CORRELATIONS BETWEEN *Schistosoma mansoni*-SPECIFIC IMMUNOGLOBULINS (IgG_1, IgG_2, IgG_3, IgM), INFECTION INTENSITY AND AGE AMONG SCHOOL PUPILS IN ASEMBO AREA, WESTERN KENYA.

BY

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DEPARTMENT OF ZOOLOGY

MASENO UNIVERSITY

DECLARATION

I declare that this thesis is my original work and has not previously been submitted for a degree in any other university. I hereby duly acknowledge the work and materials used by other authors, and any omission is highly regretted. No part of this thesis may be reproduced or transmitted in any form without written permission from Maseno University or the author.

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DEDICATION

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ABSTRACT

Schistosoma mansoni is a blood parasite that is common among school going pupils living near water bodies. It causes a disease known as schistosomiasis or bilhaziasis. Infected pupils stand higher risks of developing anaemia, absenteeism from school, retarded physical and mental growth and development, hence poor academic achievement. Human hosts respond to the infection by producing various immunoglobulins like IgA, IgD, IgE, IgG_1, IgG_2, IgG_3, IgG_4 and IgM. Some of these immunoglobulins like IgG_1, IgG_3 and IgE confer protective immunity to the host against the infection, while others like IgG_2, IgG_4 and IgM lead to susceptibility of the host to the infection. Although studies have been conducted in Asembo area indicating a prevalence rate of *S. mansoni* infection at 35-80% among school children, infection intensity of the parasite, levels of protective and susceptibility immunoglobulins, and correlations between these antibodies in relation to the infection intensity and age among the infected children have not been established. This study sought to investigate the infection intensity, levels of IgG_1, IgG_2,, IgG_3 and IgM immunoglobulins, and how these antibodies correlate with intensity of infection and age among school children suffering from *S. mansoni* in Asembo, which is an endemic area for the infection. The study followed a cross-sectional design and involved use of 350 stool and venous blood samples obtained from *S. mansoni*-infected children aged between 5-20 years, attending primary and secondary schools in Asembo area. The study area has a total of 31,293 school-going children. The participants were recruited using simple random sampling technique. The stool samples were used for determination of infection intensities by means of Kato Katz technique. The blood samples were used to obtain plasma for determination of immunoglobulin levels by means of indirect Enzyme Linked Immunosorbent Assay (ELISA) method, using soluble worm antigen preparations (SWAP) and soluble egg antigens (SEA) of the parasite. Multivariate analysis of variance (ANOVA) was conducted to determine if mean levels of immunoglobulins and infection intensities were significantly different between the age groups. Tukey's Honest Significant Difference test was used to obtain the least significant differences in the levels of the immunoglobulins and infection intensities. Spearman's Rank Correlation Coefficient was used to determine the correlations between concentrations of the IgG_1, IgG_2, IgG_3 and IgM immunoglobulins *versus* infection intensity and age of the infected school pupils. Regression analysis was done to assess the strength of associations between levels of the immunoglobulins, infection intensity and age. Levels of the immunoglobulins were dependent variables while intensity of infection and age were independent variables. The levels of anti-SWAP IgG_1, IgG_3 and anti-SEA IgG_1 peaked at the age bracket of 17-20 years. The levels of anti-SWAP IgG_2, IgM and anti-SEA IgM were highest at the age bracket of 5-8 years. The anti-SEA IgG_3 levels peaked at the age bracket of 9-12 years. Intensity of infection was highest at 184.06 eggs per gram at the age bracket of 13-16 years. Positive correlations existed between anti-SWAP IgG_1 and infection intensity (ρ_s= 0.321, p=0.001), anti-SWAP IgG_2 and infection intensity (ρ_s=0.187, p=0.001), anti-SEA IgG_1 and intensity of infection (ρ_s=0.168, p=0.002) and anti-SEA IgG_3 and infection intensity (ρ_s=0.155, p=0.005). Negative correlations were found between anti-SWAP IgM and infection intensity (ρ_s=0.115, p=0.039). No correlations existed between anti-SWAP IgG_3 and infection intensity (ρ_s=0.011, p=0.838) and anti-SEA IgM and infection intensity (ρ_s=0.097, p=0.082). Positive correlations were established between anti-SWAP IgG_1 and age (ρ_s=0.472, p=0.001), anti-SWAP IgG_3 and age (ρ_s=0.223, p=0.001), and anti-SEA IgG_1 and age (ρ_s=0.286, p=0.001). There were negative correlations between anti-SWAP IgG_2 and age (ρ_s=-0.476, p=0.001), anti-SWAP IgM and age (ρ_s=-0.436, p=0.001) and anti-SEA IgM and age (ρ_s=0.315, p=0.001). There were no correlations between anti-SEA IgG_3 and age (ρ_s=0.073, p=0.193). The ρ_s and P values were considered to be statistically significant at ρ_s>0.1 or ρ_s>-0.1, and P<0.05 respectively. It was concluded that as the school children grow older, there is an increase in levels of protective immunoglobulins like IgG_1 and IgG_3 while on the other hand there is a decrease in levels of blocking immunoglobulins like IgG_2 and IgM. The findings of this study are expected to enhance the understanding of the immunological relationship between the human host and *S. mansoni* parasite during the infection, hence provide research scientists with additional knowledge aimed at developing a vaccine against schistosomiasis. The study recommends investigations into the correlations between total IgG levels *versus* infection intensity and age of infected school pupils, and use of more sensitive protocols for assaying anti-SEA IgG_2 levels.

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LIST OF ABBREVIATIONS AND ACRONYMS

AU	-	Arbitrary Units
AWA	-	Anti-Adult Worm Antigen
CGHR	-	Centre for Global Health Research
DALYs	-	Disability Adjusted Life Years
ELISA	-	Enzyme Linked Immunosorbent Assay
EPG	-	Eggs Per Gram
ERC	-	Ethical Review Committee
Ig	-	Immunoglobulin
KEMRI	-	Kenya Medical Research Institute
KNBS	-	Kenya National Bureau of Statistics
NHS	-	Normal Human Serum
NTD	-	Neglected Tropical Diseases
ODs	-	Optical Densities
PPMCC	-	Pearson Product Moment Correlation Coefficient
RDDP	-	Rarieda District Development Plan
SEA	-	Soluble Egg Antigen
SWAP	-	Soluble Worm Antigen Preparation
SCORE	-	Schistosomiasis Consortium for Operational Research and Evaluation
TMB	-	Trimethyl Benzidyne
WHO	-	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Human Schistosomiasis is a parasitic helminth infection caused by blood flukes of genus *Schistosoma*. It affects approximately 238 million people worldwide and causes more than 250,000 deaths per year (Chitsulo, *et al*, 2007). An estimated 600 million people worldwide are exposed to the risk of infection by the parasite (Chitsulo, *et al*, 2007). At least 243 million people required treatment for schistosomiasis in 2011 globally, and 90% of these lived in Africa (WHO, 2013). The disease accounts for up to 70 million Disability Adjusted Life Years (DALYs) annually (King, 2008). With these figures, schistosomiasis is classified second only to malaria in terms of human morbidity and mortality due to parasitic diseases (Fenwick, *et al*, 2009).

Schistosomiasis is prevalent in tropical and subtropical areas, especially in poor communities without access to safe drinking water and adequate sanitation. People are at risk of infection due to agricultural, domestic and recreational activities which expose them to infested water (WHO, 2013). The disease is typically prevalent in rural areas where natural streams, ponds, rivers and lakes harboring the infected intermediate host snails are the main sources of water for domestic or occupational purposes such as washing and fishing (Stothard and Gabrielli, 2007). The highest prevalence and infection intensities in the sub Saharan Africa are usually found in school children and young adults (Van der Werf *et al*, 2003; Hotez and Fenwick, 2009). School children usually become infected during swimming or collecting water, while younger children and infants get infected when they accompany adults to the

water bodies to collect water or wash clothes, or while being bathed by the adults in these water sources (Stothard and Gabrielli, 2007).

In Kenya, *S. mansoni* tends to be confined to narrow zones along the shores of water bodies like lakes. The prevalence of the parasite along the Kenyan Lake Victoria basin ranges between 40% and 80% (Adoka *et al,* 2014). Previous studies carried out by Handzel *et al,(* 2003) in school children in Asembo area indicate a prevalence rate of *S. mansoni* of 35-80%.

The human host is able to mount cellular and humoral immune responses against schistosomiasis infection. Humoral responses involve production of various immunoglobulins which include IgG and IgM (Vereecken *et al,* 2007; Butterworth *et al,* 1987). Different human antibody isotype responses against *Schistosoma* parasite can either mediate or block *in vitro* killing of schistosomula by a number of immune effector mechanisms (Khalife *et al,* 1989; Demeure *et al*, 1993).

Longitudinal population studies have demonstrated that particular parasite-specific antibody responses correlate with human resistance to reinfection or susceptibility in areas endemic for *Schistosoma mansoni* (Butterworth *et al*, 1987; Dunne *et al*, 1992; Demeure *et al*, 1993). Some studies have shown that higher levels of IgG_2, IgG_4 and IgM are associated with a higher risk of re-infection with schistosomiasis whereas IgG_1, IgG_3 and IgE appear to be protective to the host (Vereecken, *et al,* 2007). Many of these antibodies are elicited in response to egg polysaccharide antigens (Butterworth *et al*, 1987).

Although studies concerning prevalence of *S.mansoni* infection have been conducted in school children in Asembo area, there is still lack of information about levels of protective immunoglobulins like IgG_1 and IgG_3, as well as susceptibility immunoglobulins like IgG_2 and IgM produced in response to the infection in the region. Past studies conducted elsewhere concerning the various immunoglobulins have not given exclusive and/or adequate attention to school going children in the age-bracket of 5-20 years who have been described to bear the greatest burden of the infection(Ramirez *et al*, 1996, Satti *et al*, 1996, Naus *et al*,1999, Caldas *et al*, 2000, Vendrame *et al*, 2001, Naus *et al*, 2003, Jassim *et al*, 2007, Singh *et al*, 2011, Stothard *et al*, 2011, Odongo-Aginya *et al*, 2012, Negrao-Correa, *et al*, 2014, Shawesh *et al*, 2015), but instead have dealt with a very wide age range extending between 3-88 years.

In some of the studies, important antigens crucial for measurement of levels of immunoglobulins produced against *S. mansoni* infection have not been used. For example, Badri (2011) only used soluble worm antigen preparations (SWAP) but left out soluble egg antigens (SEA) in the assessment of immunoglobulin levels in children of Elkeryab village in Khartoum state. Use of both the antigens (SWAP and SEA) gives more reliable results compared to when only one antigen is used because humoral immunity against *S. mansoni* parasite is mounted in response to the antigens on both the adult worm and eggs of the parasite. Butterworth *et al* (1987) reports that many of the antibodies produced against *S. mansoni* infection are elicited in response to egg polysaccharide antigens, hence the need for use of soluble egg antigens (SEA) in studies involving evaluation of levels of immunoglobulins.

It is would be imperative to carry out a study that exclusively involves school children *per se* and see how the results on levels of immunoglobulins IgG_1, IgG_2, IgG_3, IgM and intensity of infection in school children in Asembo area compare with those of past investigators since Asembo area is composed of a homogeneous population different from other areas previously studied . This would provide details concerning the immunobiological responses with regard to host-parasite interaction during the infection of school-children in this area.

There is lack of information about correlations between levels of IgG_1, IgG_2, IgG_3 and IgM with intensity of infection among school children in Asembo area. Scanty information also exists about these correlations in previous studies conducted in school children elsewhere e.g. by Jassim *et al* (2007), Badri (2011), Singh *et al* (2011), Stothard *et al* (2011), Odongo-Aginya *et al* (2012), Negrao-Correa, *et al* (2014) and Shawesh *et al* (2015). The wide age range extending between 3-88 years in these studies tends to limit the number of school children involved in the studies leading to scanty information about the correlations between the immunoglobulins and intensity of infection.

The results of some studies already conducted on these correlations show contrasts of those done by others. For example, a study carried out by Badri (2011) found a significant negative correlation between levels of anti-SWAP IgG_3 and intensity of infection, while that of van Dam *et al* (1996) found a positive correlation between the two variables.

The correlations between both the protective and susceptibility immunoglobulins like IgG_1, IgG_2, IgG_3 and IgM in relation to age of *S. mansoni* infected school children in Asembo area has not been established. Although studies about the same has been done elsewhere, they mainly focus on adults and pre-school children (Caldas *et al*, 2000; Jassim *et al*, 2007; Singh *et al*, 2011; Stothard *et al* (2011); Odongo-Aginya *et al* 2012; Shawesh *et al*, 2015). It is not known whether similar results can be obtained in a study that only involves school children aged between 5-20 years. Some of these studies conducted elsewhere concerning the same also appear to have contradicting findings. For example, Satti *et al* (1996) found a positive correlation between anti-SEA IgM and age, while van dam *et al* (1996) results showed no correlation between the two variables.

The present study, therefore, aimed at addressing the shortfalls mentioned herein by investigating the levels of immunoglobulins IgG_1, IgG_2, IgG_3 and IgM, and the correlations between these antibodies and infection intensity as well as age of school children infected with *S. mansoni* parasite in Asembo area, Rarieda subcounty, western Kenya. It would be interesting to find how the results of the study on this cohort of school children compare with those that have been done on other populations found elsewhere.

1.2 Statement of the Problem

Although studies have been conducted in Asembo area concerning prevalence of *S. mansoni* infection at 35-80%, the levels of protective and susceptibility immunoglobulins among infected school children have not been established in this area. Again, correlations between levels of the protective and susceptibility

immunoglobulins *versus* infection intensity in school pupils infected with *S. mansoni* in Asembo area is not known. On the same note, the correlations between levels of these immunoglobulins and age of infected school children have not been investigated in Asembo area.

1.3 Justification of the Study

S. mansoni is a neglected tropical disease that has not been given adequate attention. Asembo area has a high prevalence rate of the *S. mansoni* infection of 35-80% among school pupils. Infection by *S. mansoni* accounts for upto 70 million disability adjusted life years (DALYs) worldwide annually (King *et al*, 2008). DALYs is a measure of the years/time lost due to ill health, disability or death due to the disease, which translates to heavy economic losses in the country. This study gives exclusive attention to school-going children because they bear the greatest brunt of the infection, and constitute the largest proportion of the population of humans in the country.

1.4 Significance of the Study

The results of this study are aimed at shedding more light to research scientists about the immunobiology of *S. mansoni* infection by way of improving the knowledge about human host and *S. mansoni* parasite relationship during the infection. This will undoubtedly contribute to better understanding of resistance and susceptibility to the infection hence give an insight into possible development of a candidate vaccine against schistosomiasis, leading to improvement of public health especially of school-going children who are more vulnerable to the infection.

1.5 Objectives of the Study

1.5.1 General Objective

To determine the levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins and assess the correlations between the levels of these antibodies in relation to infection intensity and age of school children suffering from *S. mansoni* in Asembo area.

1.5.2 Specific Objectives

(i) To measure the levels of IgG_1, IgG_2, IgG_3, IgM immunoglobulins and infection intensity in school children infected with *S. mansoni* in Asembo area.

(ii) To determine the correlations between the levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins and intensity of infection by *S. mansoni* among infected school children in Asembo area.

(iii) To determine the correlations between the levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins and age of the infected school children in Asembo area.

1.6 Null Hypotheses

(i) There are no significant variations in levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins as well as intensity of infection among school children infected with *S. mansoni* in Asembo area.

(ii) There are no correlations between the levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins in relation to infection intensity among school children infected with *S. mansoni* in Asembo area.

(iii) There are no correlations between the levels of IgG_1, IgG_2, IgG_3 and IgM immunoglobulins in relation to age of the school children infected with *S. mansoni* in Asembo area.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Schistosomiasis is a collective name of parasitic diseases caused by several blood fluke species classified under phylum *Trematoda*, family *Schistosomatidae* and genus *Schistosoma* (Arora and Arora, 2007).The disease is commonly referred to as *bilhaziasis.* Snails serve as the intermediate host, contributing to the development of some stages of the parasite. It commonly infects individuals in developing countries especially those who cannot afford safe water and sanitation facilities and are, therefore, exposed to contaminated water containing the infected snails (Arora and Arora, 2007). Although it has low mortality rate, its morbidity rate is high and often results in chronic illnesses that can damage internal organs. Children carry the heaviest burden of schistosome infection (Hotez and Fenwick, 2009). School children usually become infected during swimming or collection of water, while younger children and infants get infected when they accompany adults to the water bodies. In children, the infection impairs physical growth and cognitive development (Stothard and Gabrielli, 2007).

The life cycle of human schistosomes is complex, with larval, adult worm and egg stages interacting with the human host and fresh water snail intermediate host as shown in Figure 2.1.

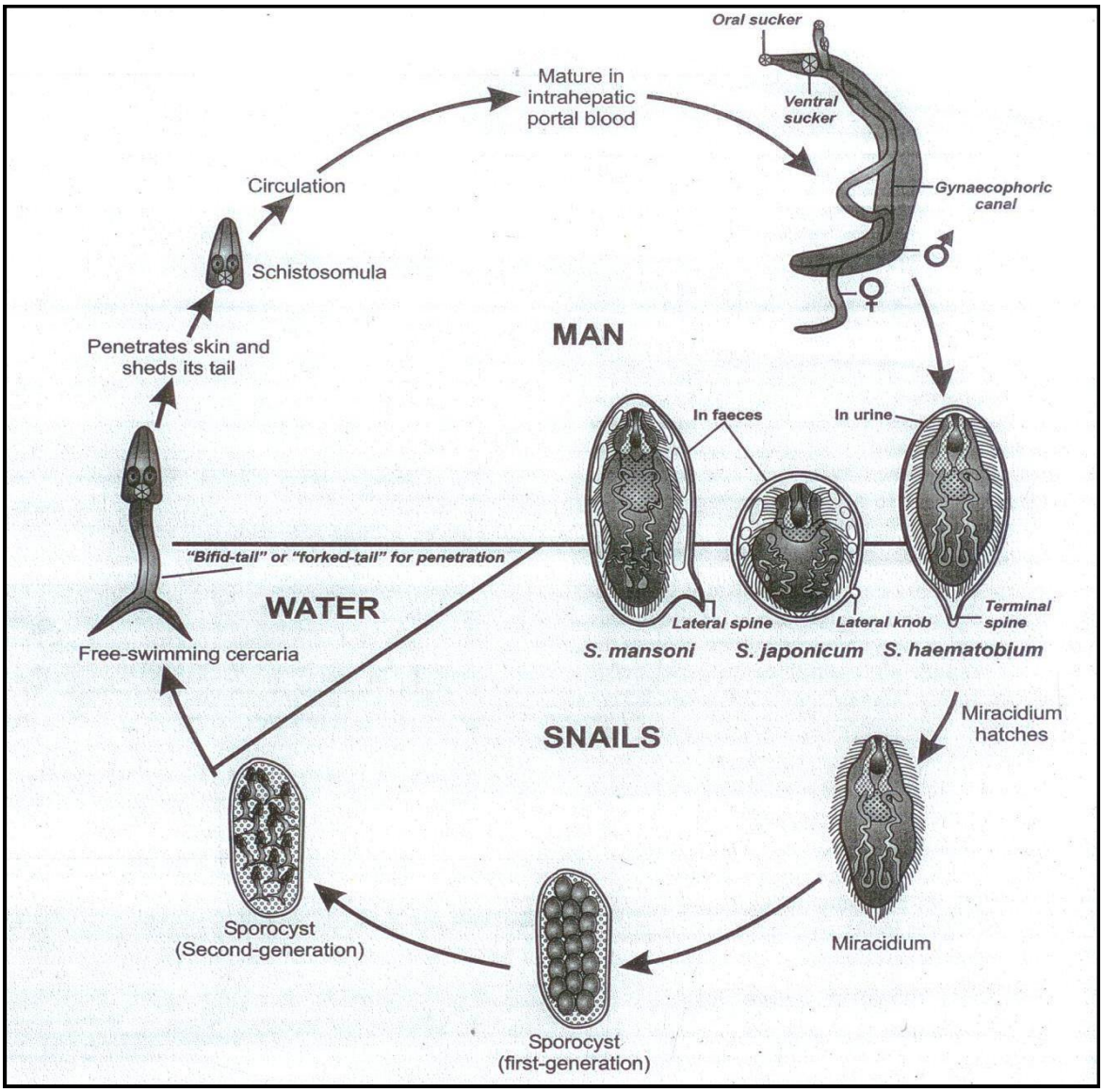

Figure 2.1: Life cycle of *Schistosoma spp*. (Source: Arora and Arora, 2007)

Adult schistosomes are white or greyish worms of 7-20mm. in length. They have a cylindrical body that has two terminal suckers, a complex tegument, a blind digestive system and reproductive organs (Arora and Arora, 2007).Unlike other trematodes, schistosomes have separate sexes, and the male is larger and shorter than the female. The body of the male forms a groove or gynaecorphoric canal, in which it holds the thinner and longer female. As permanently embraced couples, the male and female schistosomes live within the perivesical or mesenteric venous plexus, where they feed

on blood and globulins through anaerobic glycolysis. The debris is regurgitated in the host's blood (Gryseels *et al*, 2006).

The life cycle of all species of human schistosomes is complex and follows a common pathway as shown in Figure 2.1 as is described by Arora and Arora (2007). It involves the human as a definitive host and freshwater snail as an intermediate host. The females produce hundreds to thousands of eggs per day. Each egg (ovum) contains a ciliated miracidium larva which secretes proteolytic enzymes that help the eggs to migrate into the lumen of the bladder (*S. haematobium*) or intestines (other species). (Gryseels *et al*, 2006). The eggs are excreted in urine (*S. haematobium*) or faeces (other species) and can remain viable for upto 7 days. When the eggs get into contact with water, they release free-swimming larvae called miracidia. These larvae can stay in water for about 24 hours searching for a specific freshwater snail intermediate host, guided by light and chemical stimuli. After penetrating into a snail, the multicellular sporocysts then develop into human-infective larvae called cercariae. These larvae have embryonic sucker and characteristic bifid tail (Gryseels *et al*, 2006; Davis, 2009).

The cercariae start leaving the snail 4-6 weeks after infection and get into water where they stay upto 72 hours seeking the skin of a suitable definitive human host. On finding the host, the cercariae penetrate the skin and shed their tails, migrate in the blood via lungs to the liver, and transform into young worms called Schistosomulae. (Gryseels *et al*, 2006). These young worms migrate through the heart, lungs, liver, and mature and develop into male and female worms in the hepatic portal blood vessels. They then mate and migrate to their perivesicular or mesenteric destinations where

they lay eggs and the life cycle starts again. The lifespan of an adult schistosome is averagely 3-5 years, but can be as long as 30 years (Gryseels *et al*, 2006). The prepatent period between penetration of the cercariae into the human body and egg-laying is about 30-40 days in *S. mansoni* (Ukoli, 1990; Sturrock, 1987; Davis, 2009).

Differences in egg morphology can be used to distinguish between *Schistosoma* species. *S. mansoni* produces oval eggs (115-175 x 45-70µm) with a prominent sharp lateral spine. *S. japonicum* produces round eggs (70-100 x 50-70 µm) with a rudimentary lateral spine that is also referred to as lateral knob. *S. haematobium* produces oval eggs (110-170 x 40-70 µm) with a sharp terminal spine. The distribution of schisotosomiasis is linked to the snail intermediate host species. *S. mansoni* is transmitted by the snail of *Biomphalaria spp*, while in *S. haematobium* and S. *intercalatum*, the host snail is *Bulinus spp*. The intermediate host snail for *S. japonicum* and *S. mekongi* is *Oncomelania spp*. (Rollingson and Southgate, 1987).

2.2 Epidemiology of Schistosomiasis

Schistosomiasis is found in tropical countries in Africa, South America, Middle East, South East Asia, the Carribean and Venezuela, among others. As at the year 2010, it affected approximately 238 million people, 85% of whom lived in Africa. (WHO, 2013). It ranks second behind malaria among human parasitic diseases in terms of socio-economic and public health importance in tropical and sub-tropical areas (Oliveira *et al*, 2004) and contributes to a substantial share of clinical disease burden (WHO, 2013). It is estimated that it causes more than 250,000 global deaths annually (Van der Werf *et al*, 2003), and an estimated 600 million people worldwide are at risk of infection by the parasite (Chitsulo , 2007). The disease is endemic in 74-76

countries (Oliveira *et al*, 2004) and was ranked 78th in the list of causes of Disability Adjusted Life Years (DALY's) for the world , and children especially under 14 years of age carry the heaviest burden of infection (Hotez and Fenwick, 2009).

There are five major species of *Schistosoma* that cause human schistosomiasis, namely *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi* and *S. intercalatum* (Rollingson and Southgate, 1987). *S. mansoni*, *S. intercalatum*, *S. japonicum* and *S. mekongi* cause intestinal schistosomiasis, while *S. haematobium* causes urinary schistosomiasis. *S. mansoni* is common in Africa, the Middle East, South America and the Carribean (WHO, 2013). *S. haematobium* is prevalent in Africa and the Middle East while *S.japonicum* is commonly found in South East Asia, China and the Philippines (Rollingson and Southgate, 1987). *S. intercalatum* is restricted to Rain Forests of Central Africa, while *S. mekongi* is found in the central Mekong Basin in Lao and Cambodia (WHO, 2013). *S. mansoni* is the most prevalent species in Asembo area in western Kenya (Adoka *et al*, 2014).

2.3 Pathogenesis of Schistosomiasis.

Much of the pathology in schistosomiasis is due to the eggs rather than the larvae and adult worms (Caldas *et al*, 2008). The course of infection by schistosomiasis can be divided into migratory, acute and chronic phases. In the migratory phase the cercariae penetrate and migrate through the skin. This phase is often asymptomatic, though in some patients, it may cause transient dermatitis ("Swimmers' itch"), and occasionally, pulmonary lesions and pneumonitis (Caldas *et al*, 2008). The acute phase normally occurs in individuals with no previous contact with the parasite, who have recently visited an endemic area. The acute phase is characterized by symptoms such as fever,

cough, diarrhoea, anorexia, leukocytosis, fatigue, lymphadenopathy, eosinophilia, and a high cellular immune response to schistosome antigens, especially those from the parasite eggs (Caldas *et al*, 2008).The chronic phase occurs in response to the cummulative deposition of the parasite eggs in tissues and host reactions that develop against them, because not all the eggs laid by the female worms successfully penetrate the gut or bladder walls (Caldas *et al*, 2008).. Many of the eggs are swept away in the circulation and become trapped in the host's organs, where they become surrounded by inflammatory cells, resulting in granulomatous reaction (Davis, 2009; Abdel-Wahab and Mahmoud, 1987). This may result in a number of effects, including intestinal polyposis, abdominal pain, glomerulonephritis, cardiovascular problems, including heart failure, and periportal fibrosis. There may be passage of blood in stool leading to anaemia in severe cases. Portal fibrosis may lead to increased portal pressure, a condition known as portal hypertension, which often results in hepatomegally, splenomegally, ascites, and sometimes gross enlargement of oesophagial and gastric veins (varices) which may rupture/burst causing life threatening haematemesis (Lambertucci, 1993; Abdel-Wahab and Mahmoud, 1987).

Hepatomegally is caused by portal venous congestion and hyperplasia of reticulo-endothelial cells. It is usually accompanied by marked firmness or even hardness of the liver. The enlarged liver, particularly the left lobe, becomes smooth, firm and non-tender. Splenomegally makes the spleen become greatly enlarged, sometimes extending downwards past the umbilicus into the left iliac fossa (Vennervald *et al*, 2004; Davis, 2009). Studies show that the severity of hepatosplenic schistosomiasis correlates well with the intensity of *S. mansoni*, indicating a strong association between organ enlargement and schistosome infection (Vennervald *et al*, 2004).

2.4. Prevalence of *Schistosoma mansoni* among School Children

School children in this study are children attending primary and secondary schools, usually found between the ages of 5-20years. Past studies provide evidence that schistosomiasis infections are quite prevalent among school going children. For example, in a research study carried out by Stothard *et al* (2011) on *S. mansoni* infection in a sample of 242 school children with a mean age of 2.9 years in Bugoigo, Lake Albert in Uganda, it was found that there was a general prevalence of 47.5% of the infection in the area. Barbosa *et al* (2006) carried out a survey of schistosomiasis through school studies in the Forest Zone of Pernambuco in Brazil covering 271 schools in 179 different localities, where a total of 11,234 examinations were performed. The overall positivity for *S. mansoni* was found to be 14.4%, and the egg count for the parasite in faeces gave a geometric mean of 67.9 eggs per gram of faeces. These results allowed the region to be categorized as a medium schistosomiasis-prevalent region. In northwestern Ethiopia, a study was conducted by Alemu *et al* (2011) among 317 school children of Zarima town from 1st April to 25th May, 2009 to investigate soil transmitted helminths and *S. mansoni* infections among the pupils. *S. mansoni* was isolated in 37.9% of the study participants. In another study of prevalence of schistosomiasis and other intestinal helminth infections undertaken among school children in Agaie, Niger State, Nigeria between January, 2010 and September, 2011 by Banji and Babadoko (2012), 37% of the pupils were infected with urinary schistosomiasis. Deganello *et al* (2007) conducted a survey of schistosomiasis among school children in two villages in Southern Sudan, and the prevalence of *S.mansoni* infection was 51.5%. Baken *et al* (2005) conducted a cross-sectional survey on morbidity due to *Schistosoma mansoni* in 4,354 Ugandan schools across eight districts and found baseline prevalence of infection to be 44.3%.

In Kenya, schistosomiasis continues to be a significant cause of morbidity among school-going children, particularly those that live in areas near water bodies like lakes, rivers, ponds and swamps. For example, a Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) project study conducted by Samuels *et al* (2012) on *S. mansoni* morbidity among school children in Nyanza province, western Kenya, where 822 school children were investigated, *S. mansoni* infection stood at 69%. According to a study reported by Adoka *et al*, (2014) in western Kenya, the prevalence of *S. mansoni* along the Kenyan Lake Victoria basin ranges between 40% and 80%. In a research study conducted by Odiere *et al* (2012) on prevalence of schistosomiasis in Mbita and its adjacent islands of Lake Victoria in western Kenya, 4,065 school children aged 5-19 years in 84 primary schools were examined, and the mean school prevalence of *S. mansoni* infection was 60.5%. Schools in closest proximity to Lake Victoria had the highest prevalence of the infection.

In a study done by Mwinzi *et al* (2012) in East Uyoma location near Asembo area, where a total of 492 school children were involved, it was found that the prevalence of *S. mansoni* among school- going children ranged between 5% to 43.2%, with an average prevalence of 17.8% in the location. In a survey conducted by Handzel *et al* (2003) in 1,246 school children in 32 primary schools in Asembo area located within the Kenyan Lake Victoria basin, the *S. mansoni* prevalence ranged between 35-80%. The mean school prevalence was established in this study at 16.3%. A report by Ndombi, (2012) on *S. mansoni* gives a prevalence of 17% in Asembo area, which is almost similar to that of Handzel *et al*, (2003).

According to Barbosa *et al* (2006), school-going children are preferred in studies related to prevalence of schistosomiasis because schools are more accessible, and the greatest prevalence of *S. mansoni* in a given community is found within the school-going age group. Data gathered from this age group can, therefore, be used for intervention within the community as a whole.

2.5. Human IgG and IgM Immunoglobulin Responses to *S. mansoni* Infection

2.5.1. IgG and IgM Responses

Infected individuals exhibit considerable diversity of IgG and IgM responses against the *S. mansoni* infection (Dunne *et al*, 1993). It is the various antigens in the different life stages of the parasite that elicit the multiple antibody responses especially during the transition period from acute to chronic stages of the infection (Khalife *et al*, 1986).The humoral responses generally develop slowly against the antigens from all life stages of the parasite (Odongo-Aginya *et al*, 2012).The antibody responses have been studied using soluble worm antigen preparations (SWAP) and soluble egg antigens (SEA).

Different human IgG and IgM antibody isotype responses against *Schistosoma* parasite can either mediate or block *in vitro* killing of schistosomula by a number of immune effector mechanisms (Khalife *et al*, 1989; Demeure *et al*, 1993). Longitudinal population studies have demonstrated that particular parasite-specific IgG and IgM antibody responses correlate with human resistance to reinfection or susceptibility in areas endemic for *Schistosoma mansoni* (Butterworth *et al*, 1987; Dunne *et al*, 1992; Demeure *et al*, 1993). IgG antibodies and eosinophils have been shown to kill schistosomula of *S. mansoni in vitro*, and the killing effect is associated with IgG_1

and IgG_3 antibodies, which have been associated with killing of schistosomula when armed with activated eosinophils, leading to increased resistance to reinfection by the parasite (Vereecken, *et al*, 2007). IgG_2 and IgM act as blocking antibodies, preventing the expression of an effective protective immunity (Butterworth *et al*, 1987). The IgM has been found to be more highly expressed in children infected with schistosomiasis than adults suffering from the same (Naus, *et al*, 2003).

Butterworth *et al*, (1988) reports that the presence of high levels of IgG_2 anti *S. mansoni* egg polysaccharide antibodies in children may prevent development of resistance to reinfection, while children with low serum level of the IgG_2 antibodies may develop resistance to re-infection. *In vitro* studies indicate that human eosinophils can kill schistosomula if armed with appropriate antibodies such as IgG_1 and IgG_3, while presence of IgG_2 and IgM antibodies retard the eosinophil helminthotoxicity (Capron and Capron, 1994), which therefore blocks development of resistance against the infection. One hypothesis about the blocking mechanism is that IgG_2 and IgM antibodies elicited against carbohydrate epitopes on parasite eggs also react with peptide epitopes on schistosomulum surface, hence interfere with the combining sites of the effector IgG_1 and IgG_3 antibody isotypes thus preventing the latter from binding to the high affinity receptors on eosinophils involved in killing the schistosomula. This cross-reactivity results in competition between the effector antibodies (IgG_1 and IgG_3) and the blocking antibodies (IgG_2 and IgM), thereby hindering development of protective immunity against schistosomiasis (Butterworth *et al* 1988; Khalife *et al*, 1989).

2.5.2. Levels of IgG subclasses, IgM and infection intensity among school children infected with *S. mansoni*.

Humoral immune responses directed against adult worm and egg antigens of *S. mansoni* have been documented mainly in adult humans. Studies exclusively covering school children showing levels of IgG_1 and IgG_3 antibodies that contribute to resistance to *S. mansoni* parasite, and IgG_3 and IgM antibodies that lead to susceptibility to the infection are still inadequate. For example, a research study conducted by Ramirez *et al* (1996) to investigate the immunopathology of human schistozomiasis and examined immunoglobulin type profiles and responses to praziquantel in 43 participants. Only 22 children aged 8-12 years were included in the study. The low number of school children may not give adequate and reliable information about levels of the immunoglobulins among the school children previously known to be more susceptible to *S. mansoni* infection (Hotez and Fenwick, 2009). This study by Ramirez *et al* (1996) found that anti-SWAP IgG_1 levels were higher in young children. The results here contradict the findings of Naus *et al* (1999) that registered peak levels of this antibody at the age of 20 years.

Kabatereine *et al* (1999) reports that intensity of infection by *S. mansoni* peaks in the mid teen years, then shows gradual decline as the children advance in age due to development of acquired protective immunity against the infection. The development of this immunity coincides with the time when worms from early infections begin to die, given their lifespan of about 5 to 10 years (Woolhouse *et al*, 2000). Important antigens against *S. mansoni* that were not previously encountered by host immunity are then released following death of the worms. The released antigens elicit development of protective immune responses that consequently provide increased

resistance to new infections as the host matures in age (Fulford *et al*, 1995; Woolhouse *et al*, 2000). A research study only covering school children is, therefore, necessary to confirm or disapprove these findings of Kabatereine *et al* (1999).

A research study was done by Caldas *et al* (2000) to investigate susceptibility and resistance to *S. mansoni* infection, focusing on parallel cellular and isotypic immunologic assessment in Siqueira area of Minas Gerais in Brazil. A total of 102 persons aged 3 - 88 years were recruited for the study. The study found that anti-SEA IgM levels were highest while anti-SWAP IgG_2 levels were lowest in the participants. The results here appear contradictory, given that both IgM and IgG_2 antibodies are known to be blocking antibodies whose levels should not show a wide difference in a particular group of study participants.

Vendrame *et al* (2001) conducted a study on evaluation of IgG antibodies in patients with chronic *Schistosoma mansoni* before and after specific treatment on 17 chronically infected patients with ages ranging between 17 – 77 years with mean age of 33 in Brazilian endemic areas in Sao Paulo City. This age bracket, however, left out school age children who have been known to suffer most with regard to *S. mansoni* infection (Hotez and Fenwick, 2009).

A study conducted by Jassim *et al* (2007) on antibody isotypes in human *S. mansoni* on 276 study participants in the Gezira Irrigated Area of Sudan, revealed elevated total serum IgG levels in the infected people. The responses to larval and adult antigens by IgG_1, IgG_2 and IgG_3 antibodies in untreated chronic infections were poor or absent. This study did not, however, give adequate attention to school-going

children normally found in the age bracket of 5 to 20 years, and who are more vulnerable to the infection, according to Hotez and Fenwick, (2009).

2.5.3. Levels of IgG subclasses and IgM *versus* Intensity of Infection by *S. mansoni*

Longitudinal population studies have demonstrated that particular parasite-specific antibody responses correlate with human resistance to reinfection or susceptibility in areas endemic for *Schistosoma mansoni* (Butterworth *et al*, 1987; Dunne *et al*, 1992; Demeure *et al*, 1993). Some studies have shown that higher levels of IgG_2 and IgM are associated with a higher risk of re-infection with schistosomiasis whereas IgG_1 and IgG_3 appear to be protective to the host (Vereecken, *et al,* 2007). Many of these antibodies are elicited in response to egg polysaccharide antigens (Butterworth *et al,* 1987).

Correlations between IgG and IgM levels *versus* intensity of infection by *S. mansoni* in Asembo area remains unknown.There is also limited information on how the levels of IgG and IgM antibodies vary with intensity of the infection in infected school children. For example, Satti *et al* (1996) investigated specific immunoglobulin measurements related to exposure and resistance to *S. mansoni* infection in Sudanese canal cleaners, where a total of 113 study participants were investigated. The study, however recruited only 46 school children yet this is the group that has previously been described as being more vulnerable to *S. mansoni* infection compared to adult humans (Hotez and Fenwick, 2009),. The study found an insignificant negative correlation between anti-SEA IgG_1 and intensity of infection, which was a contrast of

the findings of van Dam *et al* (1996) that registered a positive correlation between the two variables.

A study was done by Naus *et al* (1999) and involved 419 participants focusing on the antibody isotype profiles in Masongaleni, Kenya involving people of different ages ranging from 5-59 years. Naus *et al* (1999) reported no correlation between IgG_2-SEA and infection intensity, while van Dam *et al* (1996) reported a weak negative correlation between the IgG_2-SEA and infection intensity. There was, therefore, a contradiction between the findings of the two studies that necessitated further investigation.

Vendrame *et al* (2001) conducted a research study in a non-endemic area in Brazil involving 17 *Schistosoma mansoni* patients, 14 of whom had low to moderate parasite burdens. The patients showed high levels of IgG antibodies in AWA and SEA-ELISA. However, the study did not correlate the levels of the immunoglobulin isotypes with intensity of infection among the subjects under investigation.

Naus *et al* (2003) conducted a research study on the relationship between age, sex, egg count and specific antibody responses against *Schistosoma mansoni* antigens in a Ugandan fishing community where 380 individuals aged 5 – 59 years were involved. Anti-SWA IgG_1 responses increased with egg count whereas anti-SEA IgG_2 decreased with egg count.

Singh *et al* (2011) conducted a study of immunoglobulin profiling of 294 patients that were infected with *S. mansoni* in Central India. They only investigated total IgG levels and found a negative correlation between the total IgG levels in relation to

intensity of infection by the parasite. The study did not also investigate the individual IgG subclasses like IgG_1, IgG_2 and IgG_3 which have been shown to contribute to resistance and susceptibility to the *S. mansoni* infection according to Butterworth *et al* (1987), Dunne *et al* (1992), Demeure *et al* (1993) and Vereecken *et al* (2007).

A research study was conducted by Badri (2011) to investigate anti- SWAP IgG isotypes against *Schistosoma mansoni* infection in school children in Elkeryab village in Khartoum state where a total of 20 infected school children aged 5 – 16 years were included as study participants. Although the study focused on the school children, the number of participating was too low to give reliable results. The study revealed that there were positive correlations between anti-SWAP, IgG_1 and age which was a contrast of the findings of van Dam *et al* (1996) that found a negative correlation between the two variables. There was significant negative correlation between anti-SWAP IgG_3 levels and intensity of infection, which does not agree with the findings of van Dam *et al* (1996) that found a positive correlation between the two variables.

A study was conducted by Shawesh *et al* (2015) on relationships between humoral responses to *S. mansoni* and age, sex and prevalence of infection in 290 sera of persons aged 5-60 years in northwestern Uganda. However only IgG_1, IgG_4 and IgE were considered thus leaving out some important immunoglobulins responsible for susceptibility and resistance to *S. mansoni* infection like IgG_2, IgG_3 and IgM. The study registered significant positive correlations between anti SWAP and anti SEA IgG_1 responses and egg count which was consisted with that of other studies (van Dam *et al*, 1996; Naus *et al*, 2003: Badri 2011).

2.5.4. Levels of IgG subclasses and IgM *versus* Age of Humans Infected with *S. mansoni*

Naus *et al* (2003) investigated the relationship between age, sex, egg count and specific antibody responses against *S. mansoni* in 380 individuals aged between 5-59 years in a Ugandan fishing community. Van Dam *et al* (1996) carried out an almost similar study focusing on antibody patterns against *S. mansoni* in a sample of 289 people aged between 5-40 years. The two studies presented contradicting results because according to Naus *et al* (2003), there was no significant correlation between anti-SWAP IgG_1 and age, while in the results of Van Dam *et al* (1996), a negative correlation existed between anti-SWAP IgG_1 and age. Again, Naus *et al* (2003) reported no significant correlation between anti-SWAP IgG_3 as well as anti-SWAP IgM with age, while Van Dam *et al* (1996) reported a positive correlation between anti-SWAP IgG_3 and age, and a negative correlation between anti-SWAP IgM and age. Naus *et al* (2003) also reported a negative correlation between anti-SEA IgG_2 and age, while according to van Dam *et al* (1996), there existed a positive correlation between anti-SEA IgG_2 and age.

A study by Negrao-Correa, *et al* (2014) was conducted on association of *S. mansoni*-specific IgG and IgE antibody production and clinical schistosomiasis status involving 97 inhabitants between 14-68 years of age in Corrego do Choro, Padre Paraiso city, Brazil. The study only correlated age *versus* infection intensity but did not investigate the correlations between age and concentrations of IgG antibodies. Again, the various subclasses of IgG like IgG_1, IgG_2 and IgG_3 were not considered under this study. Furthermore, the study left out the critical age group of between 5-13 years where most of school-going children are normally found.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area and Population

This study was conducted in Rarieda Subcounty in Siaya County, western Kenya. It is situated about 80.7km west of Kisumu town. Its geographical co-ordinates are 0° 10′ 48″ south of the equator and 34° 23″ 24″ east of Greenwich Prime Meridian, with latitude of -0.1333° and longitude of 34.3667° (Rarieda District Development Plan, 2012). It borders L. Victoria to the south and covers an area of approximately 200km^2. It has a total population of about 57,000 people. Approximately 96% of the inhabitants belong to the Luo ethnic group, and the majority of these are subsistence farmers of maize, millet, potatoes and cassava and also practice fishing (Handzel *et al*, 2003; Rarieda District Development Plan, 2012).

planPlan, 2012).

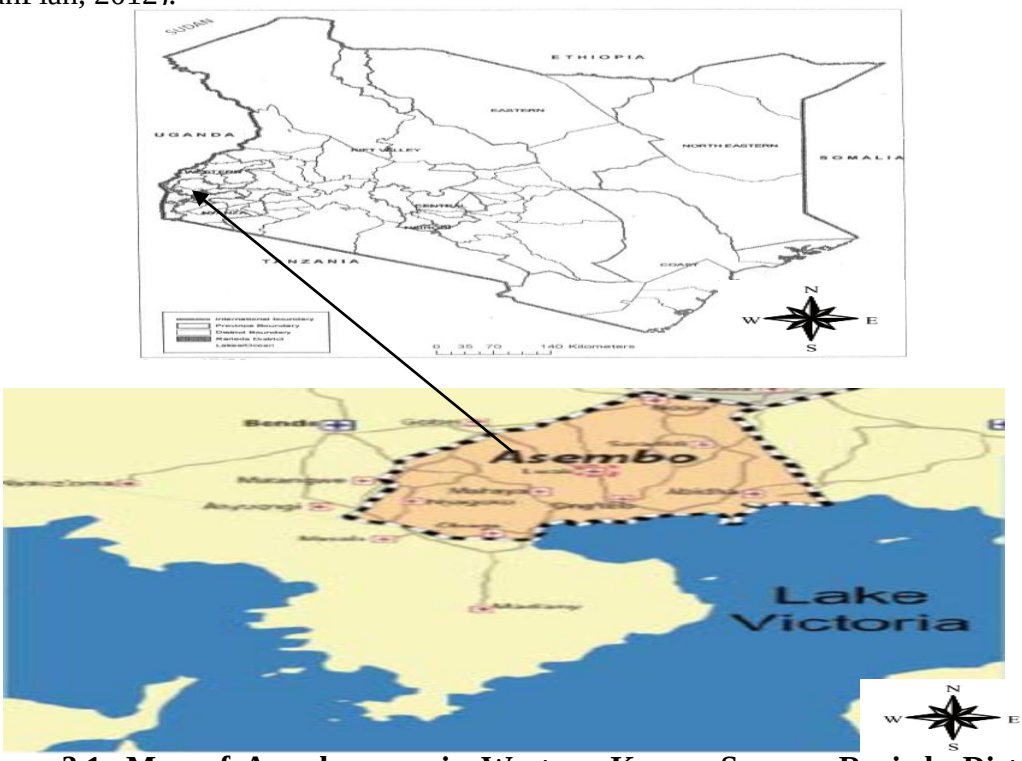

Figure 3.1: Map of Asembo area in Western Kenya. Source: Rarieda District Development Plan (2012).

Asembo area has an altitude ranging from 1,140 metres to 1,350 metres above sea level. It has modified equatorial climate with strong influence from local relief and the breeze from L. Victoria which influences rainfall amounts and distribution. The area's climate is mainly warm, dry and humid and the average temperature is 27^{o}C. The months of September to March are generally hottest whereas April to June are coldest (Rarieda District Development Plan, 2012). Rainfall ranges from 800mm to 1600mm with a mean annual rainfall of 900mm. The rainfall is bimodal with long rains occurring between March and May while short rains occurring between September and November. (Rarieda District Development Plan, 2012). Asembo area has a total of 31,293 school children. The prevalence of *S. mansoni* infection among the school children in Asembo area stands at between 35-80% (Handzel *et al*, 2003; Adoka *et al*, 2014). The target group of this study was school children aged between 5 and 20 years, attending primary and secondary schools in the *S. mansoni*-endemic areas of Asembo area. The study included a mixture of male and female pupils.

3.2 Research Design

The research followed a cross-sectional design conducted in schools within 5km from L. Victoria, involving data collection from a population of school children aged between 5 and 20 years. The children were subdivided into age groups of 5-8 years, 9-12 years, 13-16 years, and 17-20 years. The study was carved out of a larger on-going project called Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) project. The mandate of the SCORE project was to carry out research on control and elimination of schistosomiasis in the study area.

3.3 Recruitment of Participants

The participants were randomly selected for the study by the SCORE Project upon meeting the inclusion criteria and giving consent to participate in the study. These participants were drawn from two primary and two secondary schools in the areas previously established to be endemic for the *S. mansoni* infection according to Samuels *et al* (2012).

3.3.1 Inclusion Criteria

The participants to be involved in the study were included if they were students aged between 5 and 20 years, attending primary and secondary schools in the area, and willing to participate and also have parents that were willing to grant permission.

3.3.2 Exclusion Criteria

Pupils were excluded from the study if they were below the age of 5 years or above the age of 20 years, were having haemoglobin levels below 8gHb/dl, and were not willing to participate in the study.

3.4 Sample Size Determination

A total of 350 stool and blood samples were used in the present study. The samples were obtained from the infected school children already diagnosed by the larger, on-going SCORE project in the same area. The sample size for the present study was determined based on the Asembo area school children's minimum prevalence of *S. mansoni* of 35%, according to Handzel *et al* (2003). The following Fosgate (2009) formula was used to arrive at the sample size;

$$\mathbf{n = \frac{(z^2)(p)(1-p)}{e^2}}$$

where **n =** the desired minimum sample size

z = z score at z statistic of **1.96**

p = proportion in the target population expected to be infected with *S. mansoni*, in this case a minimum of 35%, i.e.**0.35**

e = the level of statistical significance, set at **0.05**

Therefore, in the present study

$$\mathbf{n = \frac{(1.96)^2(0.35)(1-0.35)}{0.05^2}}$$

= 350 samples.

3.5 Sample Collection and Processing

3.5.1 Stool Samples

The participants were asked to provide stool samples, which were used to examine the eggs of *S. mansoni* using Kato Katz technique (Katz *et al*, 1972) to establish intensity of infection by way of quantitative evaluation of eggs of *S. mansoni* on duplicate slides using Kato-Katz faecal thick smear technique (Refer appendix V). A total of 350 stool samples previously collected by the SCORE project were used in the current study. In the Kato Katz technique, a small amount of stool was placed on newspaper using a wooden applicator stick. A screen nylon was then pressed on top so that some of the faeces could filter through. The filtered faeces were collected by scrapping a flat spatula across the upper surface, then transferred to a template with a hole pre-measured to hold 41.7mg of the faeces. The template was carefully removed so that the cylinder of faeces was left on the slide. The faecal material was then covered with cellophane strip pre-soaked in 50% glycerol, 50% water and 3% malachite green.

The microscope slide was then inverted and the feacal sample pressed against the cellophane strip on a smooth hard tile, so as to spread the faecal material evenly. The slide was then carefully removed by gently sliding it sideways to avoid separating the cellophane strip and the slide. The smear was then examined under a light microscope in a systematic manner, and the number of eggs of *S. mansoni* reported and multiplied by 24 to obtain the total number of eggs per gram (EPG), which is an estimation of infection intensity (WHO, 1993). Two slides per stool were used to examine for the eggs of *S. mansoni* parasite.

3.5.2 Blood Samples

The participants were asked to provide a small amount of blood to be used to examine the concentrations of immunoglobulins IgG_1, IgG_2, IgG_3 and IgM. A total of 350 blood samples previously collected by the SCORE project were used in this study. Approximately 3mls of peripheral venous blood was obtained from each participant by venipuncture through the assistance of a qualified KEMRI phlebotomist, and placed into vacutainer tubes (BD, Franklin Lakes, NJ USA) coated with heparin that acts as an anticoagulant. On each tube was written the date of collection of the sample and sample identification code. The blood samples were then placed in an ice box that provided low temperature, then transported to KEMRI-CDC, NTD laboratory in Kisian, near Kisumu town within four hours of collection for processing.

All blood processing procedures were carried out in a sterile biological safety cabinet (NuAire, Inc. Minnesota, U.S.A). Room temperature Ficoll/ Hypague solution (Atlanta Biologicals) was placed into 50ml. centrifuge tubes (Becton Dickenson and

Co, USA). Pipettes of 10ml capacity were then used to draw blood from the Vacutainer tubes. The blood was then gently layered over the Ficoll/Hypague solution by density gradient centrifugation. The Ficoll/Hypague-blood mixture was then centrifuged at 1700 revolutions per minute for 35 minutes at room temperature and plasma obtained. Using a 5ml pipette, the plasma layer formed on top was collected and put in well labeled 15ml centrifuge tubes (Becton Dickenson and Co., USA). This was aliquoted into a 1.5ml cryotubes and stored in a freezer at -20°C to be used later for antibody ELISA assays for determining the concentrations of the immunoglobulins IgG_1, IgG_2, IgG_3 and IgM.

3.5.3 Enzyme-Linked Immunosorbent Assay (ELISA) for Immunoglobulins

Indirect ELISA was performed on plasma using soluble worm antigen preparations (SWAP) (UGA, USA) and soluble egg antigens (SEA) (UGA, USA). The anti-SWAP and anti-SEA ELISA protocol (modified February, 2006 and updated February, 2007 and June, 2013 for ELISAs in Kenya) was used (refer appendix II).

In the procedure, four ELISA microtitre 96-plates (Immulon 2HB model, USA) each for IgG_1, IgG_2, IgG_3 and IgM, were labeled for SEA. Exactly 35.78µl of SEA was added into 48ml of 0.1M coating buffer carbonate ($NaHCO_3$, Na_2CO_3 and distilled water, at pH 9.6) to make a coating mixture (Refer appendix IV for layout of ELISA plate). Exactly100µl of the coating mixture was then added into each of the wells of the ELISA plates, which were then covered using parafilm (Neenah, WI54956 USA) and incubated on a shaker (Titer Rotar Shaker, model 4625-1) at 37^0C for 2 hours.

Meanwhile, blocking buffer was prepared by mixing 60ml of 1xPBS (gibco by life technologies, USA) with 180µl of 0.3% Tween-20 (Kirkeguard and Perry Laboratories, USA) and 3g of powdered milk (New Kenya Co-operative Creameries Ltd). The resulting mixture was then shaken thoroughly and vortexed to form a homogenous mixture. A sample dilution of 1:100 was then done by adding 10 µl of each sample into 990µl of blocking buffer to make a total of 1000µl of a diluted sample mixture.

After the 2 hour period, the plates were washed four times using washing buffer (1xPBS and Tween-20), then blotted to make them dry. The diluted sample mixtures measuring 100µl was then added in duplicate to the respective wells of each of the plates according to a template set up previously prepared on a sheet of paper. The plates were then covered with parafilm and incubated on a shaker at 37^0C for 30 minutes.

Meanwhile, mouse anti-human monoclonal antibody isotypes (Southern Biotech, USA) of varying dilutions were prepared for IgG_1, IgG_2, IgG_3 and IgM, by mixing 50µl of 1 x PBS with 150µl of 0.3% Tween-20 to make a dilution solution. The mixture was then vortexed thoroughly and allowed to stand. Four small conical tubes (Corning, NY 14831, Mexico) were labeled as IgG_1, IgG_2, IgG_3 and IgM. Exactly 12.5ml of the already prepared dilution solution was then added into each of the conical tubes.

Mouse anti human monoclonal antibody isotypes were measured as follows; IgG_1(3.125µl), IgG_2(10µl), IgG_3(1.56µl) and IgM (0.78µl). The amount of the mouse anti human isotypes above were then added to the 12.5ml of dilution solutions in the

respective conical tubes to give dilutions of 1:4000, I:500, 1:8000 and 1: 16,000 for IgG_1, IgG_2, IgG_3 and IgM, respectively. The dilution mixtures of each of the conical tubes were then vortexed thoroughly. Standards were also prepared to act as positive controls by arranging flow cytometry tubes (Becton Dickinson and Company, USA) for each of the four isotypes. Blocking buffer measuring 600 µl was then added into each of the first four tubes labeled A horizontally across. Blocking buffer measuring 300µl was then added to the rest of the tubes labeled B to H vertically downwards. SEA positive plasma measuring 6µl was then added into the first four tubes. Serial dilution of the standards was then done by transferring 300µl of contents of one flow tube into the next downwards.

After the expiry of the 30 minutes period the plates were washed four times using wash buffer, to remove unbound monoclonal antibodies, then blotted to make them dry. Standards measuring 100µl were then added in duplicate to the wells in the first two columns of the plates (i.e. wells A1 and A2 to wells H1 and H2). Mouse anti human isotype dilution mixtures already prepared measuring 100µl were then added in duplicate in the respective wells of the plates from wells A3 and A4 to wells F11 and 12. Exactly100µl of 1:100 normal human serum (NHS) obtained from uninfected persons was added to each of the wells G11 and G12 to act as negative controls while 100ul of the 1 x PBS to 0.3% Tween-20 and powdered milk mixture was added to each of the wells H11 and H12 to act as blank which were also negative controls. The plates were then covered with parafilm and incubated on a shaker at 37^0C for 30 minutes.

Meanwhile TMB-A and TMB-B substrate solutions (Gaithersburg, MD20878 USA) were taken out of a 4^oC environment in a refrigerator and allowed to reach room temperature. At the same time the Molecular Devices Emax Precision Microplate ELISA Reader (Co China model) connected to Softmax Pro. 5.4 software was then set up in readiness for readings of the ODs and adjusted concentrations of the immunoglobulins. After 30 minutes the plates were washed four times using wash buffer, then blotted to make them dry. TMB-A substrate solution measuring 20ml was added to 20ml of TMB-B substrate solution in a TMB boat and both were allowed to mix. Exactly 100µl of the TMB-A and TMB-B mixture was then added into each of the wells of plates and allowed to develop for 5 minutes. Sulphuric VI acid (2N) measuring 100µl acting as a stop solution was then added into each of the wells. The plates were finally taken for OD and adjusted concentration readings of the immunoglobulins IgG_1, IgG_2, IgG_3 and IgM in the aforementioned ELISA reader.

The whole procedure was repeated for SWAP except for preparation of buffer where 40µl of SWAP was added into 48ml of 0.1m coating buffer carbonate (($NaHCO_3$, Na_2CO_3 and distilled water, pH 9.6) and dilutions of mouse anti-human isotypes which were measured and diluted as follows; IgG1(3.125µl), IgG_2(14.5µl), IgG_3(1.56µl), IgM (0.78µl).The amounts of the mouse anti-human isotypes above were then added to 12.5ml of dilution mixture to give isotype of dilutions of 1:100, 1:2000, 1:3000 and 1:16,000 for IgG_1, IgG_2, IgG_3 and IgM, respectively. The rest of the procedure was followed as was in the case of SEA (Refer Appendix III).

3.6 Data Analysis

The statistical analysis of data was conducted using Graph Pad Prism Version 5 software (Graph pad Software, Inc. San Diego, A). Multivariate ANOVA was used to determine if mean levels of immunoglobulins and infection intensities were significantly different between the age groups. Tukeys Honest Significant Difference test was conducted to obtain the least significant difference in the levels of the immunoglobulins and infection intensities. Spearman's Rank Correlation Coefficient was used to determine the correlations between concentrations of the immunoglobulins IgG_1, IgG_2, IgG_3 and IgM in relation to infection intensity and age of the infected school pupils. Regression analysis was done to assess the strength of associations between the independent and dependent variables. The independent variables in this study were infection intensity and age, while the dependent variable was immunoglobulin concentrations, which were expressed in arbitrary units per ml(AU/ml).

3.7 Ethical Considerations

3.7.1 Approval of the study

Ethical approval for this study was obtained from the Ethical Review Committee at the Kenya Medical Research Institute (Refer appendix II). Informed consent was obtained from pupils aged 18-20 years, and from the parents of the pupils aged below 18 years (Refer appendix I). The participants and their parents received adequate explanations about the study in simple understandable Dholuo language. The recruitment was purely voluntary and was to be done on condition that the students/parents were willing to grant permission.

Unique identification codes were allocated to each study participant for purposes of confidentiality. The codes were then used for purposes of sample tracking and identification during the study.

3.7.2 Risks and Benefits

There was no serious risk involved in obtaining stool from the participants. However, the process of obtaining blood samples by venipuncture exposed the participants to minimal risks of discomfort. Qualified KEMRI phlebotomists were involved in obtaining the small samples of blood to minimize the risks. Infected pupils were treated with an oral drug called Praziquantel (40mg/kg of body weight) by the main research project called Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) project from which the current study was carved out. The mandate of the SCORE project was to carry out research on control and elimination of schistosomiasis in the study area.

CHAPTER FOUR

RESULTS

4.1 Levels of IgG_1, IgG_2, IgG_3 and IgM by Age Group in School Children Infected with *S. mansoni*

Figures 4.1.1. and 4.1.2. below show bar graphs of the immunoglobulin levels in various age-groups of the school children infected with *S. mansoni* in Asembo area.

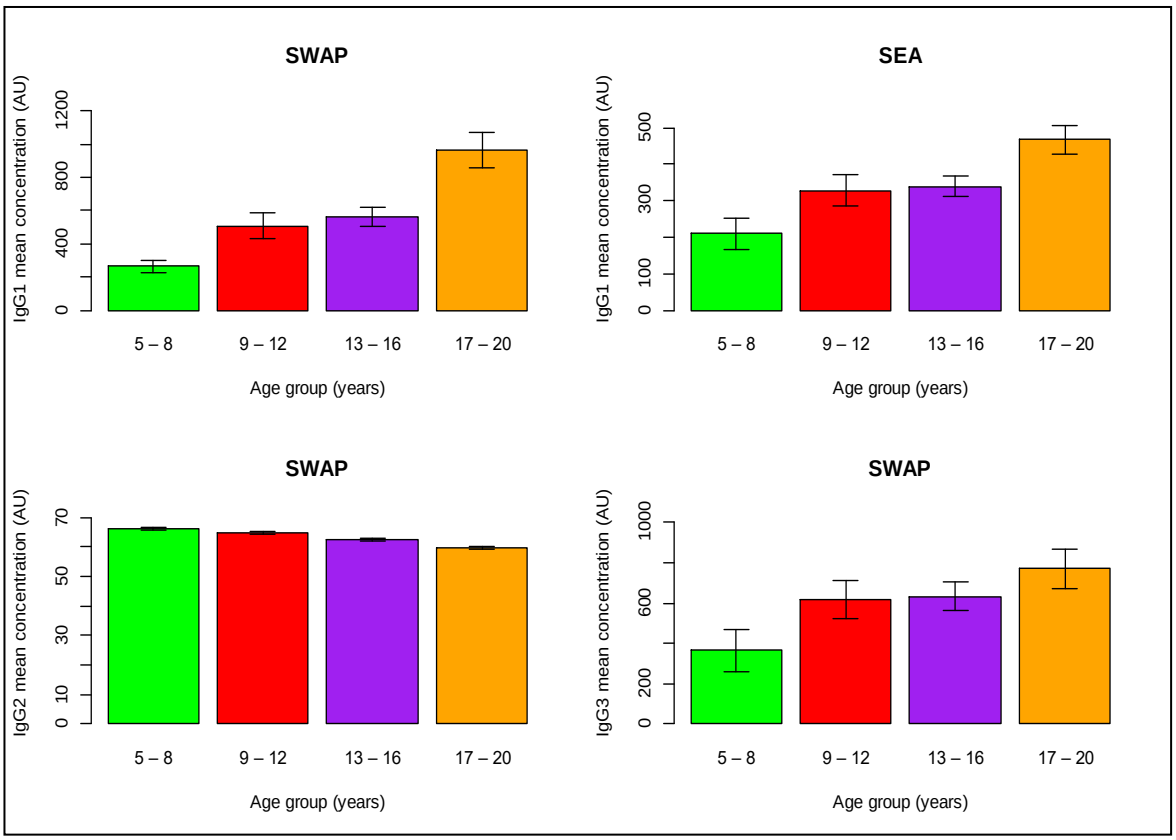

Figure 4.1.1. Bar graphs showing mean anti-SWAP and anti-SEA IgG_1, IgG_2, and IgG_3 mean levels in arbitrary units (AU)/ml by age group. The results are expressed as arithmetic means of absorbance values at OD_{450}.

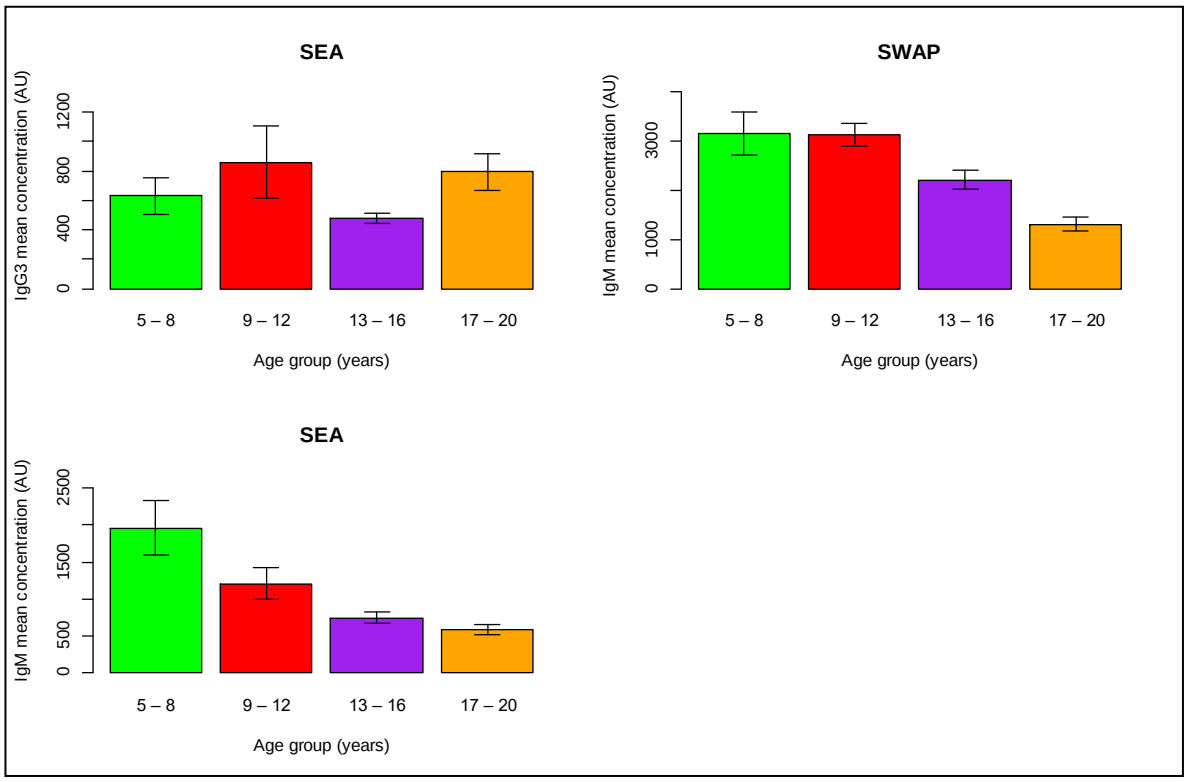

Figure 4.1.2. Bar graphs showing mean anti-SWAP and anti-SEA IgG_3 and IgM mean levels in arbitrary units (AU)/ml by age group. The results are expressed as arithmetic means of absorbance values at OD_{450}.

The levels of anti-SWAP IgG1, anti-SEA IgG_1 and anti-SWAP IgG_3 immunoglobulins increased with age of infected pupils and reached their peaks at the age bracket of 17-20 years. On the other hand, the levels of anti-SWAP IgG_2, anti-SWAP IgM and anti-SEA IgM exhibited a general decrease with age, and each of these immunoglobulins had its highest levels at the age bracket of 5-8 years. Anti-SEA IgG_3 did not follow any particular trend but reached its peak levels at the age bracket of 9-12 years. (Also refer tables in Appendix VII).

Analysis of variance (ANOVA) conducted showed that IgG_1, IgG_2 and IgM mean levels were significantly different between the age groups ($p<0.05$), while IgG_3 levels were not ($p>0.05$) (Refer Appendix VIII). Tukey's honest significant difference (HSD) test conducted demonstrated that the mean IgG_1 levels at age 17-20 years was

significantly different from those of 5-8 years, 9-12 years and 13-16 years (Refer Appendix IX).

The mean levels of anti-SEA IgG_2 antibodies were not included in the results of this study because of poor and inconsistent, hence unreliable values obtained even after conducting several optimization protocols.

4.2 Intensity of infection by *S. mansoni* among the School Children

Figure 4.2 below shows bar graphs of intensities of infection by *S. mansoni* parasite among the infected school children of Asembo area.

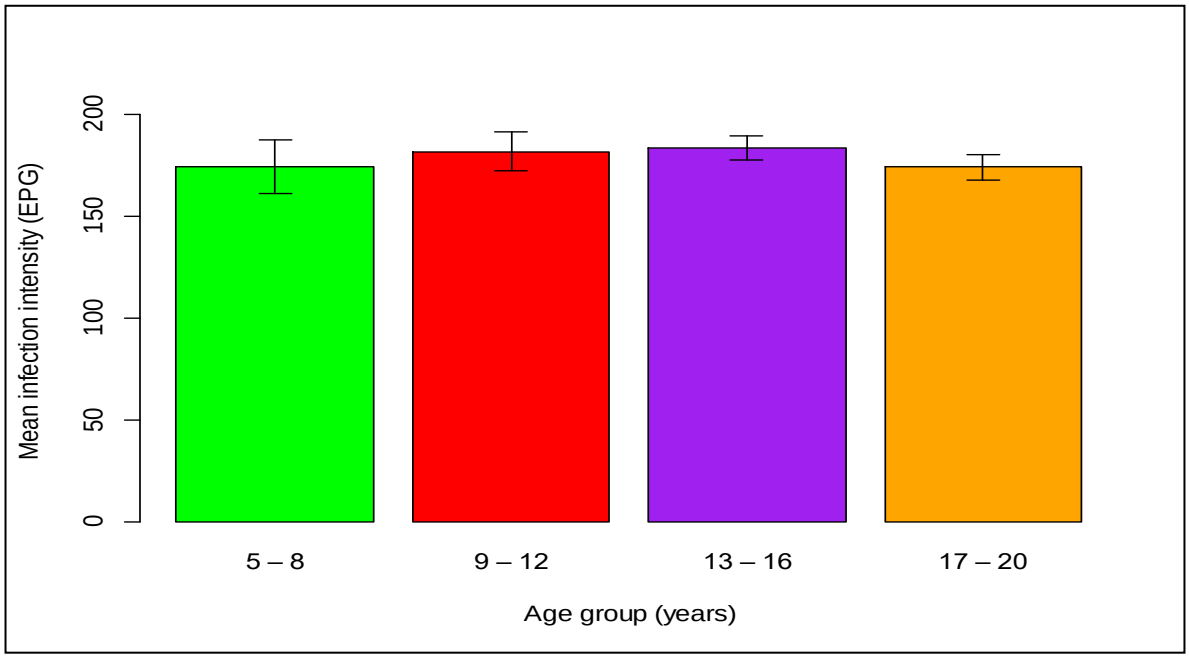

Figure 4.2. Bar graphs showing mean intensities of infection by *S. mansoni* parasite among school children in Asembo area per age group. The intensities of infection were expressed as arithmetic means of eggs per gram (EPG).

Table 4.1 below further summarises the exact parasite burden for each age group in terms of mean intensity of infection in eggs per gram.

Table 4.1: Mean intensity of infection by *S. mansoni* parasite among school children in Asembo area per age group.

	Age group (years)			
	5 – 8	9 – 12	13 – 16	17 – 20
Sample size	n=86	n=89	n=97	n=78
Mean infection intensity (EPG)	174.42±37.2	182.08±42.3	184.05±44.6	174.41±36.8

The age group of 5-8 years showed lower intensity of infection compared to 9-12 years and 13-16 years. The infection intensity increased as from 9-12 years and eventually reached peak levels at the age group of 13-16 years. The lowest intensity of infection was found at the age bracket of 17-20 years. The analysis of variance, however, indicated that there were no significant differences in infection intensities among the age groups ($p<0.05$) (Refer Appendix VII).

4.3 Correlations between Immunoglobulin levels and Infection Intensity

Figure 4.3 below shows scatter plots of correlations between levels of IgG_1, IgG_2, IgG_3 and IgM against intensity of infection by *S. mansoni* on the infected school children in Asembo area.

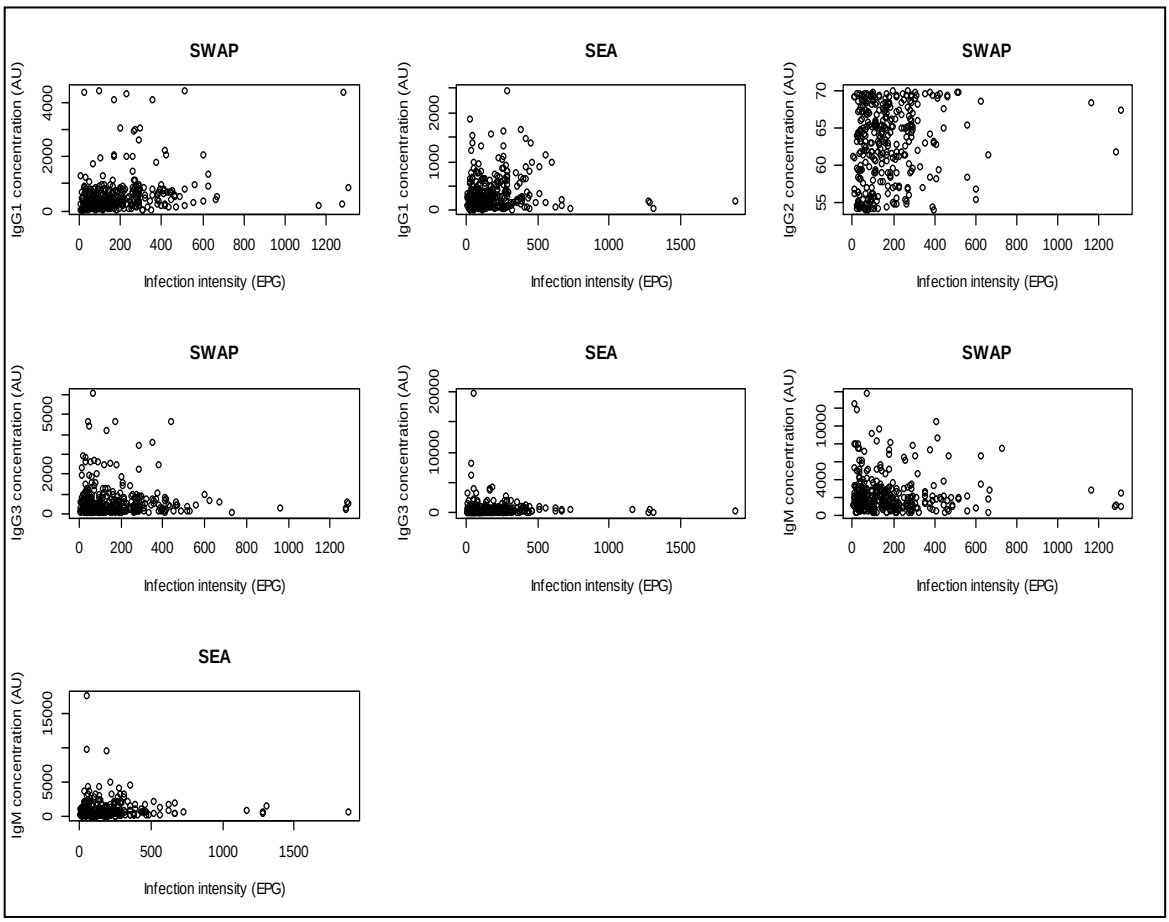

Figure 4.3. Scatter plots showing correlations between levels of anti-SWAP and anti-SEA IgG_1, IgG_2, IgG_3 and IgM immunoglobulins against intensity of infection by *S. mansoni* parasite school children in Asembo area. Results for anti-SEA IgG_2 were excluded due to the poor and inconsistent values obtained even after doing several optimization protocols.

Table 4.2 below further summarises the correlations between levels of the immunoglobulins against infection intensity of the parasite.

Table 4.2: Summary of Correlations between Immunoglobulin Levels and Intensity of Infection. Correlation coefficients were determined using Spearman's Rank correlation coefficient (ρ_s). The ρ_s and P values were considered to be statistically significant at ρ_s>0.1 or ρ_s>-0.1, and P<0.05 respectively. The values in bold were considered to be statistically significant.

Antigen	**Immunoglobulin**			
	IgG_1	**IgG_2**	**IgG_3**	**IgM**
SWAP	ρ_s **= 0.321**	ρ_s **= 0.187**	ρ_s = 0.011	ρ_s **= -0.115**
	p = 0.001	**p = 0.001**	p = 0.838	**p = 0.039**
SEA	ρ_s **= 0.168**	–	ρ_s **= 0.155**	ρ_s = 0.097
	p = 0.002	–	**p = 0.005**	p = 0.082

There were significant positive correlations between levels of anti-SWAP IgG_1 antibodies and intensity of infection by *S. mansoni* (ρ_s=0.321, p = 0.001). Significant positive correlations also existed between levels of anti-SWAP IgG_2 antibodies and intensity of infection by (ρ_s= 0.187, p=0.001). Multiple linear regression models built also indicated that levels of these antibodies significantly increased with infection intensity(Refer Appendix VI).There existed weak negative correlations between levels of anti-SWAP IgM and infection intensity (ρ_s= -0.115, p = 0.039). However, multiple linear regression models indicated no significant effect of intensity of infection on the levels of anti-SWAP IgM antibody (Refer Appendix VI). There was no correlation between anti-SWAP IG_3 levels and intensity of infection (ρ_s= 0.011, p=0.838). This was also confirmed by the multiple linear regression models built for the same (Refer Appendix VI).

The anti-SEA IgG_1 levels showed a weak positive correlation with intensity of infection (ρ_s= 0.168, p=0.002). There was also a weak positive correlation between anti-SEA IgG_3 levels and intensity of infection (ρ_s= 0.155, p=0.005). The multiple linear regression analysis conducted, however, showed no significant influence of infection intensity on the levels of these antibodies (Refer Appendix VI).

4.4 Correlations between Immunoglobulin Levels and Age

Figure 4.4 below shows scatter plots of correlations between levels of IgG_1, IgG_2, IgG_3 and IgM against intensity of infection by *S. mansoni* on the infected school children of Asembo area.

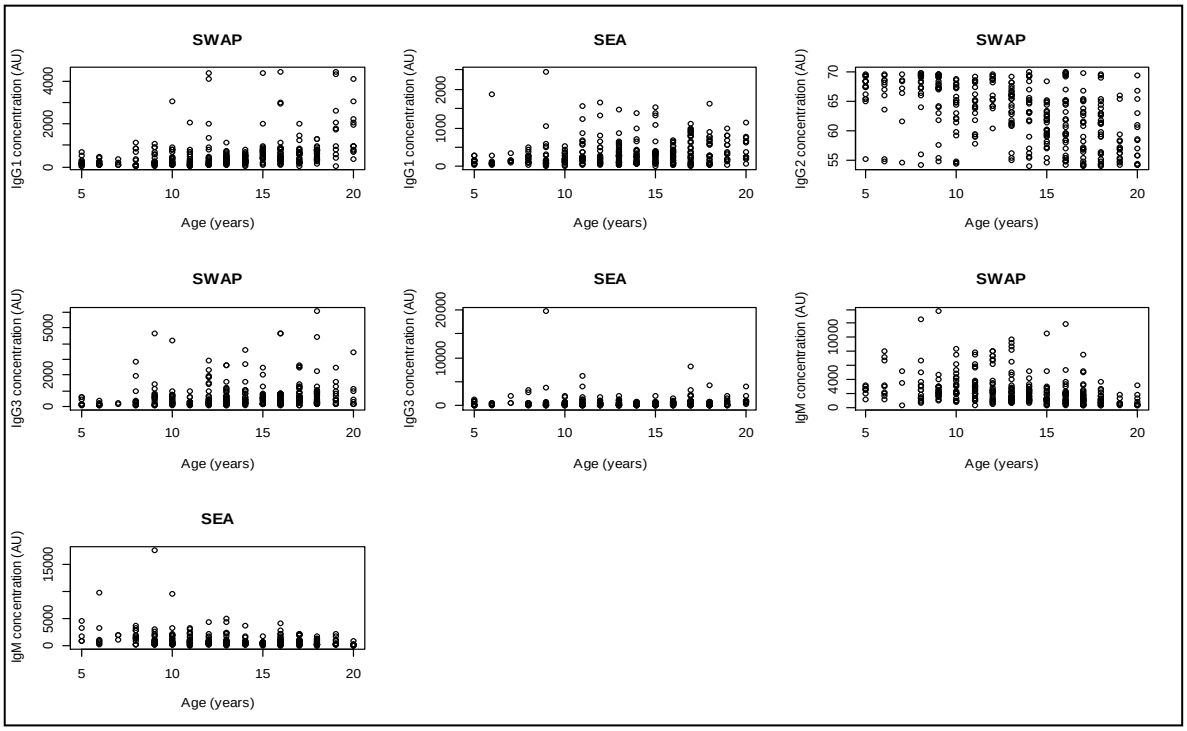

Figure 4.4. Scatter plots showing correlations between levels of anti-SWAP and anti-SEA IgG_1, IgG_2, IgG_3 and IgM immunoglobulins against age of the infected school children in Asembo area. Results for anti-SEA IgG_2 were excluded due to the poor and inconsistent values obtained even after doing several optimization protocols.

Table 4.3 below further summarises the correlations between levels of the immunoglobulins against age of school children infected by the *S. mansoni* parasite.

Table 4.3: Summary of Correlations between Immunoglobulin Levels and Age. Correlation coefficients were determined using Spearman's Rank correlation coefficient (ρ_s). The ρ_s and P values were considered to be statistically significant at ρ_s>0.1 or ρ_s>-0.1, and P<0.05 respectively. The values in bold were considered to be statistically significant.

Antigen	**Immunoglobulin**			
	IgG_1	**IgG_2**	**IgG_3**	**IgM**
SWAP	ρ_s **= 0.472**	ρ_s **= -0.476**	ρ_s = 0.0223	ρ_s **= -0.436**
	p = 0.001	**p = 0.001**	**p = 0.001**	**p = 0.001**
SEA	ρ_s **= 0.286**	–	ρ_s = 0.073	ρ_s **= -0.315**
	p = 0.001	–	p = 0.193	**p = 0.001**

The anti-SWAP IgG_1 levels were significantly positively correlated with age of the infected school children (ρ_s = 0.472, p=0.001). Multiple linear regression models built also showed that the levels of these antibodies significantly increased with age (Refer Appendix VI). There was a significant negative correlation between levels of anti-SWAP IgG_2 and age (ρ_s = -0.476, p=0.001). The multiple linear regression models for this also indicated significant decline in the IgG_2 levels with age (Refer Appendix VI). There was a significant weak positive correlation between anti-SWAP IgG_3 and age (ρ_s = 0.0223, p=0.001). Multiple linear regression showed that the IgG_3 levels significantly increased with age (Refer Appendix VI). There was significant negative correlation between anti-SWAP IgM and age (ρ_s =-0.436, p=0.001), and multiple

linear regression models also indicated that the IgM levels significantly declined with age (Refer Appendix VI).

There existed a weak positive correlation between anti-SEA IgG_1 and age of infected school children (ρ_s = 0.286, p=0.001). A multiple linear regression model for the same showed that IgG_1 levels significantly increased with age(Refer Appendix VI). There was no significant correlation between anti-SEA IgG_3 levels and age (ρ_s = 0.073, p=0.193), and regression analysis confirmed that age had no effect on levels of IgG_3(Refer Appendix VI). A significant negative correlation was found between levels of anti-SEA IgM and age (ρ_s = -0.315, p=0.001). This was confirmed by a linear regression analysis conducted for the same that showed significant decline of IgM levels with age (Refer Appendix VI).

CHAPTER FIVE

DISCUSSION

Human populations that live in areas where *S. mansoni* infections are prevalent tend to develop different immunoglobulin isotype responses which may play important roles in immunity or susceptibility to the infection. Some of the immunoglobulin isotype responses tend to rise or decline with intensity of infection and age. It is, therefore, imperative that the levels of these immunoglobulins are examined in the human host, and correlations that may exist between the immunoglobulin concentrations and infection intensity and age of the host are investigated.

The current study sought to examine the relationship between concentrations of immunoglobulins IgG_1, IgG_2, IgG_3 and IgM, infection intensity in terms of eggs per gram (EPG) and age among infected school pupils. This was in light of the fact that *S. mansoni* infection is common among school-going children living in Asembo area according to studies previously conducted in the area indicating a prevalence rate of 35-80% among school pupils, yet no studies have been conducted showing levels of antibodies that presumably contribute to immunity and susceptibility to *S. mansoni* in the infected school children in the area. Again other authors who had previously investigated these immunoglobulins based their studies in people of generally large age brackets, sometimes ranging from 5 – 59 years (e.g. Naus *et al*, 1999) but not giving exclusive and special attention to school pupils *per se*, despite the fact that they bear the greatest burden of *S. mansoni* infection (Stothard and Gabrielli 2007).

5.1. Levels of IgG_1, IgG_2, IgG_3, IgM and Infection Intensity among the school pupils

The first specific objective of this study was to investigate the levels of IgG_1, IgG_2, IgG_3, IgM antibodies, and infection intensity among school pupils infected with *S. mansoni* parasite. The present study found that there were generally elevated levels of both anti-SWAP and anti-SEA IgM immunoglobulins among the study participants compared to IgG_1, IgG_2 and IgG_3. High amounts of IgM are known to be associated with susceptibility to reinfection in children (Khalife *et al*, 1986; Butterworth *et al*, 1988) the blocking IgM compete with the protective antibodies like IgG and IgG_3 for the same antigens expressed by both eggs and surface of Schistosomulins. This competition blocks the killing of the Schistosomulun that consequently survives and perpetuates the existence of the parasite in the host (Khalife *et al*, 1986).

The anti-SWAP IgG_1, IgG_3 and anti-SEA IgG_1 levels were found to increase with age of the infected pupils, and reached their peak levels at the age bracket of 17-20 years. The two immunoglobulins (IgG_1 and IgG_3) have previously described as being protective against *S. mansoni* infection, and their levels would increase with age of human host leading to conferment of immunity against the infection in older individuals (Butterworth *et al*, 1987; Capron and Capron, 1994; Naus *et al*, 1999; Naus *et al*, 2003; Vereecken *et al*, 2007).

This study found that the intensity of infection was highest at the ages of 9-16 years. Previous studies have shown a peak of intensity of infection at around the same age (Hagan *et al*, 1991; Dunne *et al*, 1992; Fulford *et al* 1992). This could most probably be due to more exposure to the *S. mansoni* parasite as a result of more contact with

water bodies by school children at the ages of 9-16 years. Mduluza *et al* (2001) and Nmorsi *et al* (2007) explained that children at their teens have higher intensities of infection by *S. mansoni* than their younger and adult counterparts because they have more water contact during their involvement in both recreational and domestic activities in water bodies like ponds, streams and rivers compared to adults.

The generally higher intensities of infection in the children noted in the current study could also be attributed to high amounts of blocking antibodies like IgG_2 and IgM observed which make them more susceptible to infection by the parasite (Mutapi *et al*, 1997; Webster *et al*, 1997).

5.2. Correlations between IgG_1, IgG_2, IgG_3, IgM Levels and Intensity of Infection

The second specific objective of the current study was to determine the correlations between concentrations of immunoglobulins IgG_1, IgG_2, IgG_3, IgM and intensity of infection by *S. mansoni* among the infected pupils. Significant positive correlations were noted between levels of anti-SWAP and anti-SEA IgG_1 and intensity of infection ($P < 0.05$). A weak significant positive correlation also existed between anti-SEA IgG_3 and intensity of infection ($p<0.05$). The finding here are consistent with those of past investigators (van Dam *et al*, 1996; Naus *et al*, 1999; Abebe *et al* 2002, Naus *et al* 2003; Badri *et al*, 2011; Shawesh *et al*, 2015). These relationships probably explain the protective nature of IgG_1 and IgG_3 antibodies against *S. mansoni* reinfection. Their levels generally rise with increase in infection intensity to kill the schistosomula as was described by Khalife *et al* (1986) and Butterworth *et al* (1988).

The present study found a weak significant negative correlation between anti-SWAP IgM and infection intensity($P<0.05$) and insignificant correlations between anti-SEA IgM and intensity of infection ($P>0.05$). The findings here conform with those of van Dam *et al* (1996). In previous studies in school children infected with chronic schistosomiasis, the presence of high amounts of IgM antibodies has been associated with blocking of protective immunity (Butterworth *et al*, 1987; Dunne *et al*, 1988; Butterworth *et al*, 1988; van Dan *et al*, 1996). The generally highly elevated amounts of both anti-SWAP and anti-SEA IgM, reaching their peaks at the age bracket of 5-8 years then decreasing with advancement in age, probably indicates that this antibody blocks the expression of immunity against *S. mansoni* infection in Asembo area.

5.3. Correlations between Immunoglobulin levels and Age

The third specific objective of this study was to determine the correlations between the levels of immunoglobulins IgG_1, IgG_2, IgG_3, IgM and age of the infected pupils. The levels of anti-SWAP and anti-SEA IgG1 increased with age and reached peak levels at 17-20 years thus registering a positive correlation between the two variables ($P<0.05$). The results here probably confirm the postulation by past investigators (Butterworth *et al*, 1987; Vereecken *et al* 2007) that IgG_1 is a protective antibody against *S. mansoni* infection. The increase of its level with age justifies the reason why older individuals are more resistant to reinfection by *S. mansoni* (Naus *et al*, 1999; Naus *et al*, 2003). The results of the present study are in conformity with those of Hagan *et al* (1991), Dunne *et al* (1992), van Dam *et al* (1996), Webster *et al* (1997), Naus *et al* (1999) and Naus *et al* (2003).

There was a significant negative correlation between anti-SWAP IgG_2 levels and age ($P < 0.001$), which is in contrast to the findings of Badri (2011). However, the findings of the present study are not surprising because IgG_2 has previously been described as a blocking antibody that makes the host more susceptible to reinfection by *S. mansoni*. The levels of the IgG_2 are therefore highest in younger school children but decrease as the children mature in age hence allows for the expression of protective antibodies like IgG_1 and IgG_3.

The levels of anti-SWAP IgG_3 significantly increased with age ($P<0.001$) which is in agreement with the findings of other investigators (Hagan *et al*, 1991; Dunne *et al*,1992; Naus *et al* 1999; van Dan *et al* 1996; Naus *et al* 2003) . A possible explanation for the positive correlations here could be the protective nature of IgG_3 against *S. mansoni* infection. The increase of levels of this antibody with age explains the reason why older individuals in human populations are more resistant to re-infection by *S. mansoni* parasite compared to younger hosts. The results here, however, differ from that of Naus *et al* (2003) that did not find any significant relationship between the two variables.

The present study found a significant decline in levels of anti-SWAP and anti-SEA IgM with age ($P<0.001$). Highest levels of the antibody were found in younger school children aged 5-8 years. It then declined as the pupils advanced in age, thus indicating a negative correlation between IgM and age. This could probably be connected the role of IgM in preventing protective immunity in children who have previously been described to be more susceptible to re-infection by *S.mansoni*. The levels of this antibody tend to decline with age and allow for the expression protective

immunoglobulins like IgG_1 and IgG_3 that consequently confer immunity against the parasite as the host matures in age. The results here are consistent with that of Naus *et al* (1999) and Singh *et al* (2011) but differ from that of Naus *et al* (2003) which found the highest amount of anti-SEA IgM to be in the age category of children in the age bracket of 10-15 years. The differences in the results could most probably be due to the nature of populations dealt with in each case. For example, Naus *et al* (1999) studied naïve immigrant populations while Naus *et al* (2003) dealt with a stable fishing community.

The present study reveals that different immunoglobulin isotype responses are correlated in different ways with intensity of infection of *S. mansoni* parasite as well as age of the human host. One of the explanations for this is that the different isotypes are induced by antigens that have different physiochemical properties. For example, IgG_2 mainly recognizes polysaccharide antigens, while IgG_1 and IgG_3 can respond to both polysaccharide and peptide antigens. Therefore, the IgG subclasses vary in their ability to mediate effector immune responses against the infection.

Other factors may also lead to the variations in the immune responses. For example, even though both the IgG_1 and IgG_3 have similar effector functions of conferring immunity to the host, IgG_3 has more flexible hinge regions, is a more effective isotype in terms of complement-fixing ability, and has superior activity in antibody-dependent cytotoxicity than IgG_1. On the other hand, IgG_1 has longer half-life and is more effective in inducing mediator release of monocytes than IgG_3. Again, various studies have been done on different populations having different genetic compositions hence varied gene expressions of the immune responses against the *S. mansoni* parasite.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The current study found that among the immunoglobulins investigated, IgM was the most abundant in the school children infected with *S. mansoni* in Asembo area. The intensity of infection by *S. mansoni* parasite reached peak levels at around the age of 9-16 years in the infected school children in Asembo area. There were significant positive correlations between anti-SWAP IgG_1, IgG_2 anti-SEA IgG_1 and IgG_3 with intensity of infection by *S. mansoni* ($P<0.05$). There were insignificant negative correlations between anti-SWAP IgM and intensity of infection ($P=0.039$). There existed significant positive correlations between anti-SWAP and anti –SEA IgG_1 and IgG_3 with age of infected school children ($P<0.001$). Significant negative correlations existed between anti-SWAP IgG_2, anti-SWAP IgM and anti-SEA IgM with age ($P < 0.001$).

6.2 Conclusion

The current study concludes that

(i) Since IgM is most abundant in younger school children, it is a blocking antibody that hinders development of protective immunity against *S. mansoni* infection in Asembo area. The higher levels of this antibody therefore leads to susceptibility of the children to re-infection by the parasite compared to older ones in the area.

(ii) Intensity of infection has a significant influence on the production of immunoglobulins in response to *S. mansoni* infection in school children in Asembo area.

(iii) As school children grow older, there is an increase in levels of protective antibodies like IgG_1 and IgG_3 while on the other hand there is a decrease in levels of blocking antibodies like IgG_2 and IgM.

6.3 Recommendations

6.3.1 From the Present Study

a) Investigation into how duration of exposure to *S. mansoni* parasite influences levels of IgG_1, IgG_2, IgG_3 and IgM in infected school children.

b) A study of the influence of co-infection with other parasites like *Ascaris lumbricoides, Ancylostoma duodenale* and *Plasmodium sp* on the levels of IgG_1, IgG_2, IgG_3 and IgM in school children infected with *S. mansoni*.

c) Use of more sensitive anti-SEA IgG_2 optimization protocols for more reliable data to be obtained for IgG_2 against SEA.

d) Research study on how total IgG levels correlate with intensity of infection and age among school children infected with *S. mansoni*.

6.3.2 For Future Studies

a) Investigations into correlations between IgG_1, IgG_2, IgG_3 and IgM levels *versus* sex of infected school children.

b) Research study on the impact of nutritional status of school children infected with *S. mansoni* on production of IgG_1, IgG_2, IgG_3 and IgM antibodies.

c) Investigating the relationship between intensity of infection by *S. mansoni* and levels of sex hormones like testosterone and oestrogen in infected school children.

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APPENDICES

APPENDIX I: PARENT/GUARDIAN CONSENT FORM

STUDY TITLE: Correlation Between Immunoglobulin Isotypes(IgG_1, IgG_2, IgG_3, IgM), Infection Intensity and Age among Pupils Infected with *Schistosoma mansoni* in Rarieda District, Western Kenya

INSTITUTION: Kenya Medical Research Institute, Centre for Global Health Research. (KEMRI-CGHR).

PRINCIPAL INVESTIGATOR: Ajwang' J. Ombidi.

CO-INVESTIGATORS:

KEMRI/CDC	MASENO UNIVERSITY
Diana M.S. Karanja	Oyieng' P. Ang'ienda
Eric Ndombi	
Elizabeth Ochola	

Explanation of the purposes of the research

Your child is being asked to take part in a medical research study being performed by Maseno University and the Kenya Medical Research Institute (KEMRI). It is very important that you understand the following general principles that apply to all participants in our studies:

1) You and your child's participation is entirely voluntary;
2) You may withdraw from participation in this study or any part of this study at any time with no penalty, harm, or loss of access to treatment;
3) After you read about the study please ask any questions that will allow you to understand the study more clearly.

What is Bilharzia

Bilharzia, also known as schistosomiasis is a disease caused by worm parasites transmitted by snails. The snails live in different types of water including ponds,

rivers and lakes. People whose activities cause them to come into contact with water where infected snails live and where there may be transmission going on are likely to suffer from the disease. Bilharzia worm parasites enter your body through the skin when you are in the water of a lake, river, stream, or pond. Bilharzia can sometimes be serious or even cause death if not diagnosed and treated properly. In our earlier study in the Asembo Bay area, we found that children in schools closer to Lake Victoria were more likely to have bilharzia than children in schools farther away from the Lake. The most common way to find out if someone has bilharzia is to check for the eggs of the parasite in the stool and urine.

Why do we want to conduct this study:

Past studies show that antibody isotype responses against *Schistosoma mansoni* antigens vary with intensity of infection and age of host, and are associated with susceptibility or resistance to infection. There is scanty information about the relationship between the levels of various immunoglobulis, intensity of infection and age of school pupils, who bear the greatest burden of the infection. This study aims at investigating the relationship between immunoglobulin isotypes (IgG1, IgG2, IgG3, IgM), infection intensity and age among school-going pupils infected with *S. mansoni* in Rarieda District, Western Kenya. Investigation into these relationships is important to our understanding of immuno-biology of the infection and may give an insight into preparation of a vaccine against the infection.

What is important for you to know.

To do this study, we will need to study some of your child's faeces and blood. A small amount of the faeces and 2ml (about one teaspoon) of blood will be collected from the child and taken to the laboratory for preparation and other studies. Your child will be assigned a study number, and the links between the name and number, and all data collected through use of stools, urine, and blood, will be kept confidential. None of the information that we collect will be told to other people in your village.

This study is expected to last about one year. You can decide if you want your child to take part in this study. Taking part in this study will not cost you or your family anything. Your child may also leave the study at any time. You can leave for any reason without any problems.

Who Can Participate In The Study:

We can include your child in the study only if you give permission for him/her to participate, and if your child agrees to participate. We shall include children over the age of 5 and up to age17 years.

Risk involved

The risks or hazards to your child if she/he takes part in this study are minimal. There is the minor discomfort while drawing blood. To minimize any risk, hazard or discomfort during our study, the blood will be obtained from your child's finger or arm in a sterile way by well trained staff.

Questions about research

If you have any questions about this study, you may contact Mr. Ajwang' Joseph Ombidi (Tel No.0733474890) and Dr. Diana Karanja(Tel No.0722154838) at the Kenya Medical Research Institute, Kisumu during the study and in the future. If you have concerns about human rights, ethics and welfare issues you may contact the National Ethical Review Board, Kenya Medical Research Institute; Tel; 020-722541.

PARENTAL/GURDIAN PERMISSION

I, Mr./Mrs./Miss _____, being a person aged 18 years and over and being the lawful/legal guardian of: Mr/Miss (Child's name) _____ voluntarily agree that my child may be included in the study which I have read or has been read to me. I understand that I may withdraw him/her from the research at any time, for any reason, without any penalty or harm. All the above conditions have been explained to me in the _____ language in which I am fluent.

_____Age of child_____

School name _____Village_____

_____ Parent's/Guardian's signature

_____ Date

_____ Place

_____ Person Obtaining Consent

OFFICIAL STAMP

CONSENT OF PUPIL

We are requesting to take a small amount of faeces from you and obtain 2ml(about one teaspoonful) of blood from your hand. You are not forced to provide these against your wish, but there is no harm if you accept to participate. This study may hopefully help in finding a vaccine against bilhazia in future.

Do you accept to provide a small amount of stool and 2ml(about one teaspoonful) of blood from your body?

Yes……..No……

Name of pupil……

Signature/Thumbprint of pupil……Date…….

Signature of person obtaining consent……Date…….……..……

Signature of witness……Date……..……

APPENDIX II: APPROVAL BY KEMRI-ERC

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KENYA MEDICAL RESEARCH INSTITUTE
CENTRE FOR GLOBAL HEALTH RESEARCH
14 NOV 2014
P.O. Box 1578, KISUMU

KEMRI/RES/7/3/1 **November 4, 2014**

TO: **DR. AJWANG' JOSEPH OMBIDI,**
PRINCIPAL INVESTIGATOR

THROUGH: **STEPHEN MUNGA**
DIRECTOR, CGHR,
KISUMU

17/11/2014
FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

RE: SSC PROTOCOL NO. 2879 *(RESUBMISSION)*: CORRELATION BETWEEN IMMUNOGLOBULIN ISOTYPES (IgG1, IgG2, 1gG3 and IgM) INFECTION INTENSITY AND AGE AMONG SCHOOL PUPILS INFECTED WITH *SCHISTOSOMA MANSONI* IN RARIEDA DISTRICT, WESTERN KENYA.

Reference is made to your letter dated October 22, 2014. The ERC Secretariat acknowledges receipt of the revised protocol on October 27, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 231st meeting of the KEMRI ERC have been adequately addressed.

The study is granted approval for implementation effective this **November 4, 2014**. Please note that authorization to conduct this study will automatically expire on **November 3, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the SERU Secretariat by **September 22, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the SERU. You are also required to submit any proposed changes to this protocol to the SERU prior to initiation and advise them when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX III: ELISA PROTOCOL

A. SEA COATING

(i) Label 4 plates (i.e each for IgG_1, IgG_2, IgG_3 and IgM respectively) for SEA

(ii) Add 32.8 ul of SEA into 48 ml of 0.1M coating buffer carbonate (pH 9.6)

(iii) Add 100 ul per well of the coating mixture (SEA + buffer carbonate) into each of the wells of the plates.

(iv) Cover the plates with parafilm and incubate for 2 hours. MEANWHILE PREPARE BLOCKING BUFFER AND CARRY OUT DILUTION OF SAMPLES

(v) Wash the plates 4 times with wash buffer, then blot.

B. BLOCKING BUFFER PREPARATION

(i) Prepare blocking buffer by mixlng 60ml of 1 x PBS, 180 ul of 0.3% Tween 20 and 3g of powdered milk.

(ii) Shake thoroughly to obtain a homogeneous mixture.

C. DILUTION OF SAMPLES

(i.e 1:100 dilution)

(i) Add 10ul of each sample into 990ul of blocking buffer to make a total of 1000ul of diluted sample mixture.

(ii) Add 100ul of diluted sample mixture in duplicate to the respective wells of each of the plates (Refer the template set up).

(iii) Cover with parafilm and incubate for 30 minutes. MEANWHILE, PREPARE ANTI- ISOTYPES OF VARYING DILUTIONS.

(iv) Wash the plates 4 times with wash buffer, then blot

D. PREPARATION OF ANTI-ISOTYPES (IgG1, IgG2, IgG3 and IgM)

(i) Prepare dilution solution by mixing 50ml of 1 x PBS with 150ul of 0.3% Tween 20, and vertex thoroughly.

(ii) Label 4 small conical tubes as IgG1, IgG2, IgG3 and IgM

(iii) Pour 12.5ml of the dilution solution in (i) above into each of the conical tubes

(iv) Measure the anti- isotypes as shown below, and add into the respective tubes

*IgG1 (1:4000) = 3.125ul

*1gG2 (1:4000) = 3.125ul

*IgG3 (1:8000) = 1.56ul

*IgM (1:16000) = 0.78ul

(v) Vortex the mixture thoroughly in each case

(vi) Add 100 per well of the anti- isotype dilution mixture in (v) above into each of the wells of the respective plates.

E. PREPARATION OF STANDARDS

(i) Arrange 8 flow tubes labeled A – H in a rack

(ii) Add 600ul of blocking buffer into each of tube A, then 30ul of the blocking buffer into each of the tubes B-H.

(iii) Add of SWAP + ve plasma into tube A, and vortex thoroughly.

(iv) Transfer 300ul of mixture from tube A to B, then B to C ….. downwards upto G. leave H intact.

(v) Add 100ul of the standards from the tubes accordingly into the wells in duplicate from well A, (1 and 2) to H (1 and 2). Add 1:100 normal human serum (NHS) to well G (11 and 12) and blank (PBS + Tween + milk) to wells H (11 and 12)

(vi) Cover the plates with parafilm then incubate for 30 minutes.

NB: Meanwhile take TMB A and TMB B solutions out of $4^{o}C$ and allow them to reach room temperature. Also set up the soft max reader.

(v) Wash the plates 4 times with wash buffer, then blot.

F. ADDITION OF SUBSTRATE AND READING

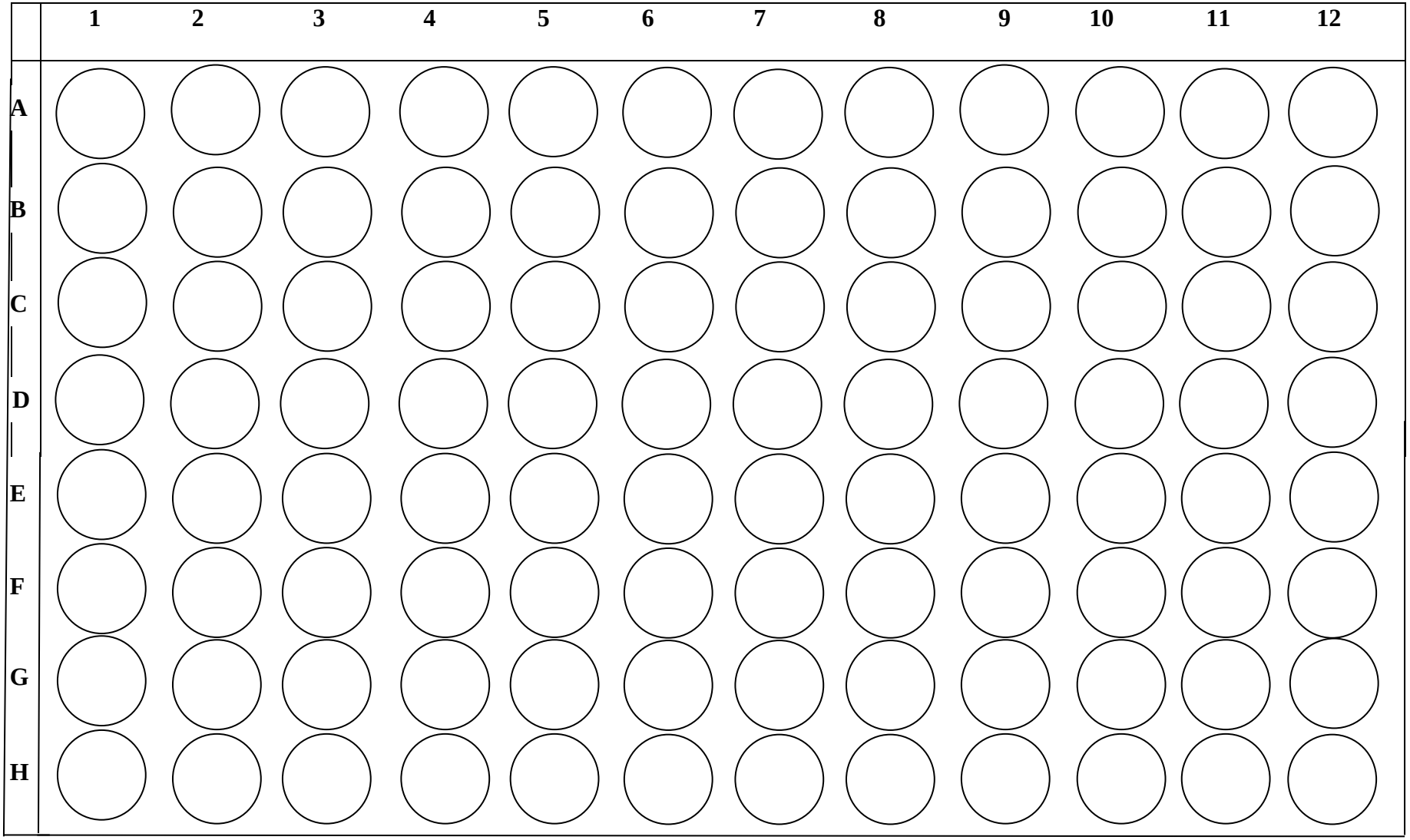
(i) Mix 20ml of TMB A and 20ml of TMB B solutions in a TMB boat.

(ii) Add 100ul per well of the TMB mixture into the wells of plates

(iii) Allow to develop for 5 minutes

(iv) Read on Emax ROM reader

APPENDIX IV: LAYOUT OF 96 – ELISA WELL PLATE



APPENDIX V: KATO-KATZ PROTOCOL

Kato-Katz technique - cellophane faecal thick smear

The Kit contains (Fig. 1)

1. a roll of nylon screen 80 mesh (20 m)
2. 400 plastic templates with a hole of 6 mm on a 1.5 mm thick template, delivering 41.7 mg of faeces
3. 400 plastic spatula
4. a roll of Hydrophilic cellophane, 34 um thick - 20 m

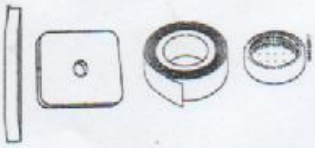


Fig. 1

To perform the technique correctly the following materials have to be procured in order

Microscope slides (75 x 25 mm).
Toilet papper or absorbent tissue
Newspaper or scrap paper
Solution of 100 ml of glycerol and 100 ml of distilled water
Flat bottom jar

Fig. 2

Preparation

- Cut an appropriate number of pieces of nylon screen 30-35 mm
- Cut an appropriate number of pieces of hydrophilic cellophane of 30-35 mm and place it in the jar
- Pour the glycerol onto the cellophane strips placed in the jar and leave for at least 24 hours

To increase the visibility of the eggs, 1 ml of 3% aqueous malachite green or 3% methylene blue can be added to the glycerol/ water solution

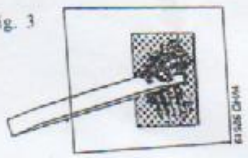


Fig. 3

Procedure

1. Place a small of mound faecal material on newspaper or scrap paper and press a piece of nylon screen on top so that some of the faeces are sieved through the screen and accumulate on top (Fig. 2).
2. Scrape the flat-sided spatula across the upper surface of the screen to collect the sieved faeces (Fig. 3.)
3. Place the template on the centre of the microscope slide and add faeces from the spatula so that the hole is completely filled (Fig. 4)
   Pass over the template using the side of the spatula to remove excess faeces from the edge of the hole (the spatula and templete may be discraded or reused if carefully washed).
4. Remove the templete carefully so that the cylinder of faeces is left on the slide.

Fig. 4

5. Cover the faecal material with a pre-soaked cellophane strip (Fig. 5). The strip must be very wet if the faeces are dry and less so if the faeces are soft (if excess glycerol solution is present on the upper surface of the cellophane, wipe it with toilet paper). In dry climates, excess glycerol will retard but not prevent drying.

Fig. 5

6. Invert the microscpe slide and press the faecal sample firmly against the hydrophilic cellophane strip on another microscope slide or on a smooth hard surface, such as a piece of tile or a flat stone. The faecal material will be spread evenly between the microscope slide and the cellophane strip (Fig. 6). It should be possible to read a newspaper print through the smear after clarification (Fig. 7)

Fig. 6

Fig. 7

7. Carefully remove the slide by gently sliding it sideways to avoid separating the cellophane strip or lifting if off. Place the slide on the bench with the cellophane upwards. Water evaporates while the glycerol clears the faeces.
8. For all except hookworn eggs, keep slide for one or more hours at room temperature to clear the faecal material, prior to examination under the microscope. To speed up clearing, the slide can be placed in a 40°C incubator or kept in direct sunlight for several minutes.
9. *Ascaris lumbricoides* and *Trichuris trichiura* eggs will remain visible and recognizable for many months in these preparation. Hookworm eggs clear rapidly and will no longer be visible after 30-60 minutes. Schistosome eggs may be recoginzable for up to several months but it is preferable in a schistosomiasis endemic area to examine the slide preparations within 24 hours.
10. The smear should be examined in a systematic manner and the number of eggs of each species reported. Later, multiply this number by 24 to obtain the number of eggs per gram of faeces (epg.)
11. The epg give an estimation of the worm burden and allow to identify individuals likely to suffer from severe consequences of the infection (i.e. those with heavy intensity infections)

The following thresholds for the classification of individuals are proposed by WHO. Some flexibility in setting thresholds may be necessary depending on local epidemiological characteristics.

	Light intensity infections	Moderate intensity infections	Heavy intensity infections
A. lumbricoides	1 - 4,999 epg	5,000 - 49,999 egp	≥ 50,000 epg
T. trichiura	1 - 999 epg	1,000 - 9,999 epg	≥ 10,000 epg
Hookworms *	1 - 1,999 epg	2,000 - 3,999 epg	≥ 4,000 epg
S. mansoni *S. japonichum*	1 - 99 epg	100 - 399 epg	≥ 400 epg

- *Control of schistosomiasis* (1993) Second report of WHO Expert Committee
- *Control of intestinal parasitic infection* (1987) Report of WHO Expert Committee
- *Guidelines for the evaluation of soil-transmitted helminthiasis and schistosomiasis at community level* (1998)

APPENDIX VI: REGRESSION MODELS OF IMMUNOGLOBULIN CONCENTRATIONS.

(a) Results of multiple linear regression model of anti-SWAP IgG1 concentration

	Coefficient	**Standard error**	**t - value**	**P - value**
Intercept	4.2370469	0.1630081	25.993	<2e-16
Age	0.1098501	0.0115274	9.530	< 2e-16
Infection intensity	0.0012673	0.0002433	5.208	3.42e-07
Residual se =0.7994, $R^2 = 0.282$, $R^2_{adj} = 0.2775$, F-stat: 62.84 on 2 and 320DF, P < 2.2e-16				

(b) Results of multiple linear regression model of anti-SEA IgG_1 concentration

	Coefficient	**Standard error**	**t - value**	**P – value**
Intercept	4.5905226	0.1818118	25.249	<2e-16
Age	0.0618566	0.0129686	4.770	2.81e-06
Infection intensity	0.0001920	0.0002585	0.743	0.458
Residual se =0.9091, $R^2 = 0.0693$, $R^2_{adj} = 0.0635$, F-stat: 11.92on 2 and 320DF, P <1.017e-5				

(c) Results of multiple linear regression model of anti-SWAP IgG2 concentration

	Coefficient	**Standard error**	**t - value**	**P – value**
Intercept	69.338849	0.823433	84.207	< 2e-16
Age	-0.561103	0.057519	-9.755	< 2e-16
Infection intensity	0.005561	0.001481	3.754	0.000207
Residual se =4.309, $R^2 = 0.2477$, $R^2_{adj} = 0.2430$, F-stat: 52.52 on 2 and 319DF, P< 2.2e-16				

(d) Results of multiple linear regression model of anti-SWAP IgG3 concentration

	Coefficient	**Standard error**	**t – value**	**P – value**
Intercept	5.0829627	0.2114957	24.033	< 2e-16
Age	0.0650661	0.0148133	4.392	1.53e-05
Infection intensity	-0.0001923	0.0002883	-0.667	0.505
Residual se =0.9709, $R^2 = 0.0571$, $R^2_{adj} = 0.05119$, F-stat:9.66on 2 and 319DF, P =8.453e-05				

(e) Results of multiple linear regression model of anti-SEA IgG3 concentration

	Coefficient	**Standard error**	**t – value**	**P – value**
Intercept	5.7095436	0.2047312	27.888	< 2e-16
Age	0.0153722	0.0140749	1.092	0.276
Infection intensity	0.0002799	0.0002659	1.053	0.293
Residual se =0.9636, $R^2 = 0.0069$, $R^2_{adj} = 0.0007$, F-stat:1.119on 2 and 319 DF, P =0.328				

(f) Results of multiple linear regression model of anti-SWAP IgM concentration

	Coefficient	**Standard error**	**t – value**	**P – value**
Intercept	8.7844883	0.1677566	52.364	< 2e-16
Age	-0.0982915	0.0118171	-8.318	2.64e-15
Infection intensity	-0.0003082	0.0002170	-1.420	0.156
Residual se =0.7740, $R^2 = 0.1820$, $R^2_{adj} = 0.1769$, F-stat:35.48 on 2 and 319 DF, P =1.217e-14				

(g) Results of multiple linear regression model of anti-SEA IgM concentration

	Coefficient	**Standard error**	**t – value**	**P – value**
Intercept	7.5655517	0.2138124	35.384	< 2e-16
Age	-0.0984650	0.0149083	-6.605	1.67e-10
Infection intensity	0.0003758	0.0002566	1.464	0.144
Residual se =0.9511, $R^2 = 0.1276$, $R^2_{adj} = 0.1222$, F-stat:23.33on 2 and 319 DF, P =3.487e-10				

APPENDIX VII: MEAN IMMUNOGLOBULIN CONCENTRATIONS AND INFECTION INTENSITIES BY AGE GROUPS.

(a) Mean anti-SWAP IgG1 concentration by age group

		Age group (in years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgG1 concentration (AU)		263.98945	509.40414	559.50194	964.04308
Standard error		40.89527	80.29038	57.92566	107.00343
95% CI	Lower	181.2709	349.76536	444.84165	750.73564
	Upper	346.70794	669.024292	674.16223	1177.35052

(b) Mean anti-SEA IgG1 concentration by age group

		Age group (in years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgG1 concentration (AU)		209.89655	328.54214	339.73225	467.08188
Standard error		43.55267	41.38502	28.61081	39.15292
95% CI	Lower	121.94015	236.31099	283.05975	389.06799
	Upper	297.85295	410.77330	396.40475	545.09577

(c) Mean anti-SWAP IgG2 concentration by age group

		Age group (years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgG1 concentration (AU)		66.20959	64.76721	62.42159	59.74541
Standard error		0.62763	0.47498	0.42541	0.49409
95% CI	Lower	64.94895	63.82120	61.57853	58.76250
	Upper	67.47022	65.71322	63.26464	60.72831

(d) Mean anti SWAP IgG3 concentration by age group

		Age group (years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgG3 concentration (AU)		364.35213	617.40975	632.82202	770.76484
Standard error		105.39161	93.69637	71.71370	97.06888
95% CI	Lower	149.11375	430.87460	490.82173	577.94944
	Upper	579.59051	803.94489	774.82230	963.58024

(e) Mean anti-SEA IgG3 concentration by age group

		Age group (years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgG3 concentration (AU)		630.26038	861.68155	480.53654	796.10735
Standard error		128.56871	243.91811	34.25724	126.04022
95% CI	Lower	368.68537	376.62364	412.70958	545.37320
	Upper	891.83540	1346.73947	548.36350	1046.84150

(f) Mean anti-SWAP IgM concentration by age group

		Age group (years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgM concentration (AU)		3156.59044	3124.45730	2214.40920	1314.36305
Standard error		440.5861 0	235.93320	197.38070	143.30140
95% CI	Lower	2260.21122	2656.07059	1823.43604	1028.89210
	Upper	4052.96966	3592.84402	2605.38236	1599.83400

(g) Mean anti-SEA IgM concentration by age group

		Age group (years)			
		5 – 8	9 – 12	13 – 16	17 – 20
Sample size		86	89	97	78
Mean IgM concentration (AU)		1962.29343	1205.91849	741.29169	589.40701
Standard error		361.71218	211.58954	76.29446	68.18158
95% CI	Lower	1220.12134	785.86013	590.27149	453.52132
	Upper	2704.46551	1625.97684	892.31189	72529271

APPENDIX VIII: ANOVA RESULTS SHOWING EFFECT OF AGE GROUPS ON IMMUNOGLOBULIN CONCENTRATIONS.

(a) ANOVA results showing effect of age group on anti-SWAP IgG1 concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	15099527	5033176	9.967	2.68e-06
Residuals	319	161089309	504982		

(b) ANOVA results showing effect of age group on anti-SEA IgG_1 concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	1906041	635347	5.566	0.000984
Residuals	319	36412949	114147		

(c) ANOVA results showing effect of age group on anti-SWAP IgG_2 concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	1678	559.5	28.71	<2e-16
Residuals	318	6196	19.5		

(d) ANOVA results showing effect of age group on anti-SWAP IgG_3 concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	3972893	1324298	1.943	0.123
Residuals	318	216750153	681604		

(e) ANOVA results showing effect of age group on anti-SEA IgG_3 concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	8821071	2940357	1.65	0.178
Residuals	319	568509446	1782161		

(f) ANOVA results showing effect of age group on anti-SWAP IgM concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	1.632e+08	54392721	12.7	7.36e-08
Residuals	318	1.362e+09	4283725		

(g) ANOVA results showing effect of age group on anti-SEA IgM concentration

	DF	**SS**	**MS**	**F-value**	**P – value**
Age group	3	50003092	16667697	8.534	1.82e-05
Residuals	318	621107365	1953168		

APPENDIX IX: TUKEY'S HSD TESTS

(a) Tukey's HSD test result showing mean anti-SWAP IgG1 concentration differences age group pairs

Age group	Mean difference	95% CI on the mean difference		P – value
		Lower	Upper	
2-1	245.4147	-105.82632	596.6557	0.2730850
3-1	295.5125	-38.20599	629.2310	0.1032161
4-1	700.0536	339.02058	1061.0867	0.0000054
3-2	50.0978	-207.44460	307.6402	0.9584694
4-2	454.6389	162.56886	746.7090	0.0004207
4-3	404.5411	133.79641	675.2859	0.0007916

(b) Tukey's HSD test result showing mean anti-SEA IgG1 concentration differences age group pairs

Age group	Mean difference	95% CI on the mean difference		P – value
		Lower	Upper	
2-1	118.64560	-44.409660	281.7009	0.2390437
3-1	129.83570	-27.297746	286.9692	0.1446171
4-1	257.18533	89.021966	425.3487	0.0005558
3-2	11.19011	-111.377894	133.7581	0.9953787
4-2	138.53974	2.117920	274.9616	0.0449927
4-3	127.34963	-1.936161	256.6354	0.0552681

(c) Tukey's HSD test result showing mean anti-SWAP IgG2 concentration differences age group pairs

Age group	Mean difference	95% CI on the mean difference		P – value
		Lower	Upper	
2-1	-1.442380	-3.500557	0.6157960	0.2705134
3-1	-3.788003	-5.716500	-1.8595055	0.0000040
4-1	-6.464179	-8.492498	-4.4358593	0.0000000
3-2	-2.345622	-4.036375	-0.6548693	0.0022132
4-2	-5.021798	-6.825578	-3.2180178	0.0000000
4-3	-2.676176	-4.330454	-1.0218983	0.0002218

(d) Tukey's HSD test result showing mean anti-SWAP IgM concentration differences age group pairs

Age group	Mean difference	95% CI on the mean difference		P – value
		Lower	Upper	
2-1	-32.13314	-1098.915	1034.6490	0.9998309
3-1	-942.18124	-1984.634	100.2714	0.0925143
4-1	-1842.22739	-2945.110	-739.3449	0.0001256
3-2	-910.04810	-1647.583	-172.5134	0.0085586
4-2	-1810.09425	-2630.825	-989.3630	0.0000002
4-3	-900.04615	-1688.895	-111.1970	0.0180395

(e) Tukey's HSD test result showing mean anti-SEA IgM concentration differences age group pairs

Age group	Mean difference	95% CI on the mean difference		P – value
		Lower	Upper	
2-1	-756.3749	-1531.6117	18.86182	0.0587890
3-1	-1221.0017	-1976.2158	-465.78765	0.0002241
4-1	-1372.8864	-2173.7220	-572.05084	0.0000772
3-2	-464.6268	-955.3121	26.05848	0.0708107
4-2	-616.5115	-1174.8674	-58.15555	0.0238531
4-3	-151.8847	-682.0899	378.32059	0.8809237