Investigating the Inflammatory Markers Involved in *Schistosoma haematobium* Protective Immunity and Susceptibility to Infection

BY

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FACULTY OF SCIENCE

UNIVERSITY OF ZIMBABWE

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Declaration

I, the undersigned hereby declare that this thesis is my own work which was conducted at the University of Zimbabwe between the period of February 2016 - November 2016. I declare that this work has not been submitted before and that I have fully acknowledged through referencing the thoughts and ideas of others.

Signed this 2nd day of December

Maritha Kasambala: 156307B
Abstract

Schistosomiasis is a neglected tropical disease that affects more than 200 million people globally. In Zimbabwe, areas have been mapped that contain high *S. haematobium* morbidity. The effects are mostly felt in rural areas where children have higher infection rates than adults. The immune mechanisms that lead to resistance of the disease are very complex and not yet fully understood. The current control method recommended to reduce morbidity is by mass drug treatment with praziquantel. This thesis reports the work aimed at monitoring the response of protective inflammatory markers in 124 children from an *S. haematobium* endemic area, aged between 7-13 years old, under praziquantel mass drug treatment. Parasitological data was obtained using the Kato-Katz and urine filtration to diagnose infected and uninfected children by counting and identifying the presence or absence of eggs in their urine and stool samples. The enzyme-linked immunosorbent assay was used to measure the inflammatory markers IgE, IgG4, IgM and IFN-γ in the participant’s serum that have been associated with resistance and susceptibility to infection. The antibodies were measured using the Sh13 antigen which is a potential vaccine candidate. The Amplification Refractory Mutation System Polymerase Chain Reaction was used to genotype the children who had mutations at the IFN-γ +874A/T promoter site to investigate the influence of genotype in production of IFN-γ and susceptibility to infection.

At baseline 42% of the children were found to be infected and 58% were uninfected. Post treatment results showed that all the study participants were now uninfected. All the inflammatory markers in this study had no statistically significant difference in their levels between baseline and follow-up. Infected children had higher levels of IgE, IgG4 and IgM compared to uninfected children. The p values of IgE, IgG4, and IgM of infected vs. uninfected analysis were 0.242, 0.0443 and less than 0.0001 respectively. The blocking antibodies associated with susceptibility to infection: IgM and IgG4 levels were higher than the protective antibody IgE levels. IFN-γ levels on the other hand were lower in infected children and higher in uninfected children with a p value of 0.0214. The genotyping results showed that the children had 3 genotypes: AA (21 %), AT (73 %) and TT (6 %). After analysis of variance at p<0.05, it was found that the genotypes produced different levels of IFN-γ and had a p value of 0.0090. The children containing the homozygous AA were higher producers of IFN-γ compared to those containing the AT, and TT genotype, the children with the AT genotype produced the least IFN-γ. The AA and TT genotypes had low infection intensities while the AT genotype children had low, medium and high infection intensities.

The results indicate that the children’s immune response had already elicited protective markers before treatment against the Sh13 antigen found on the tegument of the worms and that praziquantel did not boost the immune system against the Sh13 antigen. Infected
children had higher antibody concentrations in response to the intensity of worm’s Sh13 antigen they were carrying whereas the uninfected children had lower levels of the antibodies probably due to previous exposure and the lack of worms in their bodies. IFN-γ levels were lower in infected children because they were in the chronic phase of infection which is associated with Th-2 cytokines that down regulate IFN-γ production. The genotypes influenced different IFN-γ production levels which were found to correlate with infection prevalence of children. The conclusion obtained was that praziquantel was effective in treating *S. haematobium* and that the children are susceptible to re infection as indicated by their antibody ratio profile. The children with the AT genotype are the most prone to schistosome infections as it produces lowest levels of IFN-γ associated with susceptibility to infection.
Acknowledgements

I would like to express my deepest gratitude to my supervisor Professor T. Mduluza for his constant encouragement, guidance and motivation throughout the time I conducted this research and especially for introducing me into the field of immunology. The skills that I obtained in his laboratory and during the field trips are priceless. I want to thank Dr Fiona Robertson and Professor Sithole-Niang for their support, advice and grooming my molecular techniques which I applied in this research. I also want to give my sincere acknowledgement to Professor Mukanganyama, Professor Chetsanga and Dr Chidzvondo who were part of the biotechnology lecturers and dedicated their time to teach me.

