not clear but antibodies IgG have been linked with protection (Kariuki *et al.,* 2004; Yole *et al.,* 1996).

Studies using antibodies from infected baboons have demonstrated their role in facilitating antibody-dependent cell cytoxicity (ADCC) and complement fixing to schistosomules (Butterworth 1984; Siddiqi *et al*., 2005). The clearance of mature worms in the tissues still remains unclear because the processes have only been proven *in vitro*. In a study carried out in chimpanzees (Matthias *et al.,* 2001), vaccination by a radiation-attenuated vaccine stimulated parasite-specific IgM and IgG production that attained a peak at challenge with the control group antibody titers comparatively low before egg deposit. In another study carried out in the *Papio anubis* model, animals vaccinated withSm-p80 based DNA vaccine induced parasite specific antibodies comprising IgG and and IgM in immunized baboons (Weidong *et al.,* 2010).

1.2.3 Portal fibrosis

Infection with majority of helminthes becomes chronic for the parasites are long-lived. The host mounts an immune response over these periods that normally lead to pathologic changes that are the chief cause of the disease. Demonstration of this phenomenon is the reaction to granuloma formation where schistosome eggs are sequestered. It is within the portal vasculature, where adult Schistosoma *mansoni* parasites live. Schistosome adult female lays around three hundred eggs each day. These eggs are meant for transmission across the wall of the intestines and into the lumen of the gut. From the gut, the eggs are then meant to be passed out of the host to the environment. However, majority of the eggs are conveyed to the liver because the flow of the blood in the portal system is headed to it. The eggs happen to lodge in the sinusoids. The eggs release antigens that

evoke a robust Th2 reaction that initiate the formation of granulomatous reaction in the liver (Cheever *et al.,* 2000).

1.2.3.1 Portal fibrosis in the mouse model

Understanding how granulomatous lesions confer protection to the host has been elucidated using a murine model infected with the human schistosomiasis. Mice deficient in CD4 T cells are not capable of granuloma formation and therefore they succumb to death due to toxic effects on their liver cells (Amiri *et al.,* 1992, Dunne & Doenhoff, 1983). The granuloma surrounds the egg lodged in the hepatocytes and separates it from the hepatic tissue allowing continued function of the liver. Eggs normally die in the cause of time causing the granuloma to resolve naturally and consequently lead to the development of fibrosis (Cheever *et al.,* 2000.). Fibrosis can cause high portal blood pressure and the development of portal varices. Death due to schistosomiasis is mainly caused by bleeding from varices. Demonstration of the importance of Th2 during schistosomiasis using mice has shown IL-13 significance in fibrosis development (Fallon *et al.,* 2000). Animals immunized with egg antigens together with IL-12 to evoke a Th1reaction before infection and therefore decrease secretion of IL-13, significantly enhances fibrosis and disease (Wynn *et al.,* 1995).

1.2.3.2 Portal fibrosis in the primate model

Development of fibrosis in primates is not well known but the role of a key gene that mapped to 6q22-q23, near to the gene that encodes the alpha chain of the IFN-γ receptor was revealed by segregation analysis that was related to acute fibrosis related to portal hypertension phenotype (Dessein *et al.,* 2004). These findings are in agreement with the

results from the mouse model of human schistosomiasis where the Th1 and IFN-γ response offered protection against acute fibrosis by barring alternate macrophage stimulation. Prevention of alternative macrophage stimulation limited the fibrosis-boosting role of Th2 response (Hesse *et al.,* 2000, Hoffmann *et al.* 1998).

1.2.4 Vaccine candidates against Schistosomiasis

Research findings have revealed that partial immunity to schistosomiasis is attained with continued exposure to schistosome parasites. Schistosome parasites do not replicate in the hosts body and therefore there is no need for full sterilizing protection. Evidence sourced from field studies give an indication that some resistance caused by schistosomiasis probably develop in a number of people. Intensity of schistosomiasis is higher in young children compared to adults. Adults treated with Praziquantel show some degree of resistance as demonstrated with lower intensities when re-infected with schistosomiasis (Colley *et al.,* 1996). The most effective way of controlling schistosomiasis is through vaccination. An efficatious vaccine would offer long term protection. Morbidity caused by schistosome infection would be reduced by immune responses which would lower egg production and parasite load (Afzal *et al.,* 2011).

Experimental animals vaccinated with radiation-attenuated cercariae showed protection to *S. mansoni* (WHO, 2009). Demonstration on how protection is expressed has been investigated in a murine model using irradiated cercarial vaccine of different regimens (Hewitson *et al.,* 2005). Immune responses that offer protection occur in sequence of actions that start in the skin, draining lymph nodes and lungs where 3 weeks post-challenge most larvae are killed (Kumar & Ramaswamy 1999). Administration of irradiated cercariae leads to the development of different effector reactions ranging from

Th1 cell-mediated reactions producing antigen-specific antibodies. All these immune responses are involved in elimination of schistosomes (Bickle, 2009; Jankovic *et al.,* 1999).

For the last twenty years, many laboratories have searched for schistosomal antigens that evoke at least partial protective immune response. There are over 100 schistosomal antigens that have been identified; a quarter of these have offered some protection. Though they appear promising they have failed to attain the protection level induced with administration of irradiated cercariae (Hewitson *et al.,* 2005). There are a number of experiments where characterizing cDNA clones that encode protective epitopes have been carried out. This has enabled the identification of quite a number of antigens that are partially protective such as glutathione S-transferase (Capron *et al.,* 2004), paramyosin (Gobert and McManus 2005), GAPDH (Argiro *et al.,* 2000), 14 kDa fatty acid binding protein Sm23 (Da'dara *et al*., 2002), triose phosphate isomerase (TPI) (Harn *et al,*. 1994), and Sm-p80 (Siddiqui et al., 2005). In animal models, administration of vaccines using recombinant or synthetic schistosomal antigens that represent selected epitopes have elicited partial immunity and reduced egg production (Balloul *et al.*, 1987; Tallima *et al*., 2003; Veprek *et al.,* 2004). High protection levels of 75% against cercarial at challenge has been attained with multiple antigenic peptides (MAPs) that have epitopes from either Sm23 or TPI and a truncated part of 200 kDa myosin-like protein (rIrV-5) found on the surface of schistosomules (Soisson *et al*., 1992). 74 kDa antigen also produced a protection level of 50–76% (Attallah *et al.,* 1999). It should be noted that none of the antigen constructs discussed above when amassed into a range of sole covalent formations that incorporates multiple-defined epitopes is able to offer protection from a

successive challenge. This implies that the immune reactions induced were either insufficient or not appropriate for the in vivo killing of the parasite (Yang *et al.,* 2000). There are 2 schistosome antigens that are currently undergoing clinical trials, glutathione-*S*-transferase of 28 kDa from *S. haematobium* and the fatty acid-binding protein of 14 kDa from *S. mansoni*, Sm14 (Riveau *et al.,* 2012). Another schistosome antigen recombinant to be soon tested in phase 1 clinical trial is the *S. mansoni* tetraspanin 2, TSP-2 (Curti *et al*., 2013).

All of the aforementioned vaccine candidates with exception of Sm-p80 have only offered a relative reduction in worm burdens in experimental animals when compared to the control animals (non- immunized animals. Formulations of Sm-p80 candidate vaccine have shown protection based on reduced egg production, protection against acute schistosomiasis in mice and baboons and worm reduction (Zhang *et al.,* 2011). At the moment no human Schistosomiasis vaccine exists in the market.

1.2.5 Common antigens between *Biomphalaria* snails and *Schistosoma* parasites

Snails have been found to have shared proteins with the *S. mansoni* parasite. Studies have demonstrated that different species of *Schistosoma* have common antigens with their intermediate hosts (Chacón *et al., 2000*). Using Western-blot, it was demonstrated that agglutination occurred between sera derived from mice vaccinated with S*Bg*A and homologous snail molecules. It was also noted from ELISA results that sera derived from individuals infected with schistosomiasis agglutinated with soluble rudimentary *Biomphalaria glabrata* antigen (S*Bg*A) (Chacón *et al., 2000*).

Immunoelectrophoretic studies on shared antigens between *Biomphalaria* snails and the Puerto Rican strain of *Schistosoma mansoni* using sera from rabbits immunized with the

Puerto Rican strain of *Schistosoma mansoni* adult worms or eggs and antigens of several adult *Biomphalaria* snails have been conducted. *S. mansoni* adult worm extracts produced 8 bands both with extracts of *B. glabrata* pigmentation and *B. glabrata pigmentado.* There were *3* to 4 bands with those of *B. glabrata* albino and 1 to 2 bands with those of *B. straminea* . On the other hand, *S. mansoni* egg extracts produced 5 bands with extracts of *B. glabrata*, 4 bands with those of *B. glabrata pigmentado*, 2 bands with those of *B. glabrata* albino and 1 band with those of *B. straminea*. In the experimental infection of adult snails of *Biomphalaria* species with five miracidia of *S. mansoni*, the infection rate in *B. glabrata* pigmentation was 78.8%, and 71.2% in *B. glabrata* pigmentado, whereas the infection rate in *B. glabrata* albino was 10.3%, and *B. straminea* was not susceptible to *S. mansoni*. The infectivity of each snail corresponded with the number of bands representing common antigenicities between host and parasite. Crude antigens of *Biomphalaria* snails were fractionated by Sephadex G-100 column, and each antigen fraction was tested with anti-*S. mansoni* adult worm and egg sera by immunoelectrophoresis. The common antigenicities between fractionated antigens of Biomphalaria snails and of anti- *S. mansoni* adult worm or egg sera mostly existed in the first fraction 1 with M > 45 kDa (Iwanaga, 1994).

In a study of common tropomyosins between *S. mansoni* infecting humans and *B.* glabrata snails, characterization of protein determinants common to *S. mansoni* and *B. glabrata* snails were carried out (Dissous *et al*., 1990). Parasite (Sm39) and mollusc (Bg 39) cross-reactive proteins were identified and shown to induce in rabbit and mouse, antibodies specific for invertebrate determinants. Ultra structural studies demonstrated that antibodies to Sm39 specifically bound to muscular structures of parasite and mollusc.

Molecular cloning and sequencing indicated that Bg39 corresponded to a muscular isoform of tropomyosin. The mollusc sequence showed a 51-65% homology with seven different muscular tropomyosins from vertebrate and invertebrate species. The highest score of homology was observed with *S. mansoni* tropomyosin, suggesting that cross-reactive determinants could be specific for the trematode and its intermediate host. In miracidia, Sm39 epitopes were also shown to be contained in the vesicles present in epidermal ridges and cellular bodies. Such vesicles are involved in the formation of a protective tegument around sporocysts, suggesting a possible role of cross-reactive tropomyosins in miracidia and/or sporocyst-snail interactions (Dissous *et al*., 1990).

1.2.6 Use of shared antigens in vaccine development

Snails have been found to have shared proteins with the *S. mansoni* parasite. Studies have demonstrated that different species of *Schistosoma* have common antigens with their intermediate hosts (Chacón *et al., 2000*). Using Western-blot, it was demonstrated that agglutination occurred between sera derived from mice vaccinated with S*Bg*A and homologous snail molecules. It was also noted from ELISA results that sera derived from individuals infected with schistosomiasis agglutinated with soluble rudimentary *Biomphalaria glabrata* antigen (S*Bg*A) (Chacón *et al., 2000*).

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1.2.7 Toxicity test

Schistosomiasis is a parasitic disease that is of great significance to a billion individuals globally. The main way of controlling the disease has been mass chemotherapy but still it has not been fully managed. Vaccination would offer a long term solution but still no human vaccine for schistosomiasis is available (Afzal *et al.*, 2011). It is important to ensure that substances being used as vaccines are both efficacious and non-toxic and therefore are safe. There are a number of common product safety tests and the simplest being brine shrimp lethality test.

The brine shrimp also known as *Artemia salina* Leach has been used for toxicity tests. Availability of eggs, the rapid maturation of the larvae, the simplicity of hatching the eggs into larvae and the moderate ease of sustaining a population under laboratory conditions have rendered the brine shrimps effective and simple animal experiment in toxicology. Combined with a reference benchmark, the brine shrimp lethality test provides a bioassay, which is reproducible, inexpensive, bench-top, simple, and more importantly, rapid (Pointier *et al.*, 2000).

1.2.8 Identification of proteins by spectroscopy

When a substance has been found to be both efficacious and safe, it is important to deduce its structure. No one technique in the present arsenal of protein structural methods is able to provide information on all aspects of protein structure. Therefore, a rational strategy is to employ a concerted approach in which the protein is examined using several structural techniques. Information obtained from different techniques can be cross-correlated to provide a more complete picture of the chemical and physical state and or bioactivity of the protein under different conditions.

Spectroscopy involves measurement of the interaction of molecules with electromagnetic radiation. Light in the near-ultraviolet (UV) and visible range of the electromagnetic spectrum has energy of about 150– 400 kJ mol. Electrons are promoted from the ground state to an excited state by the energy of the light. When the absorption of light is measured as a function of its frequency or wavelength, a spectrum is obtained. Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV (150–400 nm) or the visible (400–800 nm) region (Parvez & Severcan 1999). Electromagnetic radiations are produced by the oscillation of electric charge and magnetic field residing on the atom. Different forms of electromagnetic radiation exist; for example light (visible), ultraviolet, infrared, X-rays, radio waves, cosmic rays and microwaves,

1.2.9 Problem statement

Human schistosomiasis afflicts at least 238 million people, mostly children, in 76 tropical countries with another 500-600 million people. It is also estimated that 85% of the total number of those infected worldwide live in Africa (Vos *et al.,* 2012). In 2012, 249

million people were in need of treatment (WHO, 2014). The main mechanism of controlling its morbidity is by mass drug administration. However, this is challenged with re-infection of treated masses calling for further administration of drugs. Long term strategies therefore need to be put in place to control this debilitating disease. A vaccine would be a better option.

Shared antigens between schistosomules and schistosomes have been studied and fronted as possible candidate vaccines for *Schistosoma* infections. The candidate vaccines comprise of; the 26 and 28 kD glutathione-S-transferases (GSTs), the 63 kD parasite myosin, a 23 kD integral membrane protein (Sm23), the 97 kD paramyosin and the 28 kD triose phosphate isomerase (TPI). The 28 kD *S. haematobium* GST (Sh28GST) underwent Phase I and II clinical trials in humans and was found to be safe and elicited immune responses in human volunteers in Senegal, France and Niger (WHO, 2009). However, none of the candidate vaccine is in current use in humans.

1.2.10 Justification and significance of the study

It is possible to develop a vaccine against schistosomiasis for results from the field studies have shown that partial protection is induced in hosts that are continually exposed to schistosome infection. A full sterilizing protection is unnecessary for schistosomiasis because schistosome worms do not replicate in the hosts body. The evidence from field studies indicates that partial resistance as result of schistosomiasis most likely develops in majority of infected individuals (James & Colley, 2001). Generally, the intensity of schistosomiasis is higher in children than adults. The presence of at least a partly defensive protection in exposed humans would make vaccination a rational supplement to medication.

An emerging concept of controlling parasitic diseases like schistosomiasis is by adoption of multiple and integrated approaches. One of the modern methods for the control of these parasitic infections is immune- intervention. Schistosomiasis vaccine is needed to complement chemotherapy. In addition, mathematical models have showed that administration of drugs together with a vaccine would be helpful, profitable to the health sector as a whole even though immunity offered by the vaccine are not absolute (Berquist, 1998). Strategies for controlling pathology and infection in schistosomiasis are fully pegged to the hosts' immune responses (Capron, 1992). Female fecundity, severity of the disease, formation of the granuloma, efficacy of the drugs and resistance to re-infection are dependent on immunological factors (McCarthy & Nutman, 1996). Vaccination stratergies therefore play a key part in control of schistosome infections.

Snails have been found to have shared proteins with the *S. mansoni* parasite. Chacón and others (2000) reported sharing of proteins between schistosomes and snails. Using western blot method, they revealed that crude *Biomphalaria glabrata* antigen (S*Bg*A) agglutinated with several homologous snail molecules and that soluble crude S*Bg*A agglutinated with sera from schistosome-infected persons by ELISA with a 100% of sensitivity (Chacón *et al*., 2000).

A study conducted in the mouse model by Kobia *et al*., (2011), evaluated two soluble proteins; DG and RT prepared from the *Biomphalaria pfeifferi*. Worm reduction in DG and RT was above 40%. Both proteins stimulated cellular response as demonstrated by production of interferon gamma. The response was higher in RT. In addition, the two proteins also stimulated the humoral response as demonstrated by production of Interleukin-5 and IgG, the responses being higher in RT. They also reduced the

pathology, again with RT having most reduced pathology. Although RT was more efficacious based on higher humoral and cellular immune responses, higher worm reduction and minimum pathology, as mentioned above, both RT and DG met the WHO criteria of 40% protection (WHO, 1996).

The current work aims at testing the two snail proteins, DG and RT in the olive baboon, *Papio anubis*. The baboon is phylogenetically closer to man than the mouse. It has been established as a model for schistosomiasis vaccine, based on work conducted at the Institute of Primate Research (IPR) (Yole *et al*., (1996) and Kariuki *et al*., (2004). Efficacy of RT and DG will be tested in the baboon in terms of: Worm reduction, Immune responses, and reduction in pathology. The efficacious soluble protein will be tested for toxicity and then characterized. If DG and RT are found to be protective in the baboon, there will be a greater chance of them being protective in man because of their genetic closeness.

1.2.11 Research questions

1. What is the worm recovery in olive baboons immunized with snail soluble proteins DG and RT and challenged with *S. mansoni?*
2. What is the cellular and humoral response in olive baboons immunized with DG and RT and challenged against *S. mansoni*?
3. What are the pathological effects in olive baboons immunized with DG and RT and challenged against *S. mansoni*?
4. Is the efficacious soluble protein toxic?
5. What are the chemical properties of the efficacious snail soluble protein ?

1.2.12 Null hypothesis

1. Olive baboons immunized with snail soluble proteins are not protected against *S. mansoni.*
2. Olive baboons immunized with DG and RT do not show varying immune responses.

1.2.13 General objective

To evaluate the effects of immunizing the olive baboon, *Papio anubis* against *schistosoma mansoni* with snail soluble proteins.

1.2.13.1 Specific objectives

1. To determine *S. mansoni* worm recovery in olive baboons immunized with snail soluble protein DG and RT.
2. To determine cellular and humoral immune responses to *S. mansoni* in olive baboons immunized with snail soluble protein DG and RT.
3. To examine gross pathology and histopathology of liver tissues from olive baboons immunized against *S. mansoni* with snail soluble protein DG and RT.
4. To determine toxicity and the chemical properties of the efficacious snail soluble protein.

CHAPTER TWO

2.0 GENERAL MATERIALS AND METHODS

2.1 Study site

The research was conducted at IPR. This is a Biomedical Research Centre which utilizes both non-human primate models and rodents with the vision of improving the wellbeing of mankind. The current study used Olive baboons *(Papio anubis*) from the Department of Animal Science at IPR. Flow cytometry for cytokines was done at Kenya Medical Research Institute (KEMRI). Toxicology was conducted in the Department of Public health, Pharmacology and Toxicology, University of Nairobi, Upper Kabete Campus. Characterization of the snail soluble proteins was done in the Department of Chemical Science and Technology, Technical University of Kenya.

2.2 Experimental animals

2.2.1 Definitive host – Olive baboons

Olive baboons (*Papio anubis*) are permissive definitive hosts of *S. mansoni*. It has been established as a model for schistosomiasis vaccine based on studies conducted at the Institute of Primate Research (Yole *et al*., 1996, Kariuki *et al*., 2004). Wild caught Juvenile baboons from IPR Animal Science Department were used in this study. After capture, they were quarantined for three months, dewormed and tested for TB. The animals were nourished with nutrient pellets (Laboratory Cho from Unga Feeds ® CO.) which were complemented with vegetables plus fruit. Water was supplied *ad libitum.* During the experiment they were caged in pairs as an animal welfare requirement for companionship. Three animals (3) were allocated per group. Baboons are Non-human primates and therefore ethically the minimum number for this study was three as per the

3 R's (replacement, reduction and refinement) (Russel & Burch, 1959). Ethical approval to carry out the study with 3 animals per group was obtained from the Institute of Primate Research Institutional Review Committee (IRC No. 10/13). The committee is guided by International standards of animal welfare. The measured effect/ parameters comprised of the number of worms recovered. Banking on the previous studies, about 450 worms are recovered per infected baboon. This means that for 3 baboons per group, an average of 1350 worms were expected to be recovered. Statistically this is acceptable and would provide the required statistical power for significant comparisons. Similar measured effects involved cytokines, antibodies, gross and histopathological parameters being measured.

2.2.2 Intermediate host – Snails

Biomphalaria pfeifferi snails were collected from canals in Mwea Irrigation scheme, by scooping them out of water using a scoop. Snails were carried inside plastic containers lined with damp cotton wool and transported to the snail laboratory at the IPR where they were screened for schistosomes under strong light (100 watts) for two hours for five consecutive weeks. Those that were negative were housed in the snail room whose temperature is controlled (25- 27^{o}C). Cleaning of the Plastic tanks was done thoroughly using tap water. Gravel and sand were sterilized by heating at 150^{o}C for twelve hours. They were then cooled and put in layers in the plastic tanks. Plastic tanks were 75% filled with tap water from IPR (chlorine free water from the IPR well). The screened snails were transferred into the tanks for maintenance according to Yoles' method (Yole *et al.,* 1996). Replacement of Water was done in a fortnight. Lettuce (steamed and dried) in oven was added for feeding the snails.

2.3 Preparation of RT and DG from the *S. mansoni* intermediate host *Biomphalaria pfeifferi*

Two hundred colony bred *B. pfeifferi* snails were dissected under the dissecting microscope to extract the digestive gland leaving behind the rest of the snail tissues. The rest of the snail tissue and digestive gland were placed in separate Nunc tubes containing 2 ml of phosphate buffered saline (PBS). They were placed on ice. The tissues were crashed in a glass mortar with a pestle to obtain a homogenate which were centrifuged in a microfuge for 1 h at 14,000/rpm at 4^0C to obtain the soluble protein. Protein concentration was assayed using Bradford method (1976). This method utilized bovine serum albumin, BSA, (Biorad Co.) as a standard protein. BSA and the soluble proteins were serially diluted. The dye was added and incubated. The samples were read using Enzyme Linked Immunosorbent Assay (ELISA) reader at 630nm wavelength. A standard curve for BSA was drawn, various concentration of the soluble proteins were read off the curve and average concentration obtained. Protein concentration was adjusted to 1mg/ml. The soluble protein was aliquoted and sterilized by exposure of UV light (10 minutes, 5cm from a 30 watt ultra violet OSRAM bulb). The aliquots were stored at -20^0C.

2.4 Immunization of baboons

At the beginning of the Study (-9 wk), 1 ml of DG and RT antigens were placed in a nunc vial. 1 ml of montanide-ISA 51 adjuvant (Cytrx Corporation, Los Angeles CA), was drawn by the syringe and added to 1000 µg (1mg) of the specific antigen in the nunc vial. The mixture was vortexed for 30 minutes until an emulsion was obtained. A mixture of Rompun and Ketamine 20:1 was used to provide a combined effect of anaesthesia. Anaesthesia at 0.1 ml/kg body weight was injected intra-muscularly in each of the

animals in DG and RT. The two experimental groups, RT and DG, were each injected on each of the two legs in the quadriceps muscles just above the knee with the prepared antigens intra-muscularly. Three and six weeks after the initial vaccination, DG and RT groups of baboons were boosted with 500 µg of their specific antigens in 1 ml of Montanide-ISA 51 adjuvant.

2.5 Harvest of *S. mansoni* eggs from baboon faeces and infection of snails

Feacal samples where from Olive baboons (*Papio anubis*) with chronic *S. mansoni* infection. Feacal samples were thoroughly mixed with IPR well water (high in salt content) in a plastic jar. The slurry was poured on a standard test sieve (Arthur Thomas Co. USA) of 600µm placed on another sieve of 250 µm; the latter had a collecting tray placed under it. IPR well water was poured on the feacal sample. After sieving, the feacal debris was discarded, while the feacal suspension was poured into urine glass jars. The urine jars were placed in the dark and left to sediment for at least 30 minutes. The supernatant was poured out and the pellet re-suspended in saline and then given time to sediment in the dark. This procedure was repeated thrice until the supernatant became clear. The clear supernatant was poured out. The sediment was placed on Petri dish with water. This petri dish was placed under a 100 Watts lamp for at least 30 minutes to allow hatching of free swimming miracidia from the eggs. Snails were placed independently in wells of a 24 microwell plate (Nunclon, Denmark), and 3-5 miracidia dispensed into each well to penetrate the snails. After 30 minutes, snails were transferred to snail tanks. Four weeks post-infection (pi), snail tanks were enclosed with a black cloth to avoid light from stimulating the trickle shedding of cercariae. Five weeks pi, the snails were carefully removed from tanks using forceps and placed in 100 ml beakers containing snail water.

They were exposed to 100 Watt lamp shaded with glass for 1 hr to shed cercariae. Cercariae suspensions were pooled together. Cercariae in three 50 µl aliquots of the suspension were counted under the dissecting microscope. An average of cercariae number was taken and used to calculate cercarial concentration per milliliter. This was used to determine the volume containing 600 cercariae that was used to infect one baboon.

2.6 Challenge of baboons

The experimental groups, DG and RT, and the control group, IC, were anaesthetized as described in section 2.4. The groin area was shaved and pegs were used to create a poach at the groin area. Wet cotton wool was used to moisten the poach at the groin area, to enable easy penetration of cercariae. A suspension containing 600 live cercariae was dispensed in the groin pouch using 1 ml micropipette. A period of 30 minutes was allowed for cercariae to penetrate into the baboons.

2.7 Perfusion and adult worm recovery

At week 6 post-challenge, baboons from each group (DG, RT and IC) were perfused to retrieve the mature worms. Each baboon was anaesthetized with mixture of Ketamine and Rompon 20:1, 0.1 ml/kg body weight. Each baboon was also euthanized using a mixture of 1ml heparin anticoagulant in 4 ml of sodium pentobarbitone (Euthatol, May and Baker Ltd, UK). The abdomen of each baboon was opened up. The hepatic portal vein was incised. Perfusion catheter having perfusion fluid (0.85% Sodium chloride and 1.5% Sodium nitrate) was injected into the aorta and perfusion conducted until the liver and mesenteries were clear. The perfusate was collected in a 10 litre buckets. The perfusate

was then sieved using 180µm sieve into a tray. The worms on the sieve were then transferred into petri dish having PBS and then counted. The worm maturation, percentage worm recovery and reduction for each group was calculated as shown in the formulae below.

$$Worm\ maturation = \frac{Number\ of\ worms\ recovered\ in\ infected\ control}{Initial\ number\ of\ infected\ cercariae} \times 100$$

$$Percenatge\ worm\ recovery = \frac{Mean\ of\ total\ worms\ in\ experimental\ group\ [MECG]}{Mean\ of\ total\ worms\ in\ infected\ control\ [MINC]} \times 100$$

$$Percentage\ worm\ reduction = \frac{MINC - MECG}{MINC} \times 100$$

2.8 Statistical analysis

Data was analyzed using Student t-test (T- test) and Analysis of variance (ANOVA) - Dunnet test. *t*-test was used to compare two means while ANOVA is used to compare more than two means. Significance level/probability level used in calculation was $p<0.05$.

CHAPTER THREE

3.0 WORM RECOVERY IN OLIVE BABOONS IMMUNIZED AGAINST *S. mansoni* WITH SNAIL SOLUBLE PROTEIN

3.1 INTRODUCTION AND LITERATURE REVIEW

Schistosomes are dioecious with separate male and female. The males are significantly larger than females. The male has a ventral groove called gynaecophoric canal where the female is sheltered (Cheesbrough, 1987). The eggs of *S. mansoni* are passed out in the faeces of the definitive host. On reaching fresh water, light and warmth stimulates them to hatch within few minutes into highly motile larvae called miracidia. They move about in the water for about 24 hours where on coming into contact with the snail host, they infect them. Within the host, they develop into mother sporocysts within 96 hours. On the 8^{th} day, they become non-motile convoluted tube from which daughter sporocysts are developed. These daughter sporocysts migrate to the visceral mass where they now develop to cercariae. The cercariae have bifurcated tail that helps in swimming to locate the definitive host (Farrar *et al.,* 2013). It penetrates it within few minutes and loses its tail in the tissues to become a schistosomule. The schistosomules enter the lymphatic or venous system where they are transported to the right side of the heart and lungs. Growth and pairing takes place in the liver and paired worms can be found in the liver 26 days after cercarial penetration. Majority of the worms leave the liver when they are sexually mature and have mated and migrate to the mesenteric veins where they begin egg laying.

Recovery of adult schistosome worms is carried out by perfusion. There are different perfusion methods. The method described by Pellegrino and Siqueira (1956) involves injecting saline solution into the descendent aorta using a needle that is attached to a

Brewer's automatic pipette. Thereafter, the portal vein is sectioned and saline is injected into the mice's hepatic hilum. This allows the perfusion of the mesenteric veins and the portal system. The recovered worms are then counted.

The other method was described by two researchers, Smithers and Terry in 1965. In this method, the portal vein of the mice is cut and the perfusion fluid mildly introduced into the bottom of the left ventricle of the heart. The disadvantage of this method is that it generates a lower pressure injection and therefore it impossible to recover all the mature worms. The advantage of this method is that the tegument of the parasite is left intact. The other method was described by Yole *et al.,* (1996) involves opening up of the baboons' abdomen, and the incision of the hepatic portal vein. The perfusion tube catheter containing perfusion fluid (0.85% Sodium chloride and 1.5% Sodium nitrate) is inserted in the aorta and perfusion conducted until the liver and mesenteries are clear. The perfusate is collected in a 5 litre conical containers and worms allowed to settle down. The worms are transferred to urine jars for further settling. The urine jars are topped up with PBS. Once the worms have sunk, the supernatant is drawn out, and this procedure is done thrice. Once the supernatant is clear, the worms are put in Petri dish containing PBS and then counted.

3.1.1 Egg maturation

Upon infection with *S. mansoni,* adult parasites finally settle in the mesenteric veins. Schistosome worms have a life period of ten years but have been known to live a few more years. In a day, the worms lay hundreds of eggs. Not all of the eggs are passed out of the gut lumen. In microvasculature of the liver, a number of eggs are trapped and once there, they release antigenic products that evoke a robust immune response that result to

granuloma formation (Pearce & MacDonald, 2002). Schistosome worms do not cause much pathology per se but the eggs they produce. The higher the number of worms, hence the higher number of eggs, the greater the pathology.

3.1.2 Protection with other vaccines

Vaccine-induced protection is defined as the decrease in worm load between immunized animals and the control groups. In non-human primates that are phylogenetically close to man, experiments using radiation-attenuated (RA) cercariae vaccine provided comparable data from challenge control animals. A worm maturation of 82 % was noted in a single experiment using vervet baboons, *Cercopithecus aethiops* (Yole *et al.,* 1996). Further 3 experiments by Yole and others 1996 using the baboon model *Papio anubis* resulted to a mean of 80.5 %.

Approximately a quarter of over 100 schistosome vaccine antigens have so far been identified. They have shown certain level of immunity in the murine model (Siddiqui *et al.*, 2011). There are only three molecules that have gone into clinical trials in humans; *S. mansoni* tetraspanin (*Sm*-TSP-2), *S. mansoni* fatty acid binding protein (Sm14), and *S. haematobium* glutathione S-transferase (Sh28GST). The other molecule Smp80 (calpain) is currently being tested in non-human primates (Merrifield *et al*., 2016).

Many studies have revealed that schistosomes and snails have shared proteins (Dissous *et al.* 1986; Weston *et al.* 1994; Gamal-Eddin *et al.* 1996, 1997; Chacón *et al.* 2000). Using western blot method, they revealed that crude *Biomphalaria glabrata* antigen (S*Bg*A) agglutinated with several homologous snail molecules and that soluble crude S*Bg*A agglutinated with sera from schistosome-infected persons by ELISA with a 100% of sensitivity (Chacón *et al*., 2000).

3.2 MATERIALS AND METHODS

3.2.1 Experimental plan

The experimental plan is shown in Table 3.1. The study was carried out in the olive baboon, *Papio anubis,* as the model for human schistosomiasis (Wilson, 1990). The baboons were in three groups, each comprising of three baboons: Two experimental groups, DG and RT and one control IC. The study involved vaccination of baboons and then challenging them with *S. mansoni* cercariae. In the beginning of the experiment (minus week 9, -9wk) of the experiment DG and RT were vaccinated each with their specific protein. In week -6wk and -3 wk, DG and RT were boosted with their specific protein. Three weeks after final vaccination (0 wk), baboons in the 3 groups were infected with 600 *S. mansoni* cercariae. At Week six post challenge, baboons from three groups were perfused to recover adult worms (Yole *et al.*, 1996).

Table 3.1: Experimental plan for immunization, challenge and perfusion

Groups according to treatment	*n*	Sampling Time Points				
		-9wk	-6wk	-3wk	0wk	6wk
DG	3	IM	Bo	Bo	Ch	P
RT	3	IM	Bo	Bo	Ch	P
IC	3	-	-	-	Ch	P

KEY

- wk – Week(s)
- DG- Baboons immunized with snail soluble proteins from the digestive gland and then infected
- RT- Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected
- IC- infected control

- n-Total number of baboons per group
- IM-Immunization
- Bo-Booster
- Ch-Challenge/ infection
- P-Perfusion
- - No activity

3.2.2 Baboon procedures

Definitive host, intermediate host, preparation of immunizing proteins, RT and DG, immunizing of baboons, and preparation of cercariae, challenge of baboons and perfusion and adult worm recovery are as described in 2.2 to 2.7.

3.2.3 Statistical analysis

Data analysis was done using *t*- test and ANOVA test. *t*- Test was used to find out whether there were variances in worm recovery between DG and RT. ANOVA was performed with the help of SPSS to determine whether there were differences in worm recovery among DG, RT and IC. *t*-Test is used to compare two means while ANOVA is used to compare more than two means. Significance level/probability level used in calculation was $p<0.05$.

3.2.4 RESULTS

The mean number of worms that were recovered from baboons among the three groups, percentage worm recovery and percentage worm reduction are shown in Table 3.2. The mean number of worms for RT group was 443 ± 10.69, DG group 418 ± 11.37 and IC was 473 ± 7.51. Worm maturation was 78.8%. This indicates that DG had a lower mean

number of worms than RT and also the infected control. The percentage worm reduction was 11.44% in DG while in RT it was 6.14%.

A t-test analysis showed that DG had significantly lower mean worm reduction than RT ($p<0.05$). Mean worm reduction in RT and IC was similar ($p>0.05$). However, a significant difference in the mean worm reduction was noted between DG and IC ($p<0.05$).

Table 3.2: Worm recovery and reduction in baboons immunized with soluble proteins from *Biomphalaria pfeifferi* and challenged with *Schistosoma mansoni*

Groups according to treatment	MEAN NUMBER OF WORMS ($\bar{x} \pm SE$)	% WORM RECOVERY	% WORM REDUCTION
DG	418±11.37	88.55	11.44
RT	443±10.69	93.85	6.144
IC	473±7.51		

Key: DG-Baboons immunized with snail soluble proteins and then infected**; RT-** Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected.

3.2.5 DISCUSSION

Schistosomiasis still remains a challenge in endemic regions and a great burden to the public health sector. Administration of mass chemotherapy especially by use of praziquatel has not yielded a solution especially given that we still observe a number of drawbacks to its use. Integrated strategies that include vaccination therefore need to be laid down to mitigate the debilitating effects of schistosomiasis (Biniam *et al.,* 2016). Baboons in DG group were immunized with snail soluble proteins from the digestive glands and then infected with 600 *S. mansoni* cercariae. Baboons in RT were immunized

with snail soluble proteins from the rest of the body tissues and then infected, while baboons in IC group were infected only, i.e. the infected control. The dose used to challenging baboons was 600 cercariae. In the IC, 473 of the infecting cercariae were recovered as adults, a worm maturation of 78.8%. This compares well with a worm maturation of 80% in Olive baboons (*Papio anubis)* that were immunized by irradiated cercariae (Yole *et al.,* 1996).

The mean number of worms for RT group was 443±10.69, DG group 418±11.37 and IC was 473±7.51. This indicates that DG had a lower mean number of worms than the other immunized group, RT and the infected control. The percentage worm reduction was 11.44% in DG while in RT it was 6.14%.

Mean worm reduction results in DG was significantly lower than RT ($p<0.05$). There was no significant difference in the mean worm reduction between RT and IC ($p>0.05$). However, there was a significant difference in the mean worm reduction between DG and IC ($p<0.05$). These results indicate that DG was efficacious against *S. mansoni* while RT was not.

A study done on BALB/c mice immunized with snail soluble Proteins derived from digestive gland and foot and then infected with *S. mansoni* by Kuria *et al.,* (2012) showed protection of 50% of the group immunized with the digestive gland. Kobia *et al*., (2011), immunized mice with two soluble proteins, DG and RT. Worm reduction was 60.5% in RT and 43.3% in DG. In above mice studies, DG was efficatious against *S. mansoni* and this is in agreement with this baboon study. The protection is higher in the mouse model compared to the baboon, most probably because of genetic differences.

DG was efficacious in this study with a protection level of 11.44%. Since the baboon is genetically close to man than the mouse, there is a high probability of DG being protective in man. However the protection level is below the WHO threshold of 40% (WHO, 1996). Further work should be done to find out how the efficacy of DG can be increased, for example using more refined proteins like molecules from the digestive gland.

CHAPTER FOUR

4.0 CELLULAR AND HUMORAL IMMUNE RESPONSES TO *S. mansoni* IN OLIVE BABOONS IMMUNIZED WITH SNAIL SOLUBLE PROTEINS

4.1 INTRODUCTION AND LITERATURE REVIEW

Schistosomiasis is an acute and chronic parasitic infection initiated by blood flukes belonging to the genus *Schistosoma*. Estimates show that at least 258 million individuals required preventive treatment in 2014 and more than 61.6 million people were reported to have been treated for schistosomiasis in 2014 (WHO, 2016). Animal experiments together with field studies in schistosome endemic regions have shown that infection intensity, host genetics, mixed infection status and in utero sensitization to schistosome antigen all determine the acuteness of the disease along with the development of the immune reactions (Pearce and MacDonald, 2002).

4.1.1 CD4 T cells

The major players involving adaptive immune reactions to pathogens are the CD4 (+) T cells. On recognition of pathogen-derived antigens, naïve CD4 (+) T cells differentiate into effectors. These effectors control pathogen replication in two main ways; either by directly killing pathogen-infected cells or by assisting with generation of cytotoxic T lymphocytes (CTLs) or pathogen-specific antibodies (Gesham *et al.*, 2013). Native T lymphocytes differentiates into either CD4+ Th1 cells that produce IL-2, IFN-γ, and lymphotoxin which promote cell-mediated immunity, or into Th2 cells that produce IL-4, IL-5, IL-6, IL-10 and IL-13, which stimulate antibody production and humoral protection. These T cell subsets reciprocally regulate one another since one of the Th1 products, IFN gamma, inhibits the proliferation and functions of Th2 cells, whereas the

Th2 products, IL-4 and IL-10, suppress cytokine production by Th1 cell (Kasakura, 1998).

4.1.2 CD8 T cells

The main role of CD8+ cells (cytolytic T lymphocytes) is to mount immune resistance against intracellular pathogens, including bacteria and viruses but recently its function in schistosome elimination has been described (Zhou *et al.*, 2012). A significant low parasite load which was attributed to elevated activated CD8+ cells was elicited in mice that were immunization with the *S. japonicum* 22.6/26GST combined with Sepharose 4B bead. The activated CD8+ cells caused mortality to parasites expressing MHCI molecules of the host in its surface (Zhou *et al.*, 2012).

4.1.3 CD25 T cells

Human CD25 expressing B cells belong to Memory B cell subset. They normally show a highly mature and activated phenotype. CD25 expressing B cells are involved in the pathogenesis of autoimmunity

and also act in the physiology of the immune system. CD25 deficiency in humans is severe. Patients suffer from recurrent infections and lymphocyte infiltration in multiple tissues. It has been shown that humans deficient in CD25 have a decreased apoptosis of developing T cells in thymus which affects the negative selection and results in release of auto reactive T cells causing inflammation in different tissues (Roifman, 2000). CD25 expressing B cells display a mature and class switched phenotype, having high expression of membrane bound IgA and IgG, and low expression of IgD and IgM when compared to depleted CD25B cells (Sylvie, 2008).

4.1.4 Cytokines in schistosomiasis

There are twofold discrete forms of cytokine production that have been identified from CD4+ helper T-cell clones in mouse and humans. CD4 T 1 helper (Th1) lymphocytes cells that produce Interferon- gamma (IFN-γ), tumor necrosis factor-beta (TNF-*B*) and interleukin-2 (IL-2) and CD4 T 2 helper (Th2) lymphocytes that produce interleukin-4 (IL-4), IL-5, IL-9 and IL-13 cytokines. There are other cytokines that are secreted by both Th1 and Th2 cells. These include; IL-3, IL-6, GM-CSF, or TNF-alpha. A third subset of Th known as Th0 cells, exhibits secretion of both Th1- and Th2-type cytokines. The various cytokine patterns are related to diverse roles. Th1 cells are involved in delayed type hypersensitivity responses and are cytolytic for autologous antigen-presenting cells, plus B cells while Th2 cells offer an outstanding helper task for B-cell antibody secretion, principally of the IgE class (Del Prete, 1992).

Depending on signals received from antigen- presenting cells and the cytokine environment, naïve T-helper lymphocytes (Th-cells) differentiate upon stimulation to produce a controlled set of cytokines that are either classified as Th-1 or Th-2 cells (Abbas *et al*., 1996). Interleukin 12 (IL-12) stimulates Th-1 cells to differentiate and produce interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-αand lymphotoxin (LT).The Th-2 cells develop in an IL-4 dependent manner and secrete IL-4, IL-5, IL-6, IL-10 and IL-13 respectively. Particular cytokines made by Th-1 or Th-2 cells have a tendency to to hinder the expression or role played by the alternative subset (Abbas *et al*., 1996).

4.1.5 Antibodies in schistosomiasis

Studies in the baboon model have revealed the major function carried out by antibodies in the establishing defense against schistosomes. Experiments conducted earlier demonstrated that some factors in the serum of infected baboons inhibited egg laying and caused un-pairing of *S. mansoni in vitro* (Bosshardt & Damian, 1986). In primary response the antibodies involved are classically of the IgM isotope that is followed by a secondary response that involves other classes and subclasses such as IgG and IgE (Nyindo *et al.,* 1999).

4.1.6 Role of the liver, lymph node and spleen

The blood from all the body parts including the intestines passes through the liver. The liver also receives blood from the sinusoids. The blood passes over a high number of macrophage cells that are referred to as Kupffer cells (Ian, 2009).

The liver cells are exposed to many pathogens that come from the intestines. This has led to a unique localized immune surrounding. Numerous innate lymphocytes comprising both natural killer T cells plus natural killer cells are found within liver. Numerous non-hematopoietic hepatocytes, including sinusoidal endothelial cells, liver parenchymal cells and stellate cells located in the sub-endothelial space; act as antigen-presenting cells. The aforementioned cells present antigen in the context of inhibitory cell surface ligands and immunosuppressive cytokine (Ian, 2009).

The lymph nodes are tiny bean shaped glands which tend to occur in clusters. Lymph nodes filter the toxic materials in the lymph fluid that is transported through the lymphatic channels. The lymph node is comprised of two main parts: the cortex and the medulla. Within the cortex region, numerous lymphocytes comprised mainly B-

lymphocytes and few T-lymphocytes are found. The maturation of B lymphocytes totally occurs in the bone marrow while the T lymphocytes first leave the bone marrow in immature state and achieve maturity in the thymus. Lymph nodes act as important structures of defense against the invasion of microorganisms entering the body via the blood stream and lymphatic channels Antigen presenting cells (APC) from periphery present antigens in conjunction to Major Histocompatibility Complex (MHC) 11 to T lymphocytes. This leads to proliferation of B cells, production of cytolytic T cells and antibody producing B cells (Rastogi, 2002).

The spleen manufactures antibodies within the white pulp and eliminates infected blood cells and pathogens by way of blood and lymph node circulation. Studies in the murine model have shown that the red pulp of the spleen forms a reservoir that contains over half of the body's monocytes. The monocytes change into dendritic cells and macrophages when they move to an infected tissue where they cause healing of the tissue. It serves as a nerve centre for the mononuclear phagocyte system activities. Animals lacking spleen are vulnerable to certain infections (Mebius & Kraal, 2005).

4.2 MATERIALS AND METHODS

4.2.1 Study site

Baboon work and antibody ELISA were carried out at IPR, a biomedical Research Centre which utilizes both non-human primate models and rodents with the aim of improving human health. The facility has both outdoor and indoor housing facilities for breeding colonies of about 270 primates. IPR is recognized as a WHO collaborating Centre in Human Reproductive and Tropical Diseases Research.

Flow cytometry for cytokines was conducted at Kenya Medical Research Institute (KEMRI).

4.2.2 Experimental design

Specified groups of baboons were immunized and challenged as described in 2.4 and 2.6. The baboons were in four groups, three groups comprising of three baboons: Two experimental groups, DG (Baboons immunized with DG and then challenged), RT (Baboons immunized with RT and then challenged with *S. mansoni*), one control IC (Baboons only infected with *S. mansoni*) and one naïve group comprising of two baboons. At weeks 9, 6 and 3 before challenge; and at week 0, 2, 4 and 6 after challenge, each baboon was bled. The 3 weeks interval between time points before challenge was meant to give the immune system of the immunized groups to respond to the snail soluble proteins. The two week intervals between the time points after challenge was based on the developmental stages of the schistosome worms and the antigens produced at each stage. Some of the blood was used to prepare peripheral blood mononucleocytes (PBMC) for CD4, CD8 and CD25 flow cytometry and the rest of the blood was used for preparation of serum. The serum was used in Schistosome specific IgG and IgM Enzyme linked Immunosorbent Assay (ELISA); and cytokine flow cytometry (Th1 and Th2 cytokines). The experimental plan is shown in the Table 4.1 below.

Table 4.1: Experimental plan for immunization, challenge and sampling for serum and cells, and perfusion

Groups according to treatment	n	Sampling Time Points						
		-9wk	-6wk	-3wk	0wk	2wk	4wk	6wk
DG	3	V1 S	Bo S	Bo S	Ch/Serum S	S	S	S
RT	3	V1 S	Bo S	Bo S	Ch/Serum S	S	S	S
IC	3	-	-	-	Ch/Serum S	S	S	S
NAÏVE	2	S	S	S	S	S	S	S

KEY

- Wk – Week(s)
- DG- Baboons immunized with snail soluble proteins from the digestive gland and then infected
- RT- Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected
- IC- infected control
- n-Total number of baboons per group
- V1-Immunization
- Bo-Booster
- Ch-Challenge
- S-Sampling for serum and cells
- - No activity

4.2.3 Baboon procedures

Definitive host, intermediate host, preparations of immunizing proteins, RT and DG, immunizing of baboons, preparation of cerceriae, and challenge of baboons are as described in 2.2 to 2.6.

4.2.4 IMMUNOLOGICAL ASSAYS

Antigens for the immunological assays were purchased from Theodor Bilharzia Research Institute (TBRI), El Nil Road, Warrak el Hadar,Imbaba P.O.Box 30 Imbaba, Egypt**.** The preparation of SLAP, SWAP and SEA was done by TBRI according to their guidelines.

4.2.4.1 Schistosome Lung Antigen Preparation (SLAP)

Schistosomes were maintained in hamsters and snail species for more than 10 years according to the guidelines at TBRI. Thoroughly cleaned schistosomules were preserved at -70^{o}c in autoclaved buffers (Dulbecco pH 7.4 and 15MPBS pH 7.2 respectively) till use. The crude homogenate was utracentrifuged, and the Lipid- free clear supernatant was utrafiltered under N2 pressure and then lyophilysed in bidistilled water.

4.2.4.2 Schistosome Egg Antigen Preparation (SEA)

Schistosomes were maintained in hamsters and snail species for more than 10 years according to the guidelines at TBRI. Thoroughly cleaned parasite eggs were preserved at -70^{o}c in autoclaved buffers (Dulbecco pH 7.4 and 0.15M PBS pH 7.2 respectively) till use. The crude homogenate was utracentrifuged, and the Lipid- free clear supernatant was utrafiltered under N2 pressure and then lyophilysed in bidistilled water.

4.2.4.3 Schistosome Worm Antigen Preparation (SWAP)

Schistosomes were maintained in hamsters and snail species for more than 10 years according to the guidelines at TBRI. Thoroughly cleaned worms were preserved at -70^{o}c in autoclaved buffers (Dulbecco pH 7.4 and 0.15M PBS pH 7.2 respectively) till use. The

crude homogenate was utracentrifuged, Lipid- free clear supernatant was utrafiltered under N2 pressure and then lyophilysed in bidistilled water.

4.2.4.2 Collection of blood and preparation of serum

Baboons were anaesthetized as described in 2.2.5. Blood was collected from the femoral vein of each of the anaesthetized baboon. The first 10 ml of blood was collected in a 60 ml syringe containing 10 ml of Alseviers solution (anticoagulant; 0.055% citric acid, 0.8% sodium citrate, 0.42 sodium chloride and 0.2% dextrose). Another 5 ml of blood was collected and placed in a 15 ml centrifuge to be spent in preparation of serum. The blood for preparation of serum, was permitted to coagulate on the bench (3 h), and then stored at 4^{o}C overnight. It was centrifuged at 450 g for 10 minutes, and the clear supernatant (serum) on the top, obtained, aliquoted and stored at -20^{o}C, till use in the IgM and IgG ELISA and in cytokine flow cytometry.

4.2.4.3 Preparation of Peripheral Blood Mononucleocytes

The blood collected in anticoagulant was layered on 10 ml of Ficoll paque gradient in a 50 ml sterile tube. It was centrifuged for 30 minutes at 450 g at room temperature. The interphase band containing lymphocytes and other mononuclear cells was collected and washed two times using Incomplete medium [RPMI 1640 (Gibco BRL, Life Technologies Ltd, Scotland), containing, Gentamyicine 2mM (Sigma Co.) and 1% β-mercaptoethanol] at 300g, room temperature. After the final wash, the pellet was resuspended in 1 ml of Complete media (Incomplete media fortified with 10% Foetal Calf Serum). Lymphocyte viability was determined by the Trypan blue exclusion test. (The plasma membrane of viable cells does not permit the entry of non – electrolyte dye

substances such as Trypan blue. This phenomenon is used to distinguish dead lymphocytes from living ones). Lymphocyte suspension was mixed with Trypan blue at 1:10 ratio and incubated at room temperature for at least 5 minutes to facilitate dye uptake. After incubation, lymphocyte-Tryphan blue suspension was charged on to the haemocytometer chamber and counting was done under light microscope at x40. Cells for CD4, CD8 and CD25 flow cytometry were made up to 3 $x10^6$ cells/ml in the complete media.

4.2.4.4 Sampling procedure for mesenteric lymph nodes and spleen

Baboons were anaesthetized and the abdominal cavity opened as described in 2.7. After perfusion, mesenteric lymph nodes were excised and put in a Petri dish containing incomplete media. In addition, the spleen was removed using sterile forceps. Sections of the spleen were placed on Petri dish containing incomplete media.

4.2.4.5 Preparation of Lymph Node Cells

Mesenteric lymph nodes were picked using a pair of sterile forceps and placed in a sterile petri dish containing incomplete media. They were transferred to a sterile petri dish that had sterile medium in a sterile culture hood. The lymph nodes were teased using a pair of sharp sterile forceps and the suspension dispensed into a 15 ml tube. The cells were washed two times in incomplete media by centrifugation at 300g, room temperature for 10 minutes. The supernatant was discarded with the pellet being re-suspended in 1 ml of the complete media. Cells were counted as described in 4.2.4.3.

4.2.4.6 Preparation of Spleen Cells

A piece of Spleen from each baboon was transferred to sterilized wire gauze, in a Petri dish which had sterile incomplete media in sterile culture hood. A 10ml syringe piston was utilized to squeeze the spleen. A sterile Pasteur pipette was used to disperse the cells. The cells were then dispensed in 15ml tube and topped up with incomplete media. The cell suspension was centrifuged at 300g for 10 minutes at room temperature, supernatant discarded, and pellet re-suspended and fresh sterile incomplete media was added. This washing process was repeated. After the final wash, supernatant was discarded and cells re-suspended in 4 ml of complete medium. Lymphocyte count was carried out as described for PBMC (4.2.4.3).

4.2.4.7 CD4, CD8 and CD25 flow cytometry

Four 5 ml BD Falcon ^{TM} polysterene round bottomed tubes, 12×75 mm style, were used per each baboon. Three tubes were labelled with their specific antibody (CD4, CD8 and CD 25) respectively. The other tube was labelled blank. 4 µl of the specific antibodies were dispensed into the labelled tubes except the one labelled blank. Then 200 µl of 3×10^{6} baboon cells in complete media were added to all the four tubes. This procedure was repeated for all the animals. Each tube was mixed using vortex. The rack containing the tubes was then covered with aluminium foil, placed on ice and incubated for 30 minutes. After incubation, 200 µl of PBS was added to the suspended cell in the tubes. They were centrifuged at 300 g for 5 min for cells to pellet down at room temperature. The supernatant was discarded and the pellet was re-suspended by brushing and 200 µl of PBS added. The tubes were then centrifuged at 300 g at room temperature for 5 min. the supernatant was discarded and washing repeated. PBS was discarded and 200 µl of the

fixative added. The suspension was then loaded on BD FACSCalibur™ flow cytometer for analysis.

4.2.4.8 Determination of cytokine profiles (Th1 and Th2) using flow cytometry

All materials used in this section unless otherwise stated, were obtained from BD Biosciences (California, USA) BD cytometric bead array (CBA), human Th1/Th2 cytokine kit. This contained all the necessary reagents and capture antibodies specific for IL-2, IL-4, IL-5, IL-6, IFN-γ and TNF proteins. BD FACSCalibur™ flow cytometer, BD CellQuest™ Pro version 5.2.1 and FCap Array software version 1 were used in the analysis of cytokine profiles. BD FalconTM 12 x 75mm sample acquisition tubes for the flow cytometer and 15 ml conical propylene tubes were used.

4.2.4.8.1 Reconstitution and serial dilution of the standards

Reconstitution and serial dilution was done according to manufacturer instruction. Briefly, the lyophilized standard spheres were transferred into a 15ml conical propylene tube and labeled "Top standard". 2 ml of Assay Diluent was added to the spheres and allowed to stand at room temperature for 15 min then gently mixed using a pipette. Eight acquisition tubes were labeled in the order of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 300µl Assay Diluent was added to each. To perform the serial dilutions, 300 µl of 'top standard' was added to tube marked 1:2 and mixed using pipette, 300µl of 1:2 mixture was transferred to tube marked 1:4 and mixed. This process was continued until tube marked 1:256. One acquisition tube was reserved containing only Assay Diluent as a negative control.

4.2.4.8.2 Mixing of baboon Th1/Th2 cytokine capture beads and cytokine assay

Ten tubes were labeled and 50µl of the Th1/Th2 cytokine standard solutions prepared as described above were added to control tubes as shown in Table 4.2.

Table 4.2: Reconstitution and serial dilution of the cytokine standard solution

Tube label	Concentration (pg/ml)	Cytokine standard solution
1	0	Negative control (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8
8	1250	1:4
9	2500	1:2
10	5000	Top Standard

Baboon serum samples were retrieved and 50µl of each added to labeled acquisition tube. 50µl of human Th1/Th2 PE Detection Reagent was added to all the tubes (i.e. Th1/ Th2 cytokine standards and serum samples). This mixture was incubated in darkness for 2 h at room temperature. 1ml of wash buffer was then added to each tube and the tubes centrifuged at 200 g for 5 min then supernatant discarded. 300µl of wash buffer was then added to resuspend the bead pellet and suspension loaded on BD FACSCalibur™ flow cytometer. The data was acquired using BD CellQuest™ Pro version 5.2.1 and analyzed using FCap Array software version 1.

4.2.4.9 Schistosome Specific IgM ELISA

Nunc-Immuno™ plates (MaxiSorp™ Surface) ELISA plates were separately coated overnight at 4°C with 50 µl of, 10 µg/ml of: adult worm antigen preparation (SWAP), Schistosomule lung antigen preparation (SLAP) and Schistosomule egg antigen (SEA), diluted in bicarbonate buffer, pH 9.6. The antigens were then dispensed off on a blotting paper. The plate was washed six times using the washing buffer (0.05 % Tween 20 in phosphate buffered saline, PBS). This was followed by blocking of the non-specific binding sites with 100 µl 3% BSA in PBS for 1 h at 37°C and washing off unbound BSA six times with washing buffer. Diluted (1:200) serum samples (50 µl) were dispensed into each well in duplicates and incubated for 1 h at 37°C, and then washed as above. After washing the unbound serum, 50 µl of 1:2000 peroxidase conjugated goat anti-monkey IgM (SIGMA) was dispensed into the wells and incubated for 1 h at 37°C. The unbound conjugate was washed off as before and 50 µl SureBlue™ microwell peroxidase substrate was added in each well. The plates were incubated at 37°C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader

4.2.4.10 Schistosome Specific IgG ELISA

The procedure was as described for the IgM (4.2.4.9) except for the conjugate which was goat anti-monkey IgG (SIGMA).

4.2.5 DATA ANALYSIS

Data for the ELISA and CD4, CD8 and CD25 percentage counts was analyzed using Students t–test (Independent samples t- test) and Analysis of variance (ANOVA) / Dunnet test. Independent sample Test was done to compare the absorbance due to DG

and RT and; CD4, CD8 and CD25 percentage counts in DG and RT at all the vaccination levels (-9 to week 6) to determine whether there were significant differences between them. Students t –test is applicable for comparing means of two treatments. ANOVA/ Dunnet test was done to compare the absorbance of DG, RT and IC; CD4, CD8 and CD25 percentage counts to determine whether there were significant differences between and within the groups. Because IC was only introduced from week 0 to week 6, Dunnet test captured data from challenge to week 6. Analysis of variance (ANOVA) is used to compare more than two means. Significance level used in calculation was $p<0.05$. The cytokine data was acquired using BD CellQuest^{TM} Pro version 5.2.1 and analyzed using FCap Array software version 1.

4.3 RESULTS

4.3.1 CELLULAR IMMUNE RESPONSES

4.3.1.1: CD4, CD8 AND CD25 COUNTS

Figure 4.1 shows the percentage, CD4, CD8 and CD25 counts for the DG, RT, IC and Naïve groups of baboons. Before vaccination the percentage CD4 count for both DG and RT was at the background and similar to the negative control. Three weeks after vaccination, there was a significant increase of CD4 for both DG and RT, which is the peak response. The response declined after the first and second booster. At week 2 pc, the counts slightly increased. At week 4 pc, the response declined but it was elevated at week 6 pc.

DG had significantly higher percentage CD4 counts than RT after the 1st, ($p<0.05$). RT had a significantly higher percentage than DG after vaccination, and at week 4 pc ($p<0.05$). At all other time points, DG and RT had similar counts ($p>0.05$).

The percentage CD4 count for IC at challenge was higher than for the negative control. There was a steady, gentle increase from the background level at challenge up to week 6 pc when a peak count was noted.The two immunized groups DG and RT had significantly lower CD4 percentage counts than IC at week 4 and 6 pc (p<0.05).

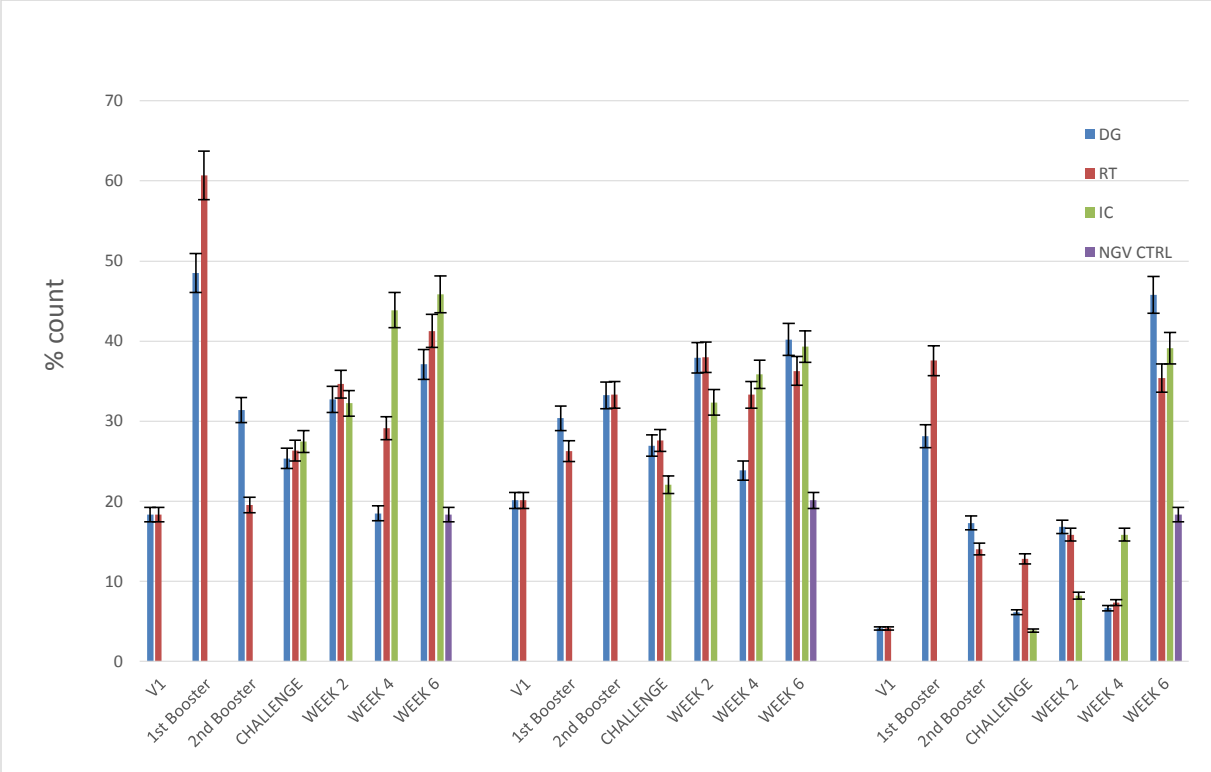


Figure 4.1: CD4, CD8 AND CD25 Percentage Counts

Key: DG-Baboons immunized with snail soluble proteins from the digestive gland and then infected**; RT-** Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC**-Infected control; **NGV CTRL**- Negative control.

The percentage CD8 count for both DG and RT before vaccination was low and similar to that of the negative control. There was a percentage increase in CD8 counts for both DG and RT at week 3 after vaccination and 3 weeks after the 1st booster. However, at week 3 after 2nd booster, the percentage counts slightly decreased. At week 2 pc, the

percentage counts slightly increased and then declined at week 4 pc. However, the percentage count was elevated at week 6 pc.

The percentage CD8 count for IC at challenge was low and slightly higher than for the negative control. There was a steady increased percentage counts from week 2 to a peak at week 6 pc. At challenge and 2 weeks pc, IC was significantly lower than DG and RT; at week 4 pc IC was significantly higher than DG ($p<0.05$), but at week 6 pc, the 3 groups had similar counts ($p>0.05$). DG CD8 counts after vaccination was significantly higher than RT ($p<0.05$) while at week 4 post challenge, RT counts were significantly higher than DG at week 4 pc ($p<0.05$). At all the other time points, they had similar counts ($p>0.05$).

The percentage CD25 count for DG and RT before vaccination was lower than that of the negative control. The percentage counts increased sharply three weeks after vaccination. There was a steady decline up to challenge i.e 3 weeks post 2^{nd} booster. The percentage count increased at week 2 pc and then declined markedly at week 4, but there was a sharp increase at week 6 pc. CD 25 counts were significantly higher in RT than in DG at 3 weeks after vaccination and 3 weeks after the 2^{nd} booster ($p<0.05$). CD 25 counts were significantly higher in DG than RT at 3 weeks after 1^{st}booster and week 6 pc.

The percentage CD25 count for IC at challenge was at the background level and below that of the naive. The percentage count increased steadily at week 2, 4 and 6 pc. Week 6 pc was the peak percentage count. There was a significantly higher difference noted between the two immunized groups and IC at challenge and week 2 pc ($p<0.05$). CD25 percentage count in DG was significantly higher than that of RT and IC at week 6 pc

($p<0.05$). IC CD25 was significantly higher than the two immunized groups at week 4 pc ($p<0.05$).

Figure 4.2 shows the percentage CD4, CD8 and CD25 counts in the blood, spleen cells and mesenteric lymph node cells in DG, RT and IC at week 6 pc. The percentage CD4 blood count for DG was lowest in the mesenteric lymph node cells and highest in the blood. There was no significant difference in CD4 cell counts between the blood, the spleen and the mesenteric lymph nodes ($p>0.05$). In the RT group, the percentage CD4 cell count was lowest in the spleen cells and highest in the mesenteric lymph node cells. There was a significantly higher difference in CD4 cell counts between the blood and the spleen cells, and between mesenteric lymph node cells and the spleen cells ($p<0.05$).

In the IC group, the percentage CD4 blood count was lowest in the spleen cells and highest in the blood. There was a significant difference in CD4 cell counts between the blood and the spleen cells, blood and the mesenteric lymph node cells and between mesenteric lymph node cells and the spleen cells ($p<0.05$). CD4 counts in spleen were similar for DG and RT ($p>0.05$) and both were significantly higher than IC ($p<0.05$).

The CD4 counts for mesenteric lymph node cells were significantly different for the 3 groups, with highest being RT and lowest DG.

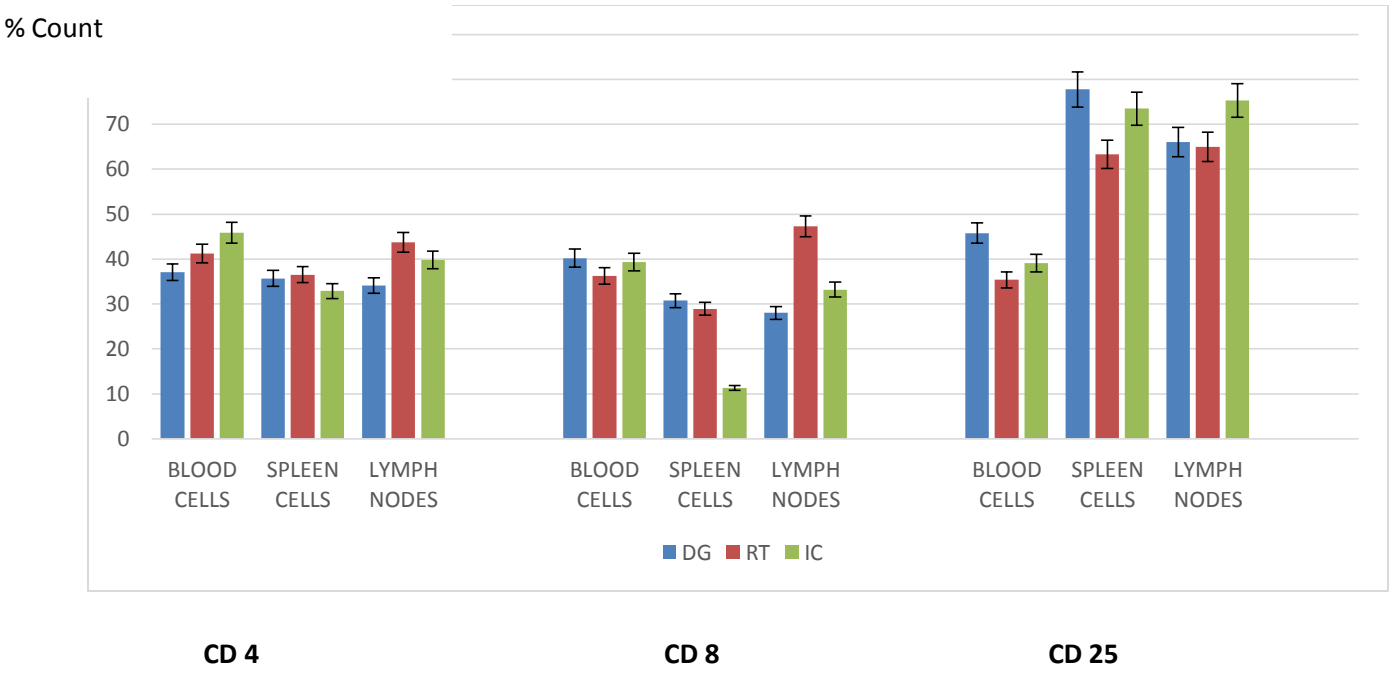


Figure 4.2: CD4, CD8 and CD25 Percentage Counts in the Blood, Spleen and Lymph Node Cells at Week 6 Post Challenge

Key: **DG**-Baboons immunized with snail soluble proteins from the digestive gland and then infected**; RT-** Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC**-Infected control.

The percentage CD8 counts in the blood, spleen cells and mesenteric lymph node cells in DG, RT and IC at week 6 post challenge. In the DG group, the percentage CD8 blood count was lowest in the mesenteric lymph node cells and highest in the blood. There was a significant difference among the 3 tissues ($p<0.05$). In the RT group, the percentage CD8 cell count was lowest in the spleen cells and highest in the mesenteric lymph node cells. There was a significant difference in CD8 counts among the 3 tissues ($p<0.05$). In the IC group, the percentage CD8 blood count was lowest in the spleen cells and highest in the blood. There was a significant difference in the percentage CD8 cell counts among the 3 tissues ($p<0.05$).

Blood CD8 cell counts were similar in the 3 groups (p>0.05). Spleen CD8 counts were similar between DG and RT (p>0.05) and the two were significantly higher than IC (p<0.05). Lymph node CD8 cells in mesenteric lymph node were significantly different among the groups (p<0.05) with RT having the highest count and DG having the lowest.

The percentage CD25 counts in the blood, spleen cells and mesenteric lymph node cells in DG, RT and IC at week 6 pc. In the DG group, the percentage CD25 count was lowest in the blood and highest in the spleen cells. There was a significant difference in the percentage CD25 cell counts among the 3 tissues (p<0.05). In the RT group, the percentage CD25 cell count was lowest in the blood and highest in the mesenteric lymph node cells. CD25 cell counts in spleen and mesenteric lymph node were similar, (p> 0.05) and these 2 were significantly different from CD25 cells in blood (p<0.05). In the IC group, the percentage CD25 cell count was lowest in the blood and highest in the mesenteric lymph node cells. CD25 cell counts in spleen and mesenteric lymph node were similar, while the two were significantly different from CD25 counts in blood (p<0.05). CD25 counts in blood were similar between RT and IC (p>0.05) and both significantly lower than DG. CD25 counts in the spleen were similar between DG and IC (p>0.05) and both were significantly higher than RT. CD25 cells in mesenteric lymph nodes were significantly similar between DG and IC (p>0.05), but different from RT ((p<0.05).

4.3.1.2 Cytokine level

Figure 4.3 shows Interleukin 5 (IL-5) and Interleukin 6 (IL-6) concentration in the three groups of baboons; DG, RT and IC. IL-5 levels for the two experimental groups, DG and RT and the control group IC were low at all the sampling points, except at week 6 pc

when the concentration was greatly elevated in all the three groups. The IL-5 concentration in the two immunized groups was significantly similar to each other ($p>0.05$) but significantly higher than IC at week 6 post challenge.

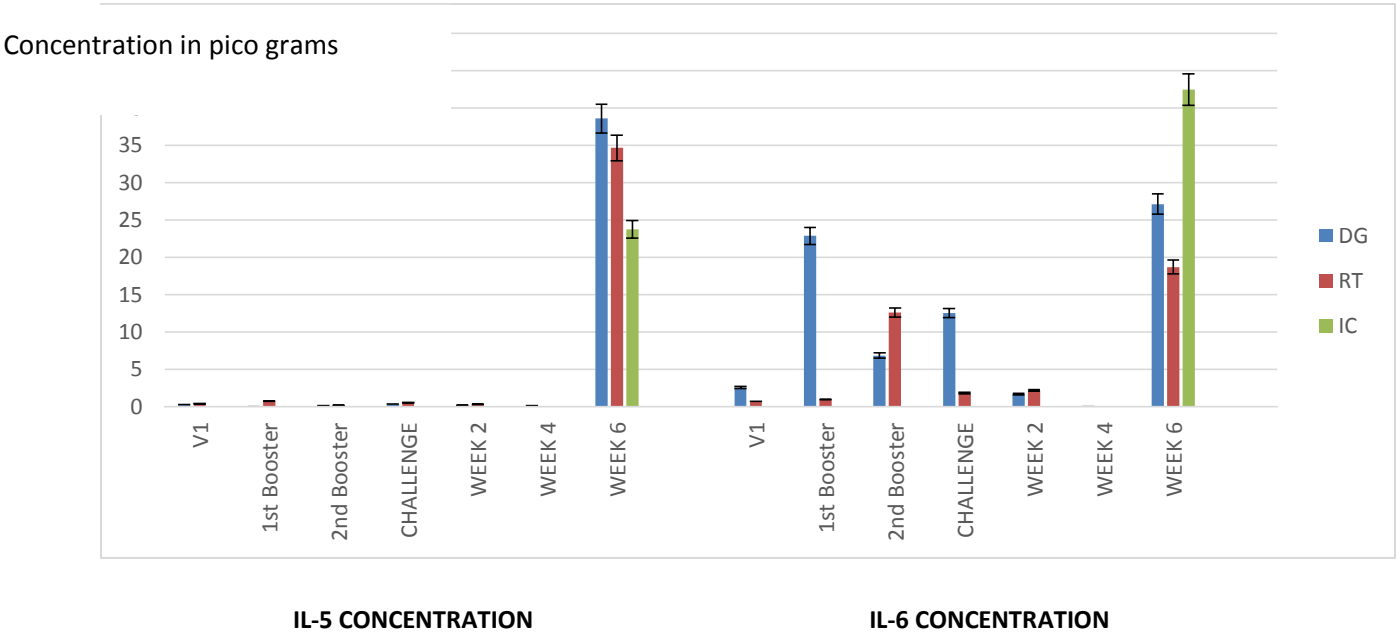


Figure 4.3: IL-5 and IL-6 Concentration

Key: DG- Baboons immunized with snail soluble proteins from the digestive gland host and then infected; **RT-**Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC**- Infected control.

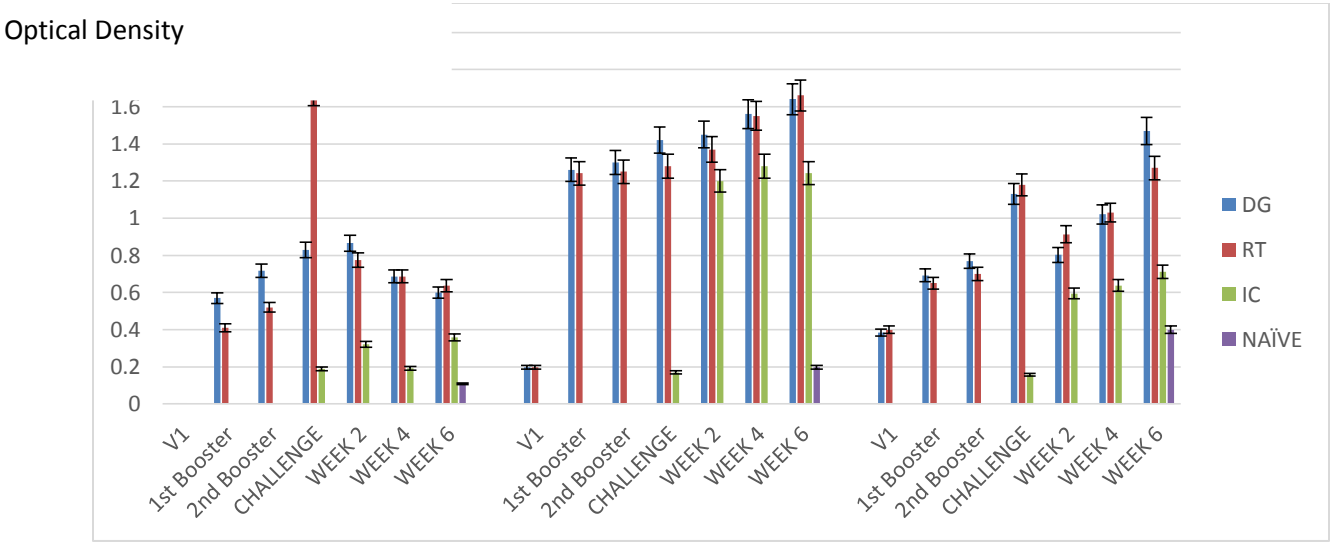
The IL-6 concentration for DG was low before vaccination but increased remarkably three weeks after the vaccination. Three weeks after the1st booster, IL-6 concentration had declined, but it increased slightly after the 2nd booster. At Week 2 pc, the IL-6 concentration sharply decreased. At week 4 post challenge, the IL-6 concentration was not detectable. However, at week 6 post challenge, the IL-6 concentration was greatly elevated.

In RT group, the IL-6 concentration was at the background before vaccination and at three weeks after vaccination. However, the concentration was elevated at three weeks after the first booster. Three weeks after the 2^{nd} booster the concentration declined to low level and the concentration remained low at week 2 pc. At week 4 pc the IL-6 was not detectable. However, at week 6 pc, the concentration was greatly elevated. In the IC group, IL-6 concentration remained at the background at challenge and week 2 pc and was not detectable at week 4. However at week 6 pc, the concentration was greatly elevated. DG IL-6 concentration was significantly higher than RT at 3 weeks post vaccination ($p < 0.05$), 3 weeks post 2^{nd} booster, and week 6 post challenge. RT IL-6 concentration was significantly higher than DG at week 3 post 1^{st} booster only ($p<0.05$). IC was significantly higher than DG and RT at week 6 pc ($p<0.05$). IL-2, IL-4, INF-γ, and TNF_B although tested, they were not detected.

4.4 HUMORAL IMMUNE RESPONSES

Figure 4.4 show SLAP, SWAP and SEA specific IgM response for the four groups of baboons; DG, RT, IC and Naive. The SLAP IgM response for both DG and RT was at the background level but higher than that of the negative control before vaccination. The SLAP IgM response was elevated three weeks after vaccination, and then there was a sharp increase to a peak at week 2 post 2^{nd} booster for RT; and a gentle increase to a peak at week 2 pc for DG. A gentle decline was observed for DG but still a remarkable level at week 6. In the RT, there was a sharp decline at week 2 pc, and the decline continued gently to week 6.

The SLAP IgM trends for the two immunized groups were similar; however, DG responses were significantly higher than RT at week 3 post vaccination, and at week 3 post 1st booster. RT responses were significantly higher than DG at week 3 post 2nd booster ($p>0.05$). IC SLAP IgM responses were significantly lower than the 2 immunized groups at all sampling points ($p<0.05$).



SLAP Schistosome **SWAP Schistosome** **SEA Schistosome**

Figure 4.4: SLAP, SWAP and SEA Schistosome Specific IgM Responses

Key: DG-Baboons immunized with snail soluble proteins from the digestive gland and then infected**; RT-** Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC**-Infected control; Naive- Negative control.

The SWAP specific IgM response for both DG and RT was marked by a sharp increase from background level at 3 weeks post vaccination. For both groups, there was a gentle increase to a peak at week 6 pc. There was no significant difference ($p>0.05$) when the DG and RT were compared at all sampling time points. In the IC group, there was a sharp increase in SWAP specific IgM response from the background level at challenge to week 2 pc. This response remained more or less the same up to week 6 pc. The response for the

2 immunized groups were significantly higher at all sampling time points post challenge, when compared to IC ($p<0.05$).

SEA specific IgM responses for DG and RT before vaccination were higher than that of the Naïve. For both DG and RT, there was a steady increase after vaccination, to the first lower peak at 3 weeks post 2nd booster. There was a decrease noted at week 2 pc then again a steady rise and the second higher peak at week 6 pc. There was no significant difference in response between DG and RT at all sampling time points except at 2 weeks pc where RT had significantly higher responses and at week 6 pc when RT had higher responses ($p<0.05$). In the IC group, SEA specific IgM responses rose sharply at week 2 pc from the background level at challenge; there was a gentle increase to week 6 pc. There was a significant difference between the two immunized groups and IC ($p<0.05$) at all-time points post challenge.

Figure 4.5 shows SLAP, SWAP and SEA specific IgG response in the 4 groups of baboons; DG, RT, IC and Naïve. Before vaccination, DG and RT responses were similar to Naïve. For both DG and RT, there was an extremely sharp increase in response at week 3 post vaccination from a very low background. The responses rose gently to a peak at week 4 pc, then there was a gentle decline, to still high response at week 6 pc. There was no significant difference noted between DG and RT ($p>0.05$) at all the sampling points. In the IC group, the IgG responses rose sharply from the background at challenge to week 2 pc. The level then slightly rose to a peak at week 4pc and gently declined at week 6 post challenge. The 2 immunized groups had significantly higher responses than IC at all sampling time points ($p<0.05$).

Optical Density

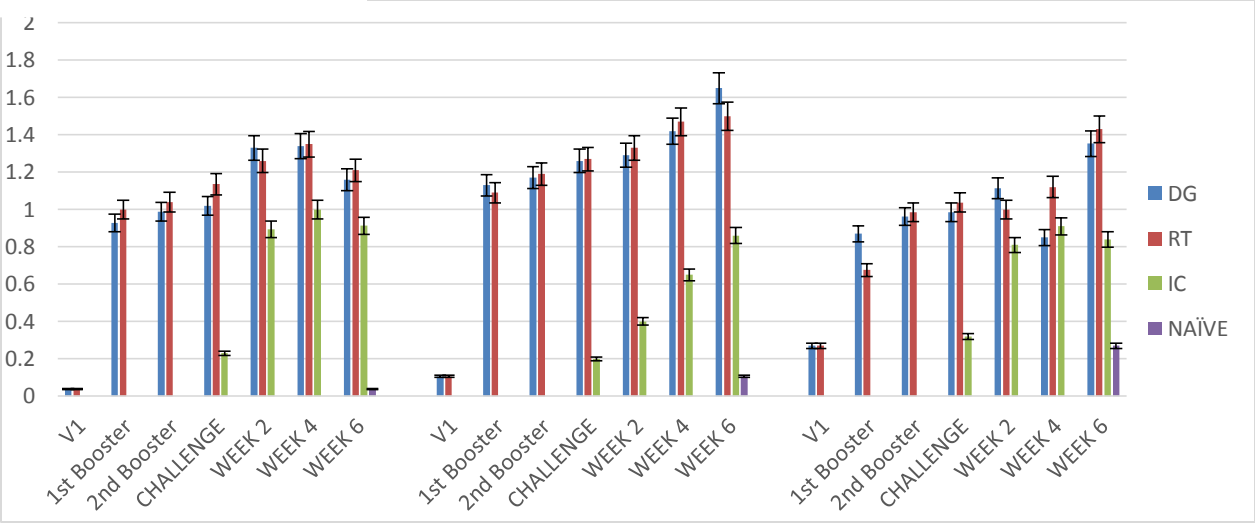


Figure 4.5: SLAP, SWAP and SEA Schistosome Specific IgG Responses

Key: DG-Baboons immunized with snail soluble proteins from the digestive gland and then infected**; RT-** Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC-**Infected control; Naive**-** Negative control

SWAP specific IgG response in DG and RT responses were slightly higher than that of the Naïve before vaccination. For both DG and RT, there was an extremely sharp increase to 3 weeks post vaccination, then a gentle increase to a peak at week 6 pc. No significant difference was noted between DG and RT at all sampling time points (p>0.005) except at week 6 post challenge where DG was significantly higher than RT (p<0.05). In the IC group, there was a gentle and remarkable SWAP specific IgG increase from week 2 pc to a peak at week 6 pc. The IgG response was significantly higher in the two immunized groups when compared to IC at all sampling time points (p<0.05).

SEA specific IgG responses for DG and RT were higher than that of the Naïve control before vaccination. For both DG and RT, there was a sharp increase 3 weeks after vaccination. There was a gentle, steady increase in response to a first peak for DG at week 3 post 2nd booster. RT attained a peak at week 6 pc. DG response declined at week

4 pc then rose to a second peak at week 6 pc. DG responses were significantly higher than RT at week 3 post vaccination only, while RT was significantly higher than DG at week 4 pc ($p<0.05$).

In the IC group, there was an elevated SEA specific IgG response from the background level at challenge to week 2 pc. There was then a steady gentle increased response to week 4 post challenge. This was followed by a gentle but insignificant decline ($p>0.05$) at week 6 pc. There was a significantly higher difference between the two immunized groups when compared with the IC at all sampling time points except week 4 pc ($p<0.05$).

4.3 DISCUSSION

4.3.1 CELLULAR IMMUNOLOGICAL RESPONSES

In the mouse model of human schistosomiasis, a polarized Th1 environment is necessary for induction of immunity against schistosomiasis (Zhang *et al.,* 2011, Bergquist *et al.,* 2008, Reis *et al.,* 2008, Coulson 1997). In the mouse model, it was shown that for resistance to *S. mansoni* secondary infection to develop, IL-4 and other Th2 cytokines were needed (Brunet *et al.,* 1999).

4.3.2 CD4, CD8 (Cytotoxic T cells) and CD25 (Memory B & T cells) counts.

Vaccination resulted in elevation of CD4 percentage counts in both DG and RT, with RT having a significantly higher count than DG ($p<0.05$). The two boosters did not increase the percentage CD4 counts but challenge resulted in increase in percentage counts. Vaccination resulted in sensitization of CD4 cells. This resulted into memory population as seen by the increase after challenge. CD4+ T cells are divided into two subsets; Th1

and Th 2. The role carried out by the two subsets is distinct. Th1 is involved in delayed type hypersensitivity responses and Th2 is mainly concerned with antibody synthesis (DeKruyff, 1989). CD4 cells are involved in cellular responses therefore DG and RT are inducing cellular immune responses. This work agrees with a study done in the mouse model of human schistosomiasis. The study revealed that CD4+CD25+ Treg cells played a central function in schistosomiasis because they serve as the origin for IL-10 which is associated with prevention of pathology (Hesse *et al.,* 2000).

Vaccination causes an increase in CD8 in both DG and RT and the first booster resulted into a further increase in both. An increase is further noted after challenge. However, the increase was not dramatic as compared to CD4. The role of CD8 in schistosome elimination has been recently described (Zhou *et al.,* 2012). This implies that the two soluble proteins offered protection to the animals. The current study is also in agreement with work done in murine model of human *schistosoma japonicum* that were immunized with the *S. japonicum* 22.6/26GST combined with Sepharose 4B bead. Low worm burden was linked to an elevated number of activated CD8+ cells (Zhou *et al.*, 2012).

Vaccination causes a remarkable increase in percentage CD25 cells. The first booster, second booster and challenge do not result into a similar increase. However, at week 6 pc, there was a remarkable increase in CD8 counts in all the 3 groups. This agrees with their function of memory. CD25 is the alpha chain of the IL-2 receptor.https://en.wikipedia.org/wiki/CD25 - cite_note-1 In mice, CD25 has been used as a marker to identify CD4+FoxP3+ regulatory T cells though a big part of resting memory T cells have shown to express CD25 in humans (Triplett *et al.,* 2012*).*

4.3.3 CD4, CD8 and CD25 percentage counts in the blood, spleen cells and mesenteric lymph node cells at week 6 pc

There was no remarkable difference in CD4 percentage counts among the three tissues in the 3 groups. However, RT had relatively higher counts in the 3 tissues. IC had elevated CD4 percentage counts in the peripheral blood due to egg and worm antigens released into the general circulation. In the primary lymphoid organs such as the lymph nodes and the spleen, mature naïve CD4+ T cells frequently seek for MHC II molecules, for antigen recognition (Drayton *et al.*, 2006).

The CD8 percentage count was significantly high in the blood in DG. However, the level was similar in the spleen and mesenteric lymph nodes. In RT, the CD8 count was significantly higher in the lymph nodes. Lymph nodes have a dense population of macrophages and lymphocytes along the lymphatic vessels (Pabst, 1988). They encounter the parasite antigens before they get into the general circulation and this could probably have caused the elevated response. In IC, the CD8 count was significantly higher in the blood and mesenteric lymph node cells. CD25 is high in both spleen cells and mesenteric lymph node cells in all the 3 groups. It is significantly higher in the mesenteric lymph node cells and spleen cells compared to the blood. Memory cells are found in the tissues.

4.3.4 Cytokine levels

IL-5 is elevated at week 6 pc and is significantly higher for both DG and RT as compared to IC. IL-5 is a Th2 cytokine involved in antibody production. At week 6 pc, we have worm antigens and the fact that the response for DG and RT is higher than IC, shows that vaccination is stimulating Th2 cells. In man, non- human primates and rats, protection against schistosomiasis is linked to Th2 responses that involve IL-4, IL-5 production,

IgE, IgG and eusinophils. This varies with infection in mice where protection is associated with Th1 responses (IL-1, interferon gamma and IL-12) (Pearce & Sher., 1991). There appears to be a link between IL-5 secretion and proliferation of lymphocyte to specific antigens and resistance to re-infection in man (Roberts *et al.*, 1993).

IL-5 is an IgA-promoting factor important in antibody immunity and a B-cell growth factor (Yokota *et al*., 1987). The IL-5 level for the two immunized groups was greatly elevated at week 6 pc. The IL-5 level in the two immunized groups was significantly higher than in IC. These results show that immunization stimulated IL-5 production and therefore invoked Th2 immune protection in baboons. Similar responses have been observed in primates, humans and rats (Pearce & Sher., 1991).

Results from this work agrees with findings from infected Balb/c mice that were immunized with snail soluble proteins where one of the group vaccinated with foot protein showed secretion of IL-5, an indication of immunity against the infection (Kuria *et al.,* 2012). The results further agree with those of another study where Swiss white mice were immunized with snail soluble proteins and challenged with *S. mansoni*, the experimental groups showed a significant production of IL-5 (Kobia *et al.,* 2011).

In DG, vaccination caused a higher and faster production of IL-6. However, in RT, the response was delayed and was only remarkable after the 2^{nd} booster. DG had a significantly higher response than RT at all the sampling time points. Production of egg antigens caused an elevated response in all the 3 groups at week 6 pc. IL-6 cytokine has many functions and is mainly involved in inflammatory responses and in B lymphocytes maturation into antibody-producing plasma cells (Kishimoto, 1989).

4.4. HUMORAL RESPONSES

4.4.1 IgM Responses

SLAP schistosome specific IgM response showed that vaccination resulted into an elevated response with RT having a significantly higher response at challenge than DG. For both experimental groups, the response slowly declined up to week 6 pc but not to the base line levels. IgM responses in IC were no remarkable. IgM classes of antibodies make up the largest part of the antibodies that occur naturally. They are the first antibodies that are produced in response to a primary infection. Unlike other antibodies, IgM antibodies are secreted in large numbers by B1 cells without any antigenic induction (Boes, 2000). However with time, it wanes off. This agrees with a study in chimpazees immunized with radiation-attenuated vaccine that elicited increased levels of antigen-specific immunoglobulin M (IgM) and IgG when compared with the control group (Matthias *et al*., 2001).

SWAP schistosome specific IgM responses showed that vaccination resulted to a remarkable increased response in the two immunized groups. The two boosters and challenge resulted into gradual increase to a peak at week 6 pc. This signifies shared antigens between the early stage schistosomules, adult and egg antigens. Responses for IC are significantly lower than both the two experimental groups. SEA schistosome specific IgM responses in this study showed that vaccination caused a remarkable response but not like for SLAP and SWAP for the two experimental groups. The two boosters resulted into an increase in response. Challenge did not have much effect as immunization but a peak was attained at week 6 pc due to production of SEA and adult worm antigens. IC had remarkable IgM responses at week 2 pc. This level remained the

same and a peak response was attained at week 6 but lower than the two immunized groups. There was a significant difference between the two immunized groups and the IC at all sampling time points.

The best IgM responses were obtained by using SWAP indicating shared antigens between all schistosomules stages. The highest response was recorded with SEA, this is expected, as this corresponds with egg production at week 6 pc (Grzych *et al.,* 1991). IgM SLAP responses were the lowest. This is expected because schistosomules do not share as many antigens as the other schistosome stages

4.4.2 IgG Responses

For the three antigens i.e SLAP, SWAP and SEA, there was a remarkable increase in the IgG responses in the 3 groups after vaccination and then a gradual increase to peak at week 4 for SLAP and week 6 for SWAP and SEA. DG and RT responses are stimulating the production of IgG. The boosters are giving further stimulation. The response for SWAP and SEA is highest at week 6 and this compares very well with high concentrations of IL-5 and IL-6 at this time point. The two cytokines are involved in stimulating B cells to produce antibodies.

IgG is involved in antibody-dependent cell-mediated cytotoxicity (ADDC) with neutrophils, macrophages and eusinophils which are damaging to the schistosomulea stage (Hagan *et al.,* 1998). Different antibody isotypes may have different effects in *S. mansoni* infection. IgG2 and IgG4 block or compete with IgE which is associated with resistance to infection. IgG, IgE and IgA are important in protection, acting at several stages to prevent invasion, migration, and development and egg production (Hagan *et al.,* 1998).

SLAP is prepared from the larval worms; its concentration in circulation under normal circumstances is highest soon after infection (week 2 post challenge) and low at week 4 when larval worms begin to mature into adults (Mahmood and Wanab, 1990). The slight increased response in RT and IC at week 4 could have been caused by cross-reactive antigens shared between worm tegument and schistomula surface (Yi *et al*., 1986; Simpson, 1990; Yole *et al*., 1996). In a study on the effects of immunizing Swiss white mice with snail soluble proteins and challenging them with *S. mansoni*, the group that was immunized with snail soluble proteins from the rest of the body tissues (RT) produced higher Immunoglobin G (IgG) responses than DG and IC. This indicated that it killed more schistosomes using antibody dependent cell-mediated cytotoxicity (ADCC) (Kobia *et al.*, 2011).

CHAPTER FIVE

5.0 GROSS AND HISTOPATHOLOGY OF LIVER TISSUES FROM OLIVE BABOONS IMMUNIZED AGAINST *S. mansoni* WITH SNAIL SOLUBLE PROTEINS

5.1 INTRODUCTION AND LITERATURE REVIEW

It is within the portal vasculature where mature *S. mansoni* worms are found. Each female schistosome lays about three hundred eggs in day that are intended to be passed out of the body to the environment for transmission. However, some eggs are retained in the body. All the blood from the systemic circulation passes through the liver via the hepatic portal vein. Numerous schistosome eggs carried in the blood get trapped in the sinusoids. The eggs secrete soluble antigens that elicit a robust Th2 immune response that bring about the formation of granulomas in the in the liver (Amiri *et al.,* 1992). A study conducted in a mouse model using human parasites demonstrated the protective role of granulomas in the host. Infected mice deficient of CD4 cells lack the ability to make granulomas and end up dying as a result of harmful effects on liver cells (Cheever *et al.,* 2000*;* Dunne & Doenhoff, 1983). The granuloma surrounds the egg and separates it from the uninfected liver tissue creating room for the infected liver tissue to function. In the course of time, schistosome eggs perish and the granulomas resolve. This can lead to fibrosis (Amiri *et al.,* 1992). Fibrosis can cause high portal blood pressure and finally lead to the development of portal varices.

5.1.1 Hepatic schistosomiasis

The host's granulomatous cell-mediated immune response to the soluble egg antigen of *S. mansoni* results in Hepatic schistosomiasis. The responses normally progress to

irreversible fibrosis and finally to severe portal hypertension (Van der *et al*., 2003). Schistosome eggs remain viable in the liver for approximately 3 weeks. Primarily, the eggs induce a moderate type 1 helper (Th1) response to egg antigens. However, this usually evolves to a dominant Th2 immune response to egg-derived antigens with later recruitment of eosinophils, granuloma formation and fibrogenesis of the liver (Wynn *et al*., 2004; Wilson *et al.*, 2007).

5.1.2 Granuloma formation

The formation of granuloma is advantageous to the host for it blocks the hepatocytes from the toxic antigenic effects secreted from parasite eggs. However, the hosts' immunological reactions to the soluble egg antigens may result in fibrosis with high accumulation of collagen fibres and extracellular matrix proteins within the periportal space (Morais *et al.,* 2008). The formation of granuloma is mediated by helper T cell-delayed hypersensitivity reaction. This reaction is brought about by cytokines such as interleukin-4 (IL-4) and IL-13 whereas schistosomal induced pathology is limited by IL-10, IFN-γ, and a subset of regulatory T cells. In addition, a variety of cell types have been implicated, including activated macrophages, and regulatory T cells and hepatic stellate cells (Gryseels *et al.,* 2006). The extent of the pathology and the development of fibrosis is dependent on the balance between TH1- and TH2-type cytokines (Stadecker *et al*., 2004). Eggs are detectable inside the granulomas with the subsequent formation of marked portal and peri lobular fibrosis, which is most pronounced with *S. mansoni* and *S. japonicum*.

5.2 MATERIALS AND METHODS

5.2.1 Baboon procedures

Definitive host, intermediate host, preparations of immunizing proteins, RT and DG, immunizing of baboons, preparation of cerceriae, challenge and perfusion of baboons are as described in chapter 2. 2. to 2.7.

5.2.2. PATHOLOGICAL EXAMINATION

5.2.2.1 Gross Pathology

Gross pathology examination was done before perfusion. It focused on the general and overt appearance of the liver. The observations that were considered in the liver include: inflammation, adhesions and presence of granulomas. Granulomas appear as raised pinheads sized foci spread over the surface of the liver lobes. 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and $\geq$ 10 granulomas per lobe was considered severe.

5.2.2.2 Histopathology

Baboons were anaesthetized and the abdominal cavity opened as described in 2.7. After perfusion, the liver tissues were fixed immediately in 10% buffered formalin for fortnight. The fixed tissue samples of liver sections were transferred into tissue cassettes and then immersed into 80%, 95% and 100% ethanol consecutively to achieve optimum dehydration. The tissues were cleared in toluene and then infiltrated in hot paraffin. The tissues were then embedded on tissue- embedding paraffin wax (Sherwood Medical Co. USA). The tissues were then sectioned serially at 6 microns using a Rotary microtome (Leitz, Germany). The thin tissue sections were mounted on glass slides and stained with

Haemotoxylin and Eosin. They were observed under the light microscope and only granulomas containing an ovum at the centre were enumerated and measured at x100 magnifications using calibrated ocular micrometer. Granuloma size was measured based on the vertical and horizontal diameters. The averages of the horizontal and vertical diameter were taken to be the granuloma diameter (Farah *et al.,* 2000). Only ten 10 granulomas per sample were measured. Histological liver abnormalities such as fibrosis and periportal infiltration were also noted. Photomicrographs of the thin tissue sections with granuloma and other liver anomalies were taken at x400 using a microscope (Leica ICC 50) that comes with its software in a CD drive which is installed in the computer to allow connection between the microscope and the computer.

5.2.3 Statistical analysis

Data was analyzed using Analysis of variance (ANOVA) - Dunnet test, to compare granuloma sizes among DG, RT and IC. Analysis of variance (ANOVA) is used to compare more than two means. Significance level/probability level used in calculation was $p<0.05$.

5.3 RESULTS

5.3.1 Pathological findings

5.3.1.1. Gross pathology

A summary of gross pathology is shown in Table 5.1. Gross pathology was done by physical observation of the liver surface to detect adhesions; inflammation and presence of granulomas. In DG, adhesions were absent while in RT they were present in only one animal. However, adhesions were observed in all the members of IC. Inflammation in the liver was categorized as no inflammation, moderately inflamed and severely inflamed. In

DG, two animals had moderate inflammation and the other one had no inflammation. In RT, all the baboons had moderately inflamed livers. In the IC, two baboons had severe inflammation while one had moderate inflammation. Granulomas are tiny pin head sized foci on the surface of the liver. 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and ≥ 10 granulomas per lobe was considered severe. In DG, two animals had moderate granulomas while one had few granulomas. In RT, all the baboons had moderate granulomas. In IC, two baboons had severe granulomas while one had moderate granulomas.

Table 5.1: Gross pathology of baboons immunized with soluble proteins from *Biomphalaria Pfeifferi* and challenged with *Schistosoma mansoni*

GROSS PATHOLOGY	GROUPS ACCORDING TO TREATMENT								
	DG			RT			IC		
	1	2	3	1	2	3	1	2	3
Adhesion	A	A	A	A	P	A	P	P	P
Inflammation	M	N	M	M	M	M	S	M	S
Granuloma	M	F	M	M	M	M	S	M	S

Key: DG- Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **RT**-Baboons immunized with snail soluble proteins from the rest of the body tissues and then infected; **IC**- Infected control. **A**-Absent, **P**-present, **M**-moderate, **S**-severe, **F**-few, **N**-none

5.3.1.2 Histopathology.

Figure 5.1 shows the granuloma sizes in the three groups. Among the three groups, DG had the average granuloma size of 25.7 $\pm$ 0.82 μm followed by the RT group whose

average granuloma size was 28.57 ± 1.87 μm. The IC had the largest average granuloma size of 34.7 ± 3.54μm. The average granuloma size for DG and RT were similar ($p>0.05$). There was a statistically significant difference between the two immunized groups and the IC ($p<0.05$).

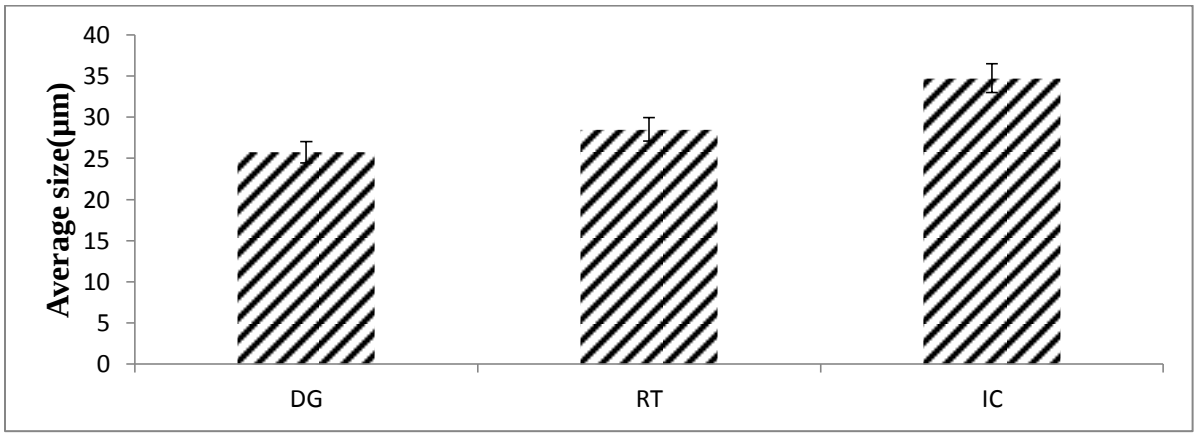


Figure 5.1: Granuloma Size in Baboons Immunized with snail soluble Proteins and Challenged with *Schistosoma mansoni*

Key: RT- Baboons immunized with snail soluble proteins derived from the rest of the body tissues and then infected; **DG-**Baboons immunized with snail soluble proteins from the digestive gland and then infected**; IC-**Infected control.

Photomicrographs of the liver (x 400) were observed from animals in DG group (Fig. 5.2.1). It shows a classical portal triad. A portal triad is composed of three major tubes. Branches of the portal vein that carry blood with nutrients from the small intestines, the bile duct which carries bile products away from the hepatocytes, to the larger ducts and gall bladder and the hepatic artery, which supplies the liver with blood from aorta.

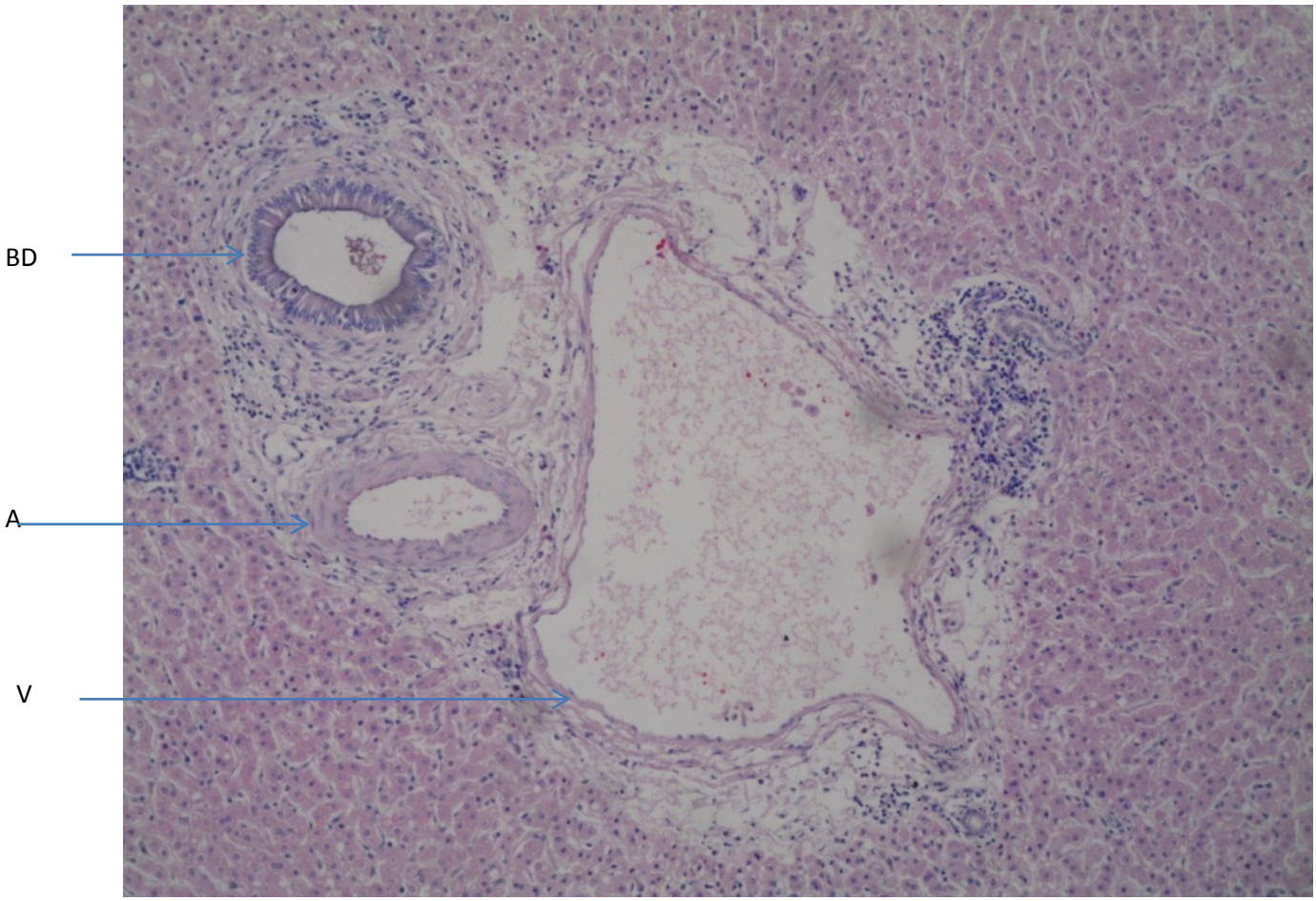


Figure 5.2.1: A section from DG showing a classical portal triad (x 400)
KEY: A-Artery **BD**-Bile duct **V**-Vein

Photomicrograph of sections of liver (x 400) from animals in RT showing a centrally placed *S. mansoni* egg and a portal triad both with cellular infiltration around the bile duct (Fig. 5.2.2). Florid granulomas with a centrally placed conspicuous egg at the centre are characteristic of the acute phase of infection of *Schistosoma mansoni.*

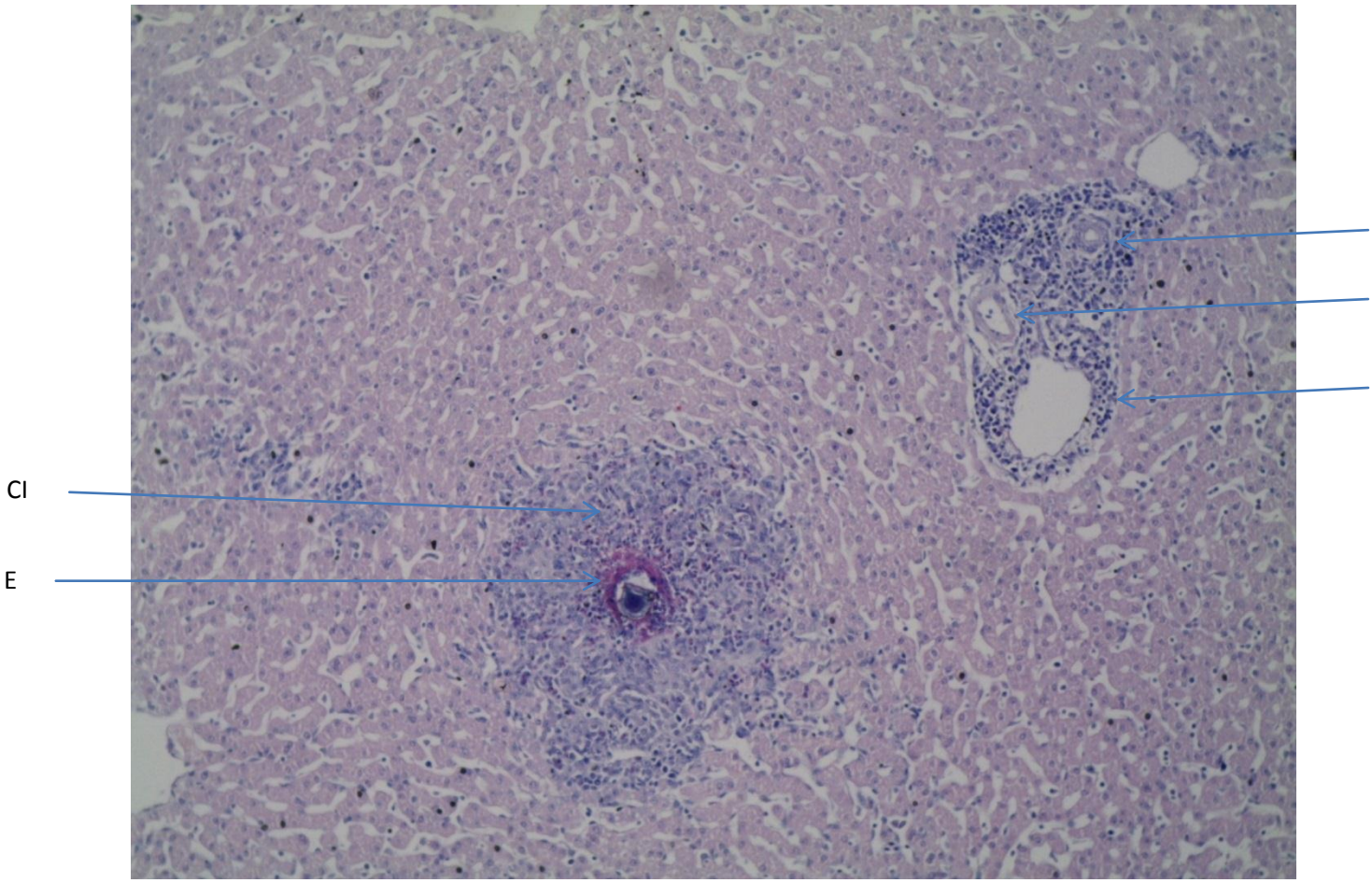


Figure 5.2.2: A section from RT showing a centrally placed *S. mansoni* egg and a portal triad with infiltrated bile duct (x 400)

KEY: A-Artery BD-Bile duct E-Egg CI-Cellular infiltration V-Vein

Figure 5.2.3 shows a photomicrograph of liver (x 400) showing a centrally placed *S. mansoni* egg with an intense cellular infiltration from IC.

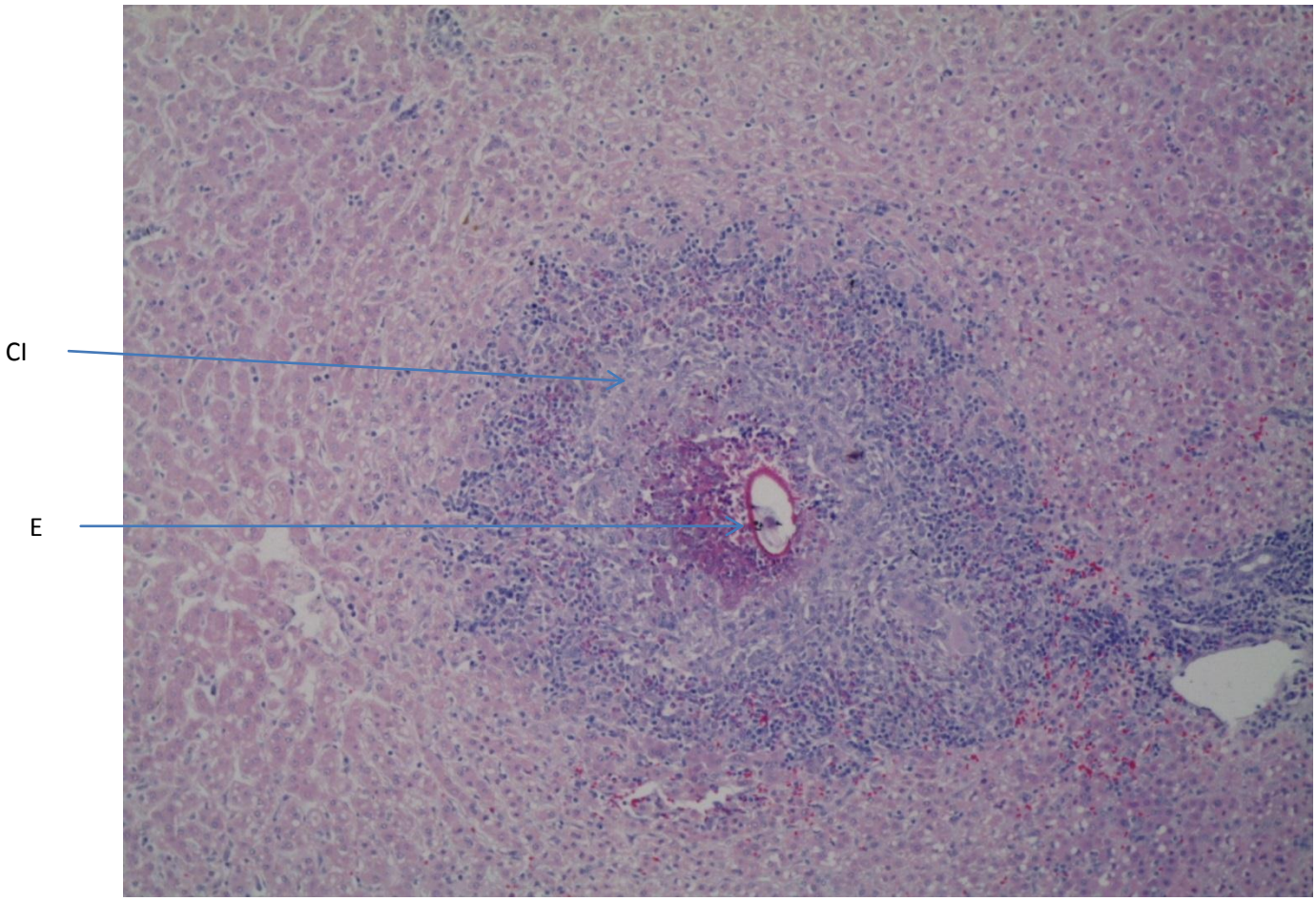


Figure 5.2.3: A section showing a centrally placed *S. mansoni* egg and an intense cellular infiltration. They were frequently observed in sections from IC (x 400)

KEY: E-Egg CI-Cellular infiltration

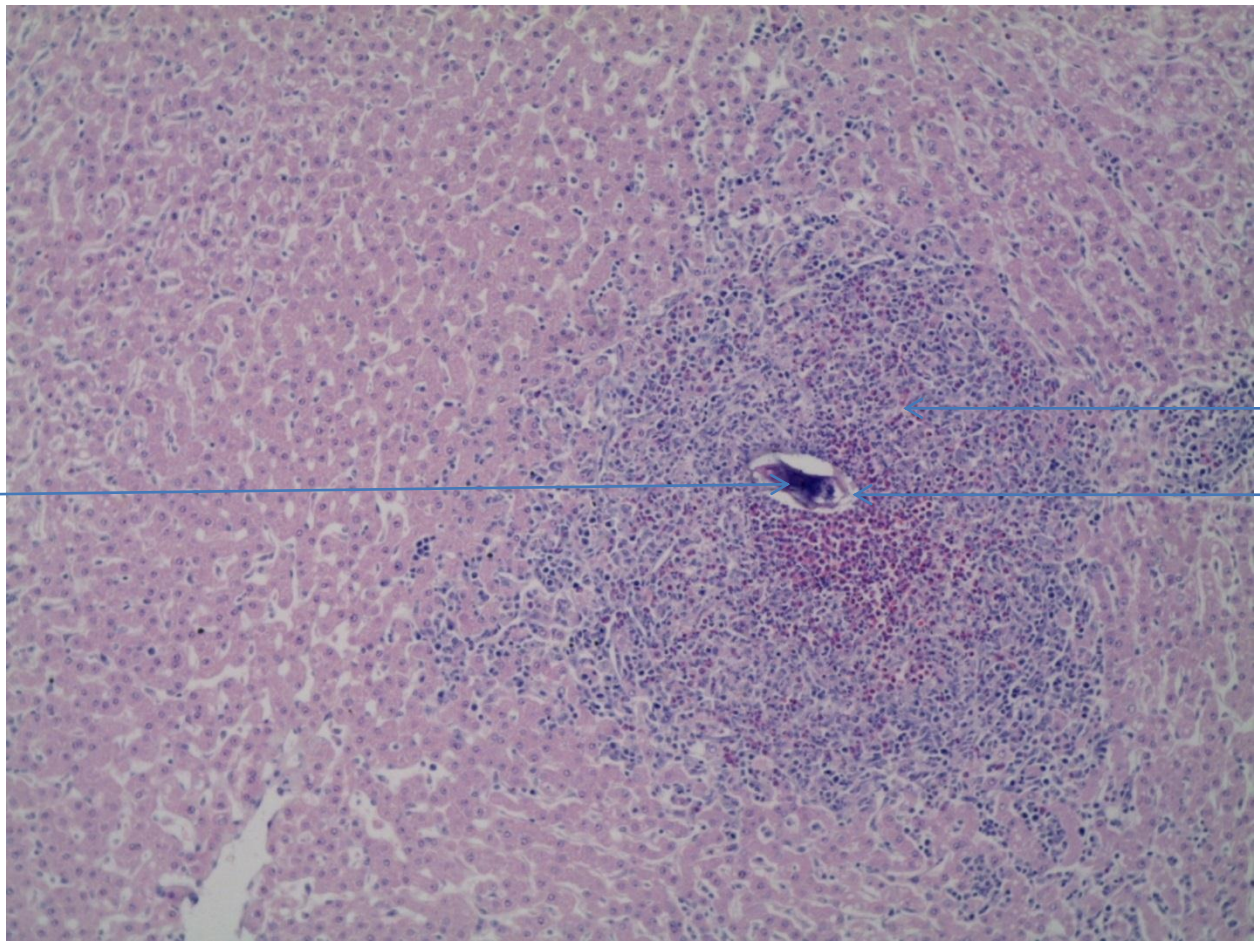


Figure 5.2.4: A section from DG showing an embroynated *S. mansoni* egg and cellular infiltration (x400)

KEY: E-Egg CI-Cellular infiltration M-Miracidium

5.4 DISCUSSION

Infection of the liver with schistosomiasis in the long run leads to chronic disease that is manifested through a wide range of clinical symptoms. The pathogenesis is associated with the hosts' immune responses. The immune responses are induced by egg antigens that results to formation of the granuloma. When granulomas finally resolve, periportal

fibrosis arises and is manifested as portal hypertension, esophageal varices and splenomegaly (Tamer & Gamal, 2013). Granulomas are regions of intense of cellular infiltration comprising of lymphocytes, eosinophils, fibroblasts, and macrophages surrounding a schistosome egg lodged in the tissues (Hagan *et al.*, 1998).

Gross pathology

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granulomas. From the observations made in this study, it was noted that none of the animals from DG had adhesions. Only one animal from RT had adhesions and all the animals from IC had adhesions. Inflammation of the liver surface was observed from all the groups. However, the extent of this inflammation varied among the groups. Two animals from DG demonstrated moderate inflammation while one had none. In RT group, all the animals had moderate inflammation. Two animals from IC demonstrated severe inflammation and the other one moderate inflammation. These results demonstrate that DG soluble protein led to reduced inflammation. In DG, two animals had moderate granulomas while one had few granulomas. In RT, all the baboons had moderate granulomas. In IC, two baboons had severe granulomas while one had moderate granulomas.

Observations on the granuloma showed that among the three groups, DG had the least number of granulomas. These results show that baboons immunized with DG soluble proteins had least gross pathology followed by those immunized with RT soluble proteins and then those of the IC. Therefore the DG baboons were better protected than those from the other two groups.

A study on the effects of immunizing mice with snail soluble proteins and challenging them with *S. mansoni,* the group that was vaccinated with the rest of the body tissues (RT) all the mice had slightly inflamed livers. DG mice had slightly inflamed livers except one female member who had an inflamed liver. In IC mice, all the sampled animals had inflamed liver tissues. However, none of the mice in all the groups had granulomas most probably due to delayed pathology development in Swiss mice (Kobia *et al.,* 2011).

Histopathology

Only granulomas containing an ovum at the centre were enumerated and measured at × 100 magnification using calibrated ocular micrometer. Granuloma size was measured based on the vertical and horizontal diameters. The averages of the horizontal and vertical diameter were taken to be the granuloma diameter. A total of 10 granulomas per sample were measured. The average size for the DG was 25.7 $\pm$ 0.82, for the RT group, the average granuloma size was 28.57 $\pm$ 1.87 μm while that of IC group was 34.7$\pm$ 3.54μm. In DG and RT, the average granuloma size was similar (p>0.05). There was a significantly higher difference between the two immunized groups and the IC (p<0.05). Murine studies have shown that larger lesions are detrimental while smaller ones are an ideal compromise between egg-sequestration and tissue pathology (Hagan *et al.,* 1998). Histological liver abnormalities such as periportal infiltration were also noted. Observations in this study showed that liver tissues from the DG and RT group had mild cellular infiltration whilst those from the IC had intense cellular infiltration as results of diffusing egg antigens.

CHAPTER SIX

6.0 BRINE SHRIMP LETHALITY TEST AND CHEMICAL IDENTIFICATION BY SPECTROSCOPY OF DG AND RT SNAIL SOLUBLE PROTEINS

6.1 INTRODUCTION AND LITERATURE REVIEW

To predict toxicity and other safety variables as well as the effectiveness of new products, use of animals has been involved. The idea that all new products such as vaccines and new drugs should be tested for safety in animal studies before being approved for human testing is based on the assumption that animals will respond to the products like human.

Spectroscopy is the analysis of the interaction between matter and any portion of the electromagnetic spectrum. Light in the near-ultraviolet (UV) and visible range of the electromagnetic spectrum has an energy of about 150–400 kJ mol21. The energy of the light is used to promote electrons from the ground state to an excited state. A spectrum is obtained when the absorption of light is measured as a function of its frequency or wavelength. Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV (150–400 nm) or the visible (400–800 nm) region (Franz-Xaver Schmid, 2001). Electromagnetic radiation occurs in various forms, for example light (visible), ultraviolet, infrared, X-rays, microwaves, radio waves and cosmic rays.

6.1.1Brine shrimp lethality test

Brine shrimp lethality bioassay is a simple, high throughput cytotoxicity test of bioactive chemicals. It is based on the killing ability of test compounds on a simple zoological organism, brine shrimp (*Artemia salina*) (Harwig J and Scott P. M, 1971). This assay was first proposed by Michael and others 1956, and further developed by several groups (Van Walbeek *et al.,* 1971; Vanhaecke *et l.,* 1981). The brine shrimp lethality bioassay is

widely used in the evaluation of toxicity of heavy metals, pesticides and medicines especially natural plant extracts (Price *et al.*, 1974). It is a preliminary toxicity screen for further experiments on mammalian animal models.

The biological or physiological impact to be examined in the screening process is vital. One of the easiest biological reactions to evaluate the lethality is a single criterion; either alive or dead. In such situation, the the statistical evaluation is moderately easy. The lethal concentration resulting in 50% mortality following 24 hour exposure is considered as the evaluation of the toxicity of the compound or extract. The selection of time, based on the solubility of the substance or extract is one of convenience because the test should be kept simple yet rapid (Pointer *et al.,* 2000). The brine shrimp lethality test has been established as a method for the past 20 years. This technique is currently applied globally with great reports of its success.

6.1.2 Mass spectroscopy (MS)

Mass spectrometry is now commonly being used to determine both the primary and higher order structures of proteins. The basis for these investigations lies in the ability of mass analysis techniques to detect changes in protein conformation under differing conditions. These experiments can be conducted on proteins alone with no modifying substance present or in combination with proteolytic digestion or chemical modification (Richard, 2004).

The extensive application of mass spectroscopy in solving structural problems only began around 1960. Mass spectroscopy is based on a single principle, i.e. it is possible to determine the mass of an ion in the vapour phase. Thus, a mass spectrometer ionizes the sample into a beam of ions in the vapour phase, separates the ions according to their mass

to charge ratios *(m/e* or *m/z* values) and records the mass spectrum as a plot of *mle* of ions against their relative abundances. Mass spectroscopy is useful for characterization of organic compounds in that it can give the exact molecular masses and thus, the exact molecular formulae and it can show the presence of certain structural units and their points of attachment in the molecule, and thus gives idea about the structure of the molecule (Yadav, 2005).

6.1.3 Ultraviolet spectroscopy

Ultraviolet spectroscopy deals with the recording of the absorption of radiations in the ultraviolet and visible regions of the electromagnetic spectrum. The ultraviolet region extends from 10 to 400 nm. It is subdivided into the near ultraviolet (quartz) region (200-400 nm) and the far or vacuum ultraviolet region (10-200 nm). The visible region extends from 400 to 800 nm. The absorption of electromagnetic radiations in the UV and visible regions induces the excitation of an electron from a lower to higher molecular orbital (electronic energy Ievel). Since UV and visible spectroscopy involves electronic transitions, it is often called electronic spectroscopy (Yadav, 2005). Ultraviolet spectroscopy is used in analytical chemistry for the quantitative determination of different analytes, such as highly conjugated organic compounds and biological macromolecules.

6.1.4 Fourier Transform Infrared (FTIR) spectrometry

Fourier transform infrared (FTIR) spectroscopy is a non-destructive technique for structural characterization of proteins and polypeptides. The FTIR spectral data of polymers are usually interpreted in terms of the vibrations of a structural repeat. The

repeat units in proteins give rise to nine characteristic FTIR absorption bands (amides A, B and I–VII). Amide I bands (1,700–1,600 cm^{-1}) are the most prominent and sensitive vibrational bands of the protein backbone, and they relate to protein secondary structural components (Huayan *et al*., 2015).

FTIR spectroscopy is most widely used for the detection of functional groups and identification of organic compounds. FTIR absorption spectra originate from transitions in vibrational and rotational energy Ievels within a molecule. On absorption of FTIR radiation, vibrational and rotational energies of the molecule are increased. When a molecule absorbs FTIR radiation below 100 cm -1, the absorbed radiation causes transitions in its rotational energy levels. Since these energy Ievels are quantized, a molecular rotational spectrum consists of discrete lines.

When a molecule absorbs FTIR radiation in the range 100-10,000 cm-1, the absorbed radiation causes transitions in its vibrational energy Ievels. These energy Ievels are also quantized, but vibrational spectra appear as bands rather than discrete lines. The energy differences between various rotational energy Ievels of a molecule are far less than that between its vibrational energy Ievels. Thus, a single transition in vibrational energy Ievels is accompanied by a large number

of transitions in rotational energy Ievels and so the vibrational spectra appear as vibrational-rotational bands instead of discrete lines (Yadav, 2005).

6.2. MATERIALS AND METHODS

Toxicology was carried out in the Department of Public Health, Pharmacology and Toxicology, University of Nairobi, Upper Kabete Campus. The source of the intermediate host and preparation of the soluble protein, DG and RT are as described in

2.2.2 and 2.3. DG is a soluble protein from the digestive gland of the intermediate host *Biomphalaria pfeiferri* while RT is a soluble protein from the rest of the body tissues of the intermediate host *Biomphalaria pfeiferri*.

6.2.1. Toxicity Test

Toxicity test for the two snail soluble proteins DG and RT was done using brine shrimp (*Artemia salina*). Brine shrimp eggs (JBL Novo Temia, Germany) were hatched in a shallow rectangular dish filled with commercial sea salt (Sera premium Brine-Sea Salt, company). A plastic divider with several 2 mm holes was clumped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was dark while the smaller one was illuminated. A Lamp was positioned above the uncovered side to attract hatched shrimps. After 48 h the eggs hatched and matured as nauplii. Three different concentrations (10 µg/ml, 100 µg/ml and 1000 µg/ml) of the two test samples, DG and RT, were prepared and dispensed into vials. Ten brine shrimp larvae (nauplii) were collected by pipette from the light side and added into each vial and incubated at room temperature for 24 h. The numbers of surviving nauplii in each vial were counted under a stereoscopic microscope after 24 h. The experiments were conducted in five replicates for each concentration.

6.2.2 Preparation of DG and RT soluble proteins

The two soluble snail proteins were prepared as described in chapter 2.3.

6.2.3 Statistical analysis

Lethality assays were evaluated by Finney computer statistical program to determine the LC50 values and 95% confidence intervals. Significance level/probability level used in calculation was $p<0.05$.

6.2.4 Analysis of DG and RT snail soluble protein using the gas chromatography-mass spectroscopy

Gas chromatography-mass spectrometry (GC-MS) analysis of soluble protein extracts isolated from *B. pfeifferi* was performed using a gas chromatography mass spectrometry (GC-MS) machine (GCMS-QP2010SE) coupled to gas chromatography (GC-2010 plus) both manufactured by Shimadzu Corporation (Kyoto, Japan). The machine had two components; the chromatography component and the mass spectrometer component, equipped with MS fused silica capillary column (30m ×0.25mm internal diameter and film thickness of 0.25µm). In GC-MS spectroscopic detection, ionization energy of 70 eV was used in the electron ionization energy. Pure helium gas (99.9%) was used as carrier gas and was set to between 5-9 bars and the chromatography vacuum for mass transfer line and interface temperature oven programmed to heat at 40^0C, 60^0C, 100^0C, 130^0C and 170^0C with 1 min interval. Diluted samples (1/100 v/v in methanol and water of 1 µl were loaded in cuvettes which were inserted in an auto injector (A0C-201) also manufactured by Shimadzu Corporation (Kyoto, Japan) in the spit and the separation performed in the gas chromatography followed by analysis using the mass spectrometer. The relative percentage of the chemical constituents in the soluble protein extracts were captured and resolved using the GC-MS real time analyser software (GC-MS Shimadzu

Lab Solution) during the process and later using GC post-run analyser with a built in library to compare the molecular masses to known compounds.

6.2.5 Analysis of DG and RT snail soluble protein using ultraviolet (UV) spectroscopy

UV spectroscopy was performed using standard process. Using soluble protein extracts, a concentration of 1.4 mg/ml of ddH_2o was prepared and each transferred into a clean cuvette (precision cells Inc., NY, USA). This was placed in a UV spectrophotometer (UV-1800, Shimadzu corporation, Kyoto, Japan) and scanned between wavelength ranges of 200nm and 700nm. The data sets obtained were used to plot the chromatogram using Origin software.

6.2.6 Analysis of DG and RT snail soluble protein using FTIR

Solid potassium bromide (KBr) was dried in an oven at more than 100^0C while the soluble protein extracts were lyophilized. The samples of soluble protein extracts were mixed with the KBr (IR grade) in the ratio of 1: 100 and ground to fine powder using glass and mortar pestle. The mixture was then used to prepare thin pellets by compressing at 20 MPa using a hydraulic press machine (Biotech engineering Management Company limited, UK). The pellets were transferred to a pellet holder. The machine was run first without the sample to collect the background. The pellets were then analysed using a 600 FTIR spectrometer (Biotech engineering management company limited, UK). The data sets obtained were used to plot the chromatograms using Origin software.

6.2.7 Analysis of DG and RT snail soluble protein using the gas chromatography-mass spectroscopy

Gas chromatography-mass spectrometry (GC-MS) analysis of soluble protein extracts isolated from *B. pfeifferi* was performed using a gas chromatography mass spectrometry (GC-MS) machine (GCMS-QP2010SE) coupled to gas chromatography (GC-2010 plus) both manufactured by Shimadzu Corporation (Kyoto, Japan). The machine had two components; the chromatography component and the mass spectrometer component, equipped with MS fused silica capillary column (30m ×0.25mm internal diameter and film thickness of 0.25µm). In GC-MS spectroscopic detection, ionization energy of 70 eV was used in the electron ionization energy. Pure helium gas (99.9%) was used as carrier gas and was set to between 5-9 bars and the chromatography vacuum for mass transfer line and interface temperature oven programmed to heat at 40^0C, 60^0C, 100^0C, 130^0C and 170^0C with 1 min interval. Diluted samples (1/100 v/v in methanol and water of 1 µl were loaded in cuvettes which were inserted in an auto injector (A0C-201) also manufactured by Shimadzu Corporation (Kyoto, Japan) in the spit and the separation performed in the gas chromatography followed by analysis using the mass spectrometer. The relative percentage of the chemical constituents in the soluble protein extracts were captured and resolved using the GC-MS real time analyser software (GC-MS Shimadzu Lab Solution) during the process and later using GC post-run analyser with a built in library to compare the molecular masses to known compounds.

6.2.8 Analysis of DG and RT snail soluble protein using ultraviolet (UV) spectroscopy

UV spectroscopy was performed using standard process. Using soluble protein extracts, a concentration of 1.4 mg/ml of ddH_2o was prepared and each transferred into a clean cuvette (precision cells Inc., NY, USA). This was placed in a UV spectrophotometer (UV-1800, Shimadzu corporation, Kyoto, Japan) and scanned between wavelength ranges of 200nm and 700nm. The data sets obtained were used to plot the chromatogram using Origin software.

6.2.9 Analysis of DG and RT snail soluble protein using FTIR

Solid potassium bromide (KBr) was dried in an oven at more than 100^0C while the soluble protein extracts were lyophilized. The samples of soluble protein extracts were mixed with the KBr (IR grade) in the ratio of 1: 100 and ground to fine powder using glass and mortar pestle. The mixture was then used to prepare thin pellets by compressing at 20 MPa using a hydraulic press machine (Biotech engineering Management Company limited, UK). The pellets were transferred to a pellet holder. The machine was run first without the sample to collect the background. The pellets were then analysed using a 600 FTIR spectrometer (Biotech engineering management company limited, UK). The data sets obtained were used to plot the chromatograms using Origin software.

6.3 RESULTS

6.3.1: Lethal Concentration 50 of DG and RT against *Artemia salina* Nauplii

Table 6.1 shows analysis of lethal concentration 50 (LD50) of DG and RT against *Artemia salina* nauplii larvae.A summary of average mortality and percentage average

mortality for both DG and RT that was recorded for nauplii that were incubated for 24 h in the concentration of 10 µg/ml, 100 µg/ml and 1000 µg/ml are shown. In DG, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%, in the concentration o100 µg/ml was 10% while it was 26% in the concentration of 1000 µg/ml. In RT, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%; in the concentration of100 µg/ml was 16% while it was 32% in the concentration of 1000 µg/ml. The average percentage mortality in all the three different concentrations for the both soluble proteins was less than 50%.The LCD50 for DG was 4158.08 µg/ml while for RT was 3988.74 µg/ml.

Table 6.1: Lethal Concentration 50 of DG and RT against *Artemia Salina* Nauplii

Protein	Concentration (µg/ml)	Total No. of Brine Shrimps	Mortality in 5 Replicates					Average Mortality	% Average Mortality	LC_{50} (µg/ml)
DG	10	10	0	0	1	0	1	0.4	4%	3988.74
DG	100	10	2	1	0	2	0	1.0	10%	
DG	1000	10	5	3	1	2	2	2.6	26%	
RT	10	10	1	0	1	0	0	0.4	4%	4158.08
RT	100	10	3	2	2	1	0	1.6	16%	
RT	1000	10	3	5	3	2	3	3.2	32%	

Key: DG- Soluble proteins from the digestive gland of the intermediate host; **RT-** Soluble proteins from the rest of the body tissues of the intermediate host

6.3.2 Establishment of chemical constituents of DG and RT snail soluble protein using GC- MS

The chemical compounds of the different soluble protein extracts isolated from *B.pfeifferi* were identified based on GC retention time on the chromatogram column, and computer matching of mass spectra with the library standards in computer.

6.3.3 Chemical identification of the snail soluble proteins

The chemical identification of compounds of DG and RT snail soluble proteins are shown on table 6.2 and 6.3 respectively according to their elution on the capillary column. The main chemical compounds in DG were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l- Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. The main chemical compounds in RT were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentynamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

Table 6.2: Chemical Composition of Snail Digestive Gland (DG) Soluble Protein Extracts

Extracts	Peak	Retention time	Similarities (%)	Name of the compound	Molecular formula
DG extract in Methanol	1	1.625	97	Butylamine S	C4H11N
	2	2.195	92	N-tert-Butylmethylamine	$C5H11NO_2$
	5	3.255	83	Valine	$C4H11NO_2$
	6	4.120	74	Aminoheptanoic acid	$C7H15NO_2$
	7	4.345	84	L-Valine	$C5H11NO_2$
DG extract in water	1	1.625	88	1,1-Dimethylamino-1 butane	C6H13N
	3	2.20	82	N-tert-Butylmethylamine	C5H13N
	4	2.265	73	Valienamine	$C7H13NO_4$
	7	3.26	69	Penicillamine	$C5H11NO_2S$
	8	4.345	82	Penicillamine	$C5H11NO_2S$

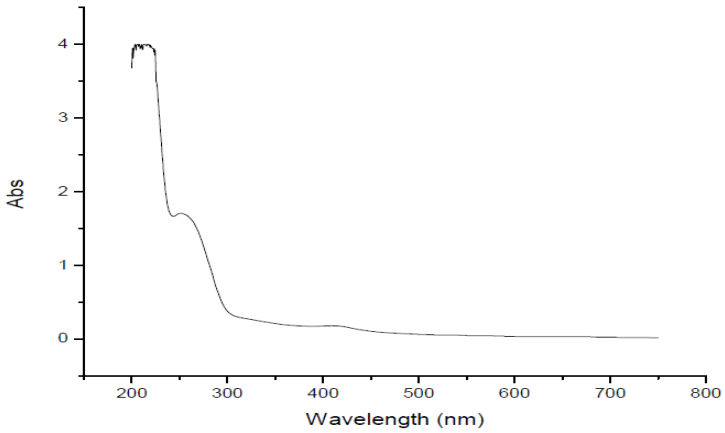
Table 6.3: Chemical Composition of the Rest of the snail Body Tissues (RT) Soluble Protein Extracts

Extracts	Peak	Retention time	Similarities (%)	Name of the compound	Molecular formula
RT extract in Methanol	1	1.625	97	Tert Butylamine	C4H11N
	2	2.195	90	N-tert-Butylmethylamine	C5H13N
	3	2.20	91	Heptylamine	C7H17N
	6	4.120	76	Pentanamine	C5H13N
	7	4.345	84	Valine	$C5H11NO_2$
RT extract in water	1	1.625	83	Cycloheptanemethylamine	C8H17N
	3	2.20	83	Pentanamine	C8H19N
	4	2.265	82	Hexadecylamine	C16H35N
	7	3.26	82	Buten 1 amine	C7H15N
	8	4.345	81	Dimopheptanol S Dimethylamine	C21H29NO

6.3.4 Ultraviolet (UV) Spectrometry

Figure 6.1(A) and (B) shows the UV spectrum of DG and RT soluble protein extracts. The peak for DG ranged between wavelengths 210- 230nm while for the RT ranged between wavelength 250-280 nm.

A.)



B.)

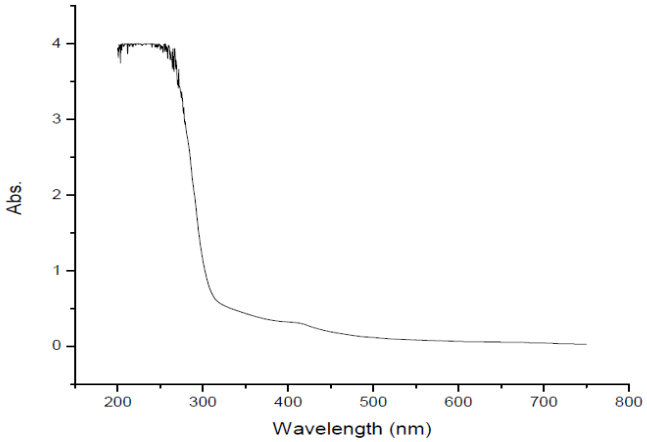
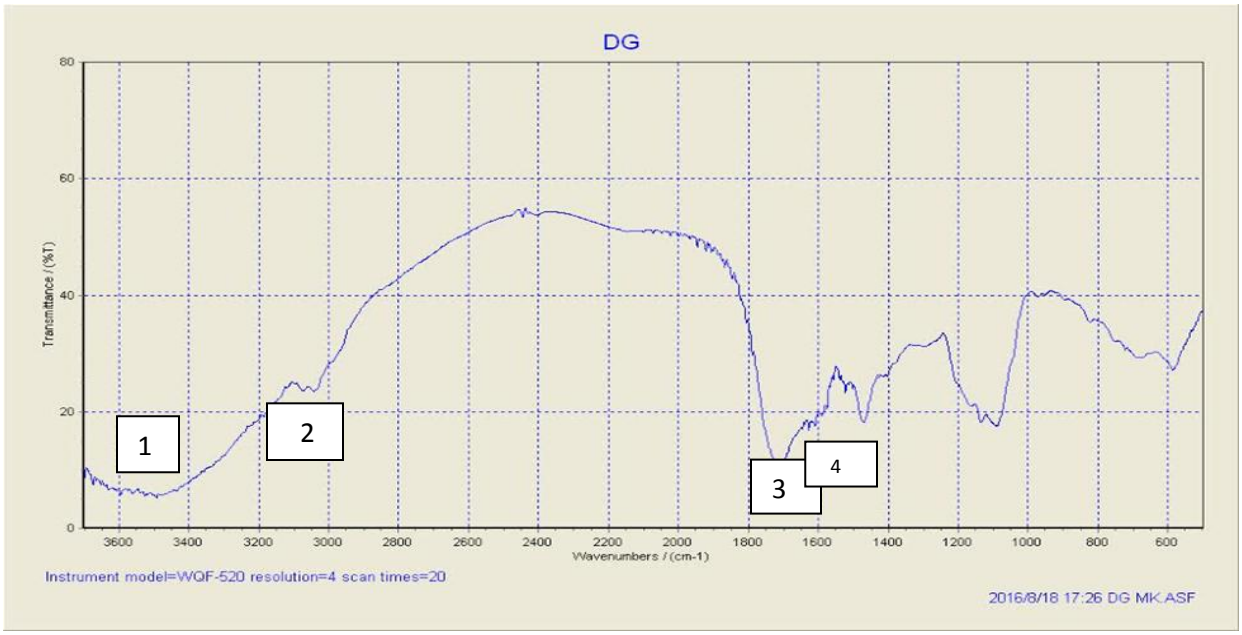


Figure 6.1: (A) UV spectrum of DG and (B) UV spectrum of RT soluble protein extracts

6.3.5 Fourier Transform Infrared (FTIR) spectrometry

Figure 6.2 (A) and (B) shows the FTIR spectrum of DG and RT soluble protein extracts.The results show that O-H stretch was at 3500 cm-1, N-H stretch was at 3100 cm-1, C=O stretch was at 1700 cm-1 (Amide I band) and N-H bending was at 1600 cm-1 (Amide II band). These results confirm the presence of proteins, evidenced by amide (peptide) bands in both DG and RT soluble protein extracts.

A)



B)

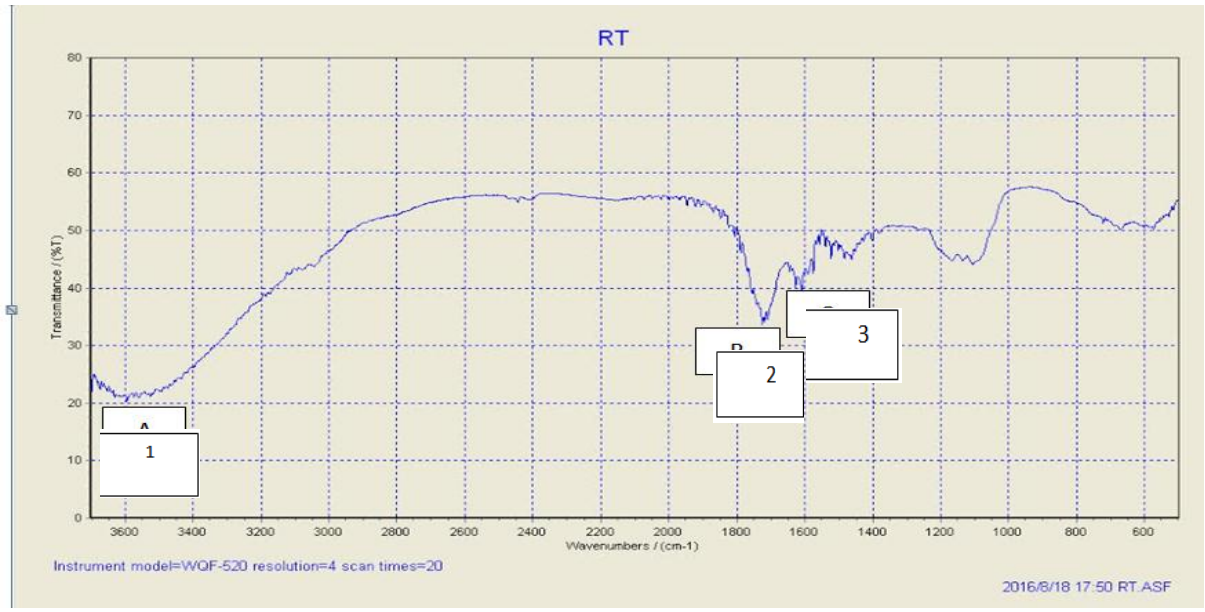


Figure 6.2: (A) FTIR Spectrum of DG and (B) FTIR Spectrum of RT Soluble Protein Extracts

KeyA: 1.O-H stretch at 3500 cm-1**. 2.** N-H stretch at 3100 cm-1. **3.** C=O stretch at 1700 cm-1(Amide I). **4**. N-H bending at 1600 cm-1(Amide II). **KeyB**: **1:** O-H stretch at 3500 **2**: C=O stretch at 1700 (Amide I band) **3**: N-H bending at 1600 (Amide II band)

6.4 DISCUSSION

DG was a soluble protein from the digestive gland of the intermediate host *Biomphalaria pfeiferri* while RT was a soluble protein from the rest of the body tissues of the intermediate host *Biomphalaria pfeiferri*. The two proteins were used to immunize olive baboons, *Papio Anubis* challenged against *Schistosoma mansoni.* In DG, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%; in the concentration o100 µg/ml was 10% while it was 26% in the concentration of 1000 µg/ml. In RT, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%; in the concentration of100 µg/ml was 16% while it was 32% in the concentration of 1000 µg/ml. The average percentage mortality in all the three different concentrations for the both soluble proteins was less than 50%.

Nguta *et al.*, (2011) gave a criteria for lethal concentration dose; LC50< 100 = Strongly/highly toxic, LC50> 100 < 500 = moderately toxic, LC50> 500 < 1000 = weakly toxic andLC_{50} > 1000 = Non- toxic. RT had LC_{50} of 4158.06 µg/ml and DG LC_{50} of 3988.73 µg/ml. DG and RT had a LC_{50} of over 1000µg/ml. The toxicity results for the two soluble proteins at the three different concentrations shows that both proteins are non-toxic and are thefore safe vaccines.

Most of the toxicity using brine shrimps has been done in plant studies. One of the studies by Musila *et al*., 2013 on in vivo antimalarial activity, toxicity and phytochemical screening of selected antimalarial plants ; aqueous extract of the stem bark of *Adansonia digitata* exhibited highest chemo-suppression of parasitaemia, ≥60% in a murine model of *Plasmodium berghei* infected mice. Aqueous and organic extracts of *Launaea cornuta* and *Zanthoxylum chalybeum* were toxic to the brine shrimp (LD50≤1000 mg/ ml) while

aqueous and organic extracts of *Adansonia digitata* and aqueous extracts of *Canthium glaucum* were not toxic to brine shrimp (LD50≥41000 mg/

This work demonstrates that both DG (LCD 50=3988.74) and RT (LCD 50=4158.08) are not toxic and are therefore safe. This is important since DG was found to be efficacious and it can go into vaccine development.

The GC-MS spectrum established the presence of different components with varied retention times. The mass spectrometer evaluates compounds eluted at various times to identify the nature and chemical structure of the compounds. These mass spectra are fingerprint of that compound which can be identified from the data library (Kanthal *et al*., 2014). Findings from this work help to predict the molecular formula and structure of the chemical compounds in the two soluble proteins. The main chemical compounds in DG were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l-Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. The main chemical compounds in RT were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentynamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

Among the compounds identified, penicillamine has been implicated in antiparasitic action against the invitro growth of *Plasmodium falciparum, Plasmodium chabaudi and Plasmodium berghei.* The compound as S-nitroso-acetyl-penicillamine, on high concentrations was cytotoxic to *Plasmodium falciparum*. At lower concentrations, it had

cytostatic effect and some parasites resumed growth and division (Balmer *et al.*, 2000). The chemical compounds identified in the DG and RT snail soluble proteins by GC-MS might have some immunological significance as DG and RT caused profound immunological responses. This is a crucial step in vaccine development.

The UV spectrum of DG and RT soluble protein extracts peaks ranged between wavelengths 210--280 nm. Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum (Stoscheck, 1990). Commonly, the optical absorption of proteins is measured at 280 nm. At this wavelength, the absorption of proteins is mainly due to the amino acids tryptophan, tyrosine and cysteine with their molar absorption coefficients decreasing in that order (Layne, 1957). The peaks observed for the both proteins are a further confirmation of presence of amino acids in the two soluble proteins.

The FTIR spectrum of DG and RT soluble protein extracts results showed that O-H stretch was at 3500 cm-1, N-H stretch was at 3100 cm-1, C=O stretch was at 1700 cm-1 (Amide I band) and N-H bending was at 1600 cm-1 (Amide II band). Characteristic bands found in the infrared spectra of proteins and polypeptides include the Amide I and Amide II (Krimm & Bandekar 1986). These arise from the amide bonds that link the amino acids. Amide I is the most intense absorption band in proteins. It is primarily governed by the stretching vibrations of the C=O and C-N groups. Its frequency if found in the range between 1600 and 1700 cm-1. The exact band is determined by the backbone

confirmation and hydrogen bonding pattern. Amide II is found in the 1510 and 1580 cm-1 region and it is more complex than Amide I. Amide II derives mainly from in-plane N-H bending. The rest of the potential energy arises from the C-N and the C-C stretching vibrations. These results confirm the presence of proteins, evidenced by amide (peptide) bands in both DG and RT soluble protein extracts. In view of the above, the results from the GC-MS can be confirmed by the FTIR results which confirm the presence of proteins. This is evidenced by the amide (peptide) bands in both cases of the DG and RT soluble protein extracts.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

Schistosomiasis is an infectious disease that affects more than 230 million people worldwide (vos at al., 2012). Many strategies such as implementation of mass chemotherapy and other mitigating measures have been undertaken to contain the spread of schistosomiasis. Despite all these measures, the disease has continued to spread to other new regions. The most potentially effective way to control the disease would be the discovery of a vaccine, which would provide long-term immunity against the disease. A vaccine would contribute to low morbidity by eliciting immune responses leading to a decline in parasite load and female fecundity (Afzal et al., 2011).

In this study, Baboons in DG group were immunized with snail soluble proteins from the digestive glands then infected. Baboons in RT were immunized with snail soluble proteins from the rest of the body tissues and then infected, while baboons in IC group were infected only, i.e. the infected control. Worm maturation was 78.8%. This compares well with a worm maturation of 80% in olive baboons (Papio anubis) that were immunized by irradiated cercariae (Yole et al., 1996). DG was protective with worm reduction of 11.6 %. This is below the WHO criteria of 40% protection. The efficacy of DG can be increased by using more refined molecules.

Vaccination resulted in specific IgM responses and the boosters resulted in an increase in response. IgM is involved in the primary response The responses were higher for SWAP and SEA as compared to SLAP indicating more shared antigens between SWAP and SEA. IgM is required in initial responses to an infection. This can be seen in an increase

in response at vaccination, boosters and challenge and at week 6 pc when egg antigens are produced.

IgG specific responses for the three antigens i.e. SLAP, SWAP and SEA, was a remarkable increase in the 3 groups after vaccination and then a gradual increase to peak at week 4 for SLAP and week 6 for SWAP and SEA. DG and RT responses are stimulating the production of IgG. The boosters are giving further stimulation. The response for SWAP and SEA is highest at week 6 and this compares very well with high concentrations of IL-5 and IL-6 at this time point. The two cytokines are involved in stimulating B cells to produce antibodies. IgG is involved in antibody-dependent cell-mediated cytotoxicity (ADDC) with neutrophils, macrophages and eusinophils which are damaging to the schistosomulea stage (Hagan *et al.*, 1998).

There was a remarkable increased response for IL-5 and IL-6 after vaccination and a gradual increase CD4 percentage counts were elevated in the two experimental groups. CD4 cells are of two sub-types; Th1 and Th2. Th1 cells produce IL-2, IFN-γ, and lymphotoxin which promote cell-mediated immunity while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which promote antibody production and humoral immunity. Th2 cells mediate host defense against extracellular parasites including helminthes (Seder and Paul, 1994). This is an indication that the two snail soluble proteins induced development and multiplication of CD4 cells.

CD8 percentage counts were elevated in DG and RT although the elevation was not dramatic as compared to CD4. CD8 cells are mainly cytotoxic. CD8+ T cell becomes activated on recognition of its antigen. It secretes cytokines, primarily TNF-α and IFN-γ and also produces and release cytotoxic granules. CD25 that are memory cells are

elevated. At week 6 pc, they are more in spleen and lymph node a cell, which is expected. This corresponds with egg production at week six. Lymph nodes are region that are densely populated with macrophages and lymphocytes along the lymphatic veins (Pabst, 1988). They encounter the parasite antigens before they get into the general circulation and this could probably have caused the elevated response.

Vaccination resulted to specific IgM responses and the boosters resulted in an increase in response. IgM is involved in the primary response The responses were higher for SWAP and SEA as compared to SLAP indicating more shared antigens between SWAP and SEA. IgM is required in initial responses when an antigen attacks. This can be seen in an increase in response at vaccination, boosters and challenge and at week 6 pc when egg antigens are produced.

IgG specific responses for the three antigens i.e SLAP, SWAP and SEA, was a remarkable increase in the 3 groups after vaccination and then a gradual increase to peak at week 4 for SLAP and week 6 for SWAP and SEA. DG and RT responses are stimulating the production of IgG. The boosters are giving further stimulation. The response for SWAP and SEA is highest at week 6 and this compares very well with high concentrations of IL-5 and IL-6 at this time point. The two cytokines are involved in stimulating B cells to produce antibodies. IgG is involved in antibody-dependent cell-mediated cytotoxicity (ADDC) with neutrophils, macrophages and eusinophils which are damaging to the schistosomulea stage (Hagan *et al.,* 1998).

There was a remarkable increased response for IL-5 and IL-6 after vaccination and a gradual increase after the second booster and challenge. A peak response was obtained at week 6 pc for SWAP and SEA. This correlates well with CD4 counts, IL-5 and IL-6

elevation at week 6 pc. In addition to worm antigens, soluble egg antigens are produced leading to high humoral responses. IC humoral responses were lower than DG and RT, therefore DG and RT increased humoral protection.

DG exhibited least pathology compared to RT. IC had severe pathology compared to RT. This is in agreement with histopathology results were DG and RT had significantly lower average granuloma size than IC. DG had an average granuloma size of of 25.7$\pm$0.82 μm RT had an average granuloma size was 28.57$\pm$1.87 μm while IC had the largest average granuloma size of 34.7$\pm$3.54μm. Pathology results are in agreement in Th2 responses that are involved in granuloma formation and that are elevated at week 6 pc. Histological liver abnormalities concerning periportal infiltration showed that liver tissues from the DG and RT group had mild cellular infiltration whilst those from the IC had intense cellular infiltration as a result of diffusing egg antigens. The brine shrimp toxicity results for the two soluble proteins were found to be safe. The LCD 50 for the two soluble proteins was >1000.

The chemical compounds found in DG usingGC-MS spectrum were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l- Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. The main chemical compounds in RT were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentynamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

Among the compounds identified, penicillamine has been implicated in antiparasitic action against the *invitro* growth of *Plasmodium falciparum, Plasmodium chabaudi and Plasmodium berghei.* The compound as S-nitroso-acetyl-penicillamine, on high concentrations was cytotoxic to *Plasmodium falciparum*. The UV spectrum of DG and RT soluble protein extracts peaks ranged between wavelengths 210--280 nm. Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm absorbance.

The FTIR spectrum of DG and RT soluble protein extracts results confirmed the presence of proteins, evidenced by amide (peptide) bands in both DG and RT soluble protein extracts. The chemical compounds identified in the DG and RT snail soluble proteins by GC-MS and further confirmed by UV and FTIR might have some immunological significance as DG and RT caused profound immunological responses. This is a crucial step in vaccine development.

7.2 CONCLUSION

The mean worm burden was higher in IC group than both DG and RT group of baboons. Worm reduction in DG was 11.44% while that of RT was 6.14 %. %. The results also reveal that DG snail soluble proteins offered a better protection than RT snail soluble proteins. However this was below the 40% protection threshold of World Health Organization (WHO, 1996).

Both DG and RT snail soluble proteins stimulated production of CD4 cells, CD8, CD 25, IL-5 and IL-6 and hence provoked Th-2 protection in baboons. However, at most sampling points DG had a significantly higher stimulation.

SLAP, SWAP and SEA schistosomule specific IgM and IgG responses were significantly higher in the two immunized groups when compared to IC. This reveals that DG and RT offered humoral protection to the baboons.

Baboons immunized with DG soluble proteins had least gross pathology and histopathology compared to those immunized with RT soluble proteins and IC an indication that DG baboons were better protected than those from the other two groups.

Brine shrimp lethality bioassay results for DG and RT snail soluble proteins at the three different concentrations showed that both proteins were non-toxic and are therefore safe vaccines.

Chemical identification of DG and RT using the GC-MS spectrum established the presence of different components with varied retention times. In DG, these were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l- Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. In RT, these were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentynamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

The results from the GC-MS were confirmed by UV spectrum and the FTIR results which confirmed the presence of proteins. This was evidenced by the amide (peptide) bands in both cases of the DG and RT soluble protein extracts.

7.3 RECOMMENDATIONS

1. Further work should be done to find out how the efficacy of DG can be increased, for example using more refined proteins like molecules from the digestive gland.

2. Further study should be done using different adjuvants to increase the efficacy of the snail soluble proteins.

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APPENDIX 1: Proposal approval form

APPENDIX 1

Institute of Primate Research

NATIONAL MUSEUMS OF KENYA

Address: P. O. Box 24481-00502 Karen Nairobi Kenya Tel: +254 02 2606235 | Fax: +254 02 2606231

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/10/13**

Dear Prof.Dorcas Yole,

It is my pleasure to inform you that your proposal entitled, "Immunizing the Olive baboon *Papio anubis*, with snail soluble proteins, R1 and DG, towards developing a human schistosomiasis vaccine", in collaboration with Dr Rebecca Waihenya of JKUAT and Eunice Kobia of the University of Nairobi, has been reviewed by the Institutional Review Committee (IRC). The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVIMS and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

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

Signed .... Chairman, IRC ....Dr. Hastings Ozwara

Signed .... Secretary IRC ....Dr. [illegible]

Date: September 9th 2013

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
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APPROVED...09/09/2013

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