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<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>ARMS</td>
<td>Amplification Refractory Mutation System</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAA</td>
<td>Circulating anodic antigen</td>
</tr>
<tr>
<td>CAP</td>
<td>Cercarial antigen preparation</td>
</tr>
<tr>
<td>CCA</td>
<td>Circulating cathodic antigen</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>MDA</td>
<td>Mass drug administration</td>
</tr>
<tr>
<td>MRCZ</td>
<td>Medical Research Council of Zimbabwe</td>
</tr>
<tr>
<td>NIHRZ</td>
<td>National Institute of Health Research Zimbabwe</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>Ortho-phenyldiamine dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PZQ</td>
<td>Praziquantel</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEA</td>
<td>Soluble egg antigen</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-Nucleotide Polymorphism</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SWAP</td>
<td>Soluble worm antigen preparation</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>Th1/2</td>
<td>T helper Cell 1/2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T (helper) regulatory cell</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>UZ</td>
<td>University of Zimbabwe</td>
</tr>
<tr>
<td>WHA</td>
<td>World Health Assembly</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Global Impact of Schistosomiasis

Schistosomiasis which is commonly known as bilhazia is an infectious disease that is caused by parasitic blood fluke worms of the genus *Schistosoma*. The three main species that highly infect people are *S. haematobium*, *S. mansoni* and *S. japonicum*. Approximately 300 million people are infected globally, 30 million suffer from morbidity and 155 000 deaths are recorded yearly (Fenwick *et al.*, 2003; Ketema and Beyene, 2003).

Transmission is through contact with contaminated water containing the infected snail intermediate host. Studies have shown that the majority of people infected by this parasitic disease reside in low income generating countries, areas that contain poor water sanitation and where HIV and malaria parasites are prevalent (Midzi *et al.*, 2008). The highest infection intensities are found in poverty and under-developed areas, where 60-80% school aged children are the ones that are greatly affected physically, mentally and emotionally by the disease while 20-40% adults are just actively infected (Werf, 2003; Bundy, 2005). Infected children suffer from the following symptoms: anaemia, slow growth rates, haematuria, impaired memory and cognitive development, and reduced physical fitness (Rujeni *et al.*, 2012). According to WHO (2014), seventy eight countries have documented schistosomiasis transmission and of these, 52 countries have been recognized as high transmission areas requiring mass drug treatment as summarised in Table 1.1. Zimbabwe is one of the countries that have been identified to have high risk
areas of infection. This project serves to contribute knowledge consolidating towards the monitoring and evaluation programme of mass drug treatment of schistosomiasis in school age children taking praziquantel.

Table 1.1: Global distribution of areas and populations requiring mass drug treatment for Schistosomiasis annually by WHO 2014

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Africa</th>
<th>America</th>
<th>South East-Asia</th>
<th>East Mediterranean</th>
<th>Western Pacific</th>
<th>Global</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endemic Countries</td>
<td>43</td>
<td>10</td>
<td>3</td>
<td>15</td>
<td>6</td>
<td>78</td>
</tr>
<tr>
<td>Countries requiring preventive chemotherapy</td>
<td>41</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>52</td>
</tr>
<tr>
<td>Total requiring preventive chemotherapy</td>
<td>236 590 060</td>
<td>1 578 367</td>
<td>27 971</td>
<td>18 642 304</td>
<td>2 216 750</td>
<td>258 875 452</td>
</tr>
<tr>
<td>Countries where bilhazia is yet to be determined</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

According to Van der Werf et al., (2003), 90% of the infected people live in Africa and are mostly infected by *S. mansoni* which is also found in the Caribbean, South America and Eastern-Mediterranean. *S. haematobium* is found in the Middle East and endemic countries in Africa including Zimbabwe where it is the most prevalent schistosomiasis causative species (Olveda *et al*., 2013). DRC and Cameroon have reported the presence of *S. intercalatum* (Tchuem *et al*., 2003). *S. japonicum* is more prevalent in Indonesia, China and the Philippines (Zhou *et al*., 2007; Gordon *et al*., 2012).

The schistosome species cause a variety of symptoms. *Schistosoma mekongi, S. mansoni* and *S. japonicum* cause bowel diseases such as ulceration, blood in stools, and
enlargement of the liver, fibrosis and pain in the abdomen. *S.mansonii* has been suggested to be the potential cause of liver cancer (Mckerrow and Salter, 2002; Wilson *et al.*, 2007). *S. haematobium* is the cause of clinical morbidity, urogenital schistosomiasis, haematuria, kidney damage and ureter fibrosis (Ross *et al.*, 2002; Olveda *et al.*, 2013).

### 1.2 The Distribution of Schistosomiasis in Zimbabwe

According to WHO (2011) schistosomiasis is categorized as heavy infection if the prevalence in a country is found to be above 50%. In Zimbabwe, surveys have been conducted to map out the prevalence of schistosomiasis until recently where the endemic areas have been identified and the appropriate treatment recommended for each area (Taylor, 1985; Chandiwa *et al.*, 1988; Ndlovu *et al.*, 1992; Midzi *et al.*, 2014). A study conducted on Zimbabwean school children aged between 10-15 years (high risk group) showed that Zimbabwe has a schistosomiasis prevalence of 25.2% and the causative schistosomiasis agents are; *S. mansoni* and *S. haematobium*. The study indicated that schistosomiasis is more dominant in rural areas (78.9%) that are in the eastern, northeast and north region of Zimbabwe (Midzi *et al.*, 2014). *Schistosoma haematobium* was found to have a higher prevalence (18%) than *S. mansoni* (7.2%) with the male children being more infected than female children.
Figure 1.1: Prevalence of schistosome species in children living in Zimbabwe, the size of the red dots indicate the intensity of the *schistosome* specie in that area; a) Endemic areas containing *S. haematobium* infected children. North, North-East and South east region are highly endemic. Western region cities such as Gokwe had low infection intensityb) Endemic areas containing *S. mansoni* infected children North,North East, Eastern and South East region are endemic areas for *S. mansoni* but with lower infection intensities in comparison to *S.haematobium* (Midzi et al., 2014).
Figure 1.2: The Life Cycle of Schistosomiasis. The course of infection by different schistosome species indicates that stages 1-6 involve the asexual reproduction stages of the life cycle in water and snails, stages 7-9 involve the maturation of schistosomes in the human host and stage 10 involves the sexual reproduction stage (Wami et al., 2014).

The life cycle of these Schistosoma trematodes are similar and involve an infected human host excreting faeces or urine containing Schistosoma eggs in a water body. When the eggs come into contact with a water body, they hatch releasing miracidia that infect snails and undergo asexual reproduction to produce many fork tailed forms known as cercariae within 4-6 weeks. Daily averages of 1500 cercariae leave the snail and enter water for a period of 18 days. The cercariae then enter a human host within a day via the skin using proteolytic enzymes (Stirewalt and Kruidenier, 1961). They develop into larvae termed schistosomulae and travel to the lungs and liver were they feed on red blood cells then mature into egg producing adult worms which have a life span of about 6-11 years.
(Fulford et al., 1995; Ross et al., 2002). Failure to find a human host within a day results in their death.

The male and female adult worms pair up in the liver and migrate to different sites depending on their species type as shown in Table 1.2 below.

**Table 1.2: The distribution of schistosome species in their definitive hosts and geographic location.**

<table>
<thead>
<tr>
<th>Schistosome Specie</th>
<th>Definite host</th>
<th>Site of infection</th>
<th>Location of egg excretion</th>
<th>Snail vector</th>
<th>Geographic location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. haematobium</em></td>
<td>Humans, primates</td>
<td>Veins of urogenital system</td>
<td>Urine</td>
<td><em>Bulinus</em></td>
<td>Africa</td>
</tr>
<tr>
<td><em>S. mansoni</em></td>
<td>Humans, rodents</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Biomphalaria</em></td>
<td>Africa, America</td>
</tr>
<tr>
<td><em>S. japonicum</em></td>
<td>Humans, ruminants, carnivores</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Oncomelania</em></td>
<td>SE Asia</td>
</tr>
<tr>
<td><em>S. intercalatum</em></td>
<td>Humans, Rodents, cattle</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Bulinus, Physopsis</em></td>
<td>Africa</td>
</tr>
<tr>
<td><em>S. mekongi</em></td>
<td>Dogs, cats, humans</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Oncomelania</em></td>
<td>South-East Asia</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td>Ruminants</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Bulinus</em></td>
<td>Africa, South-East Asia, Middle East, Europe</td>
</tr>
<tr>
<td><em>S. mattheei</em></td>
<td>Ruminants</td>
<td>Intestinal mesenteric veins</td>
<td>Faeces</td>
<td><em>Bulinus</em></td>
<td>Africa, Middle East</td>
</tr>
</tbody>
</table>

Studies on mice models have shown that adult worms are resistant to immune attack (Collins et al., 2013). These worms are not attacked by the host’s immune system as they mimic the host’ own cells and act as self cells instead of antigens that induce an immune
response (Ross et al., 2002). The eggs are excreted in urine (S. haematobium) or faeces (S. japonicum and S. mansoni) where they hatch upon reaching a water body and infect compatible host snails to continue the life cycle.

1.3 Methods Used to Diagnose and Investigate Schistosomiasis

Direct and indirect methods have been used over the years to diagnose schistosomiasis. Parasitological diagnosis falls under the direct methods and involves enumeration of schistosome eggs found in urine and stool (Katz et al., 1972; Mduluza et al., 1998). This method has been found to be cost effective and the most appropriate to use on a large scale scenario (Doenhoff et al., 2004; Stothard, 2009). Rapid diagnosis of eggs in stool is done by using the Kato-Katz thick smear technique (Ezeama et al., 2012). Eggs in urine are diagnosed by the use of the urine filtration method and microscopy (Fieldmeier and Poggensee, 1993; Olveda et al., 2013). Other methods used such as the formal aldehyde-ether and merthiolate-iodine concentration technique are more sensitive but time consuming (Olveda et al., 2013).

Indirect methods are based on detection of biochemical markers that are associated with schistosomiasis (Polman, 2000). Examples of indirect methodsthat have been found to be more sensitive and effective are serological detection of parasite specific antibodies (Sorgho et al., 2005; Kinkel et al., 2012, Wami et al., 2014). The ELISA technique is used to detect the biochemical markers and uses schistosome antigens such as SEA and SWAP to detect the antibody levels particularly IgM for diagnosis in plasma or serum (Doenhoff et al., 2004). Other antigens such as circulating antigen preparation (CAA) and circulating cathodic antigen(CCA)are gut associated antigens and can be detected in urine (Van-Lieshout et al., 2000; Midzi et al., 2009; Dawson et al., 2013). Cercarial
antigen preparation (CAP), soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) are antigens that are found to circulate in schistosome infected host and these antigens consists of a wide range of antigen molecules (Bourke et al., 2011; Wami et al., 2014). Some worm antigens are hidden from the hosts’ immune system and only exposed after the worm is killed naturally or by medication (Gryseels et al., 2006).

ELISA is done on a micro titre plate that has wells with high affinity surfaces that immobilize the ELISA reagents and antigens through hydrogen bonding. A secondary antibody used contains an enzyme that can breakdown substrates used giving off a colour change upon positive binding to the biochemical marker being tested for. The levels of the biochemical markers will be represented by the optical densities given off per well by the amounts of substrate broken down and these optical readings are read on a spectrophotometer (Crowther, 2001). According to Cavalcanti et al., (2013), DNA detection is a method that could be used in the future.

1.4 Control and Treatment Strategies for Schistosomiasis

Anti-schistosome drugs that have been produced are: niridazole, metrifonate, oxamnique, hyacanthone and antimonials. Most of these drugs only work against one type of helminth species and were found to be not effective (Olveda et al., 2013). According to Danso-Appiah et al.,(2008), metrifonate has low efficacy and is unsuitable for mass drug administration. Metrifonate is used against S. haematobium and inhibits cholinesterase enzyme resulting in the paralysis of the schistosome worms (Arnon et al., 1999).
Oxamniquine is effective against *S. mansoni* and inhibits metabolism of nucleic acids of the schistosome worms, causes worm paralysis and damage of the tegument. It is more expensive and has side effects which make it less suitable to use compared to praziquantel (Ferrari *et al.*, 2003).

In the 1970’s praziquantel (PZQ) was used in the veterinary sector and up to date has been the preferred drug treatment of choice against schistosomes. Praziquantel with the molecular formula C$_{19}$H$_{2}$N$_{2}$O$_{2}$ is a white substance that is insoluble in water and is the preferred drug globally as it works against a wide range of helminth species and is effective (Fenwick *et al.*, 2003; Koukounari *et al.*, 2010; Meurs *et al.*, 2012). Praziquantel boosts protective antibody responses and enhances acquired immunity development against parasites (Mutapi *et al.*, 2005; Mitchelle *et al.*, 2012).

Studies show that praziquantel works against cercaria a few days after infection and mature schistosome worms but is ineffective towards immature worms that are 2-4 weeks old (Keteme and Beyene, 2013). The mode of action against these worms is not yet fully understood but a few hypotheses have been made (Harder *et al.*, 2002). Praziquantel has been hypothesized to cause muscle paralysis of the schistosome by causing a rapid influx of calcium ions inside their membranes and vacuolization of the schistosome’s teguments thus, exposing antigens that the host’s immune system responds to (Kohn *et al.*, 2001; Cioli and Pica Mattoccia, 2003). Molecular studies have suggested that there are specific genes that are exclusively on the schistosomes that are targeted by praziquantel (Hu *et al.*, 2004; McManas *et al.*, 2004). Detegumented worms show little contraction indicating that the schistosomes’ tegument is the key to PZQ action (Blair *et al.*, 1992). In contrast a
study done by Greenburg et al.,(2005)suggested that PZQ interferes with the phospholipid bilayer in the membrane resulting in a change in calcium ions permeability.

Praziquantel starts working in the body within 15 minutes of being ingested. The drug is metabolized in the liver within 24 hours through cytochrome P450 pathway 3A4 and 80% of the metabolized hydroxylated compounds are excreted in urine (Valenciaa et al., 1994; Coli and Pica-Mattoccia, 2002). Increase in bio availability is enhanced by simultaneous intake of food with the drug while reduction of bio availability of PZQ in the body can be caused by simultaneous intake of the drug with other drugs such as rifampicin and anti-malarial chloroquine (Cioli and Pica-Mattoccia, 2002).

Side effects that occur after drug treatment is due to the host’s immune response towards the abrupt massive parasite antigen exposure and the side effects are dependent on the infection intensity level of the schistosomes in the host. The side effects include dizziness, stomach discomfort, diarrhoea, headache, nausea and itchy skin (Midzi et al., 2008).

Studies have been done in many different countries to find out the efficiency of praziquantel on their population groups and the average efficacy was found to be 75% (Tshuente et al., 2004; Liu et al., 2011). The use of both praziquantel and artesunate has been observed to increase protection rate to 78%-99% (Cheung et al., 1996). Ishmail in 1999 reported that there were some resistant to PZQ and concluded that prolonged use of PZQ would lead to drug resistance. Resistance was also reported in Senegal populations under praziquantel treatment by Guisse et al.,(1997) and Stelma et al.,(1995). It was also reported that upon treating the infected individuals with oxamnique a 79% cure rate
was achieved. In 2000 King and his colleagues researched on the efficacy of praziquantel on school children in Kenya and reported a yearly variation in PZQ response. Liang et al. (2001) tested the resistance theory in China as praziquantel has been used for over 10 years to treat *S. japonicum*. The conclusion was that there was no resistance and praziquantel was still effective.

As a strategy to reduce the morbidity of schistosomes, a solution of periodic mass drug administration of praziquantel was implemented although this is feared to create strains of schistosomes that are resistant. Studies are underway to find alternative permanent solutions to wipe out schistosomiasis (Olveda et al., 2013). To date there is no licensed vaccine. The vaccine on clinical trial is Bilhvx which works against *S. haematobium* (Riveau et al., 2012).

1.5 Immune Response against Schistosomes

Initial infection to schistosomes results in an acute inflammatory response that is characterized by rash (swimmers itch), Katayama fever and diarrhoea (Gryseels et al., 2006). Granulomatous reactions occur around the eggs deposited by mature worms whereby the eggs are aggregated by macrophages and T cells. Both Thelper 1 and Thelper 2 immune responses seem to be induced by schistosome species and the cytokines produced in both immune responses down regulate each other. Th1 cell inflammatory response is the first to be induced when schistosomes enter the body. This type of immune response is characterised by the production of TNF-α, IFN-γ, IL-6 and IL-1 cytokines that induce cell mediated immunity while the Th-2 immune response is recognized by the presence of IL-4, IL-5, and IL-10 that help in inducing humoral
mediated immunity. Research on murine models has reported that the initial immune response that occurs is towards the cercarea.

This Th1 inflammatory response has been reported to take place within 5 weeks of initial infection (Grzych et al., 1991). Some studies have indicated that there is an immediate inflammatory response that occurs once cercarea enter the skin (Kumar et al., 1999; Angeli et al., 2001; Wang et al., 2005). In the research that Kumar and Ramaswami (1999) did in mice, they found out that mice that were exposed to S. mansoni cercarea within 3 days induced the production of the pro-inflammatory cytokine IL-4 and the anti-inflammatory cytokine IL-10 but upon exposing mice to irradiated cercarea (candidate vaccine) they produced high levels of IFN-γ and IL-10 instead. Tolerant phenotypes have been observed to express high levels of the cytokines IL-10 and TGF-β which induce the production of IgG4.

As soon as the worms mature, mate and produce eggs a Th-2 inflammatory immune response is induced which is characterized by the presence of high levels of IgE, IL-10, eosinophils, mast cells and basophils. According to Schramm et al., (2003), glycoproteins found on the surface of schistosome eggs have been reported to induce production of IL-10 (from T regulatory cells), IL-13 (from basophils) and IL-4 (from basophils and natural killer cells). Deposition of the schistosome eggs in the liver causes liver fibrosis which is mediated by IL-13 (Pearce and MacDonald, 2002; Ramalingam et al., 2008).

A research on Brazilian individuals that were infected by S. mansoni reported that during chronic infection cytokine responses towards the eggs are reduced (Montenegro et al., 1999). The cytokines measured were IFN-γ and IL-10 with infected individuals at the
acute phase having very high levels of IFN-γ and IL-10. Various studies on the immune response to schistosomiasis have shown that schistosomes have the ability to manipulate the hosts’ defence system in order to survive by hindering the skin dendritic cells to the draining lymph node and increasing the IFN γ levels (Collins et al., 2013).

In 1990, Boctor et al conducted a research on Egyptian male individuals (12-56 years old) who were infected with S.mansoni and reported that their IgG4 levels were 20 times above normal (9000mg/l) against SWAP antigen. IgG4 levels in comparison to IgE levels were found to decrease after 12 weeks treatment with praziquantel whereas no significant changes in IgE were observed. In a research done on a Brazilian population exposed to S.mansoni, the same level of IgE was observed in individuals exposed to different parasite burdens whereas the levels of IgG4 differed in the individuals. Individuals with higher parasite intensity produced more IgG4 than those who had lower parasite intensity. It was also observed that the IgE/IgG4 ratio could be linked to resistance to re-infection (Figueiredo et al., 2012). Other immune-epidemiological studies have reported that Th2 immunity with high IgE production is associated with resistance to re-infection (Dunn et al., 1992; Jiz et al., 2009; Black et al., 2010).

1.6 The Role of Antibodies and Cytokines in Protective Immunity towards Schistosomes

Resistance to infection is considered to be age dependant and indicated by lower levels of infection before and/or after treatment (Karanja et al., 2002; Vereecken et al., 2007). Both Th1 and Th2 immune response have been reported to play a role in protective immunity (Anderson et al., 1998; Gatlin, 2002).
High levels of IgE have been suggested to play a role in protective immunity. The research that was done by Hagan (1991) reported that individuals with high levels of IgE where ten times unlikely to be re-infected than those individuals with lower levels of IgE. In the studies it was also noted that age and re infection intensity is a great confounding factor when investigating for the correlation of antibody responses to resistance to re-infection (Wami et al., 2014). IgE response in children from Kenya aged from 6-16 years was negatively correlated to re-infection whereas no correlation was found in the 17-66 years group (Dunne et al., 1992).

In a study done by Correa-Oliveira (2000), individuals who had chronic schistosomiasis were observed to produce low levels of IFN-γ. Henriet al (2002) reported that individuals who produced low INF-γ levels were susceptible to severe fibrosis. According to Russell et al., (2015) susceptibility to infection or re-infection to helminth parasites have been linked to genetic variation as studies indicate that higher infection prevalence are observed in European than African descent (Coellio and McGuire, 2006).

1.7 The Role of Single Nucleotide Polymorphisms (SNPs) of Cytokines in Protective Immunity

According to Gokce (2014), single nucleotide polymorphisms cause 90% of the human genetic variation. SNPs are variations of a single nucleotide in a DNA sequence and can occur in non-coding or coding DNA regions. The phenotype of individuals can be affected by SNPs especially if they occur on regulatory regions on DNA such as on promoter sites (Wue and Jiang, 2013).Extensive studies have been done to identify the role and effect of SNPs on susceptibility and resistance to schistosome infection (Bacharier and Geha,2000; Verra et al., 2004; Wen et al., 2006).
Most African Americans have been reported to contain the +874A allele that results in decreased IFN-γ production than in European Americans (Hoffmann et al., 2002; Hassan et al., 2003; Delany et al., 2004; Upperman et al., 2005). The human leukocyte antigen studies indicate that population genetics can contribute to disease susceptibility in Egypt and Sudanese; IFN-γ polymorphisms have been linked to Schistosoma fibrosis in these populations (Blanton et al., 2000).

Genome studies show that the 5q31-q33 chromosome region which contains Th2 cytokine genes is linked to resistance to S.mansoni and IgE production levels (Russell et al., 2015). Individuals with IL-13 polymorphisms in -1055C and -591A were reported to have high infections compared to those with -1055T (Kouriba et al., 2005). Cameron et al., (2006) also reported that polymorphisms in IL-13 -1055 and -591 are associated with resistance to S.mansoni infection. Henry et al., (2002) reported that individuals with the IFN-γ +874T produced high levels of IFN-γ and this correlated to increase in resistance to Schistosoma fibrosis. Polymorphisms in IL-4 -590 T have been reported to produce high levels of IgE which also correlates to resistance. A study done by Bacharier and Geha (2000) reported that the cytokine IL-4 plays a role in IgE class switching and polymorphisms in -590C/T alleles seem to produce different levels of IgE. The -590C allele was found to produce higher IgE levels whereas Verra et al., (2004) reported that co infected children with the -590T allele produced higher IgE quantities.
1.8 IFN-γ
IFN-γ is a cytokine that plays an important role in the activation of macrophage immune cells that are responsible for engulfing pathogens that enter the body. IFN-γ is the only type two IFN and is produced mostly by T lymphocytes (CD4 T helper cells, CD8 cytotoxic cells) and natural killer cells (Schroder et al., 2004). In the early host defence stages against pathogens, the antigen presenting cells (APC) cells secrete the cytokines IL-12 and IL-18 which are responsible for IFN-γ production while T lymphocyte cells produce IFN-γ during the adaptive immune response phase (Frucht et al., 2001). The cytokines IL-4, IL-10 and TNF-α down regulate the production of IFN-γ.

Increased IFN-γ has shown to result in the development of hepatosplenic disease in individuals infected by *S. mansoni* that can lead to death (Mwatha et al., 1998). It was also noted that increase in IFN-γ alone does not cause death, but down regulate Th2 immune responses that help fight the schistosome species. This was seen when mice given anti IFN-γ treatment produced enhanced levels of Th-2 cytokines and decreased granulomatous lesions (La Flamme et al., 2001). These granulomatous lesions are said to cause portal hypertension and liver fibrosis as a response to schistosome eggs being trapped in the liver which can lead to death (Butterworth et al., 1994). Studies on a Kenyan population revealed that there are groups of people who develop hepatosplenic morbidity and that the prevalence is significantly varied between different endemic areas and is more experienced in higher intensities of infection (Siongok et al., 1976; Mwatha et al., 1998). Kenya individuals who had similar infection intensity were observed to have different hepatosplenomegaly prevalence that suggested that perhaps other factors such as genotype was causing severe hepatosplenic disease.
1.9 Thesis outline

Due to the reports from a few countries suggesting that the current treatment method of mass drug administration of praziquantel might lead to the development of resistant schistosome species, there is thus need to develop a permanent solution to wipe out schistosomiasis. Understanding the immune response towards schistosomes is a prerequisite of vaccine development as it is of great importance for potential vaccines to induce the required immune reactions to fight off the infection. Studies that have been done so far have provided conflicting results with regard to the immune response associated with resistance to infection and a few studies have been done on *S. haematobium* which is the prevalent schistosome species in Zimbabwe. This research was done to contribute towards the understanding and monitoring of the immune response in children that are under the mass drug treatment programme. This research will address the question of whether or not praziquantel treatment in school children is beneficial in high endemic areas in Zimbabwe. The benefits expected to be observed are increased levels of protective markers and decreased levels of markers associated with susceptibility to infection against *S. haematobium*.

Chapter 2 of this thesis contains the Objectives, Study design and methods used to meet the objectives.

In Chapter 3 the thesis compares the confounding factors that may affect results (host age, sex and exposure area to infection levels) and the results of praziquantel treatment on the children. Chapter 4 contains the investigation and results of antibodies (IgM, IgE
and IgG4) that have been associated with protective immunity against schistosomes. These were compared against the *S. haematobium* specific antigen Sh13.

Chapter 5 of the thesis contains the results of the cytokine IFN-γ profiles and genotype profiles that have been associated with susceptibility and resistance to schistosome infections. The effect of praziquantel treatment on the cytokine production is also reported in this chapter.

The limitations and recommendations of this study are outlined in Chapter 6.
CHAPTER 2

METHODOLOGY

2.1 Main Objective

Investigate the Inflammatory Markers involved in *S. haematobium* protective immunity and susceptibility to infection in school children.

2.2 Study Objectives

1. Determine the infection status and efficacy of praziquantel treatment on the study participants.

2. Investigate profiles of inflammatory markers associated with resistance and susceptibility to infection in 7-13 year old children on praziquantel mass drug treatment.

3. Compare the levels of inflammatory markers associated with resistance and susceptibility to *S. haematobium* infection intensity in order to identify individuals who are resistant to infection or re-infection.

4. Identify if there is a genetic link between production of IFN-γ that leads to protective and susceptibility of infection.
2.3 Study design

This research was part of a 5 year longitudinal cross sectional survey of the immune response patterns and morbidity control of school children exposed to *S. haematobium* infection in Zimbabwe, prior to and at 6weeks follow up after praziquantel treatment.

**Survey time**

**Recruitment**

- n=284
- n=52
- Refused treatment and to give blood samples

**Baseline**

- n=232
- 59 egg positive for *S. haematobium*
- All given praziquantel after diagnosis

- n=48
- Excluded in serological studies
  - *S. mansoni* positive
  - 4 *S. haematobium* infected

- n=60
- Absent/refused to give 2nd blood samples

**6 weeks follow up**

- n=124
- F (63) and M (61)
- Blood, urine and stool samples collected for diagnosis.
- All children found negative

Figure 2.1: Flow chart of the study design. Representation of the number of children recruited at baseline and at post treatment time periods.
2.4 Ethical Approval

Permission was granted from the Medical Research Council of Zimbabwe (MRCZ/A/1976), Provincial and District Medical Directors, chiefs, village headmen, councillors and parents of the voluntary participating children. The participants were allocated an ID, case number and were told to feel free to withdraw at any point of the survey. Explanations and instructions were given to the participants in their native language (Shona).

2.5 Study Population and Area

Blood samples that were used in this research where collected from school children aged between 7-13 years, residing in Mashonaland Central (Mt Darwin) and Manicaland (Makoni) region in Zimbabwe. Schistosomiasis has been found to have the highest infection intensities in these regions with a prevalence of 39.3% and 31.2% respectively and school children are the most infected (Midzi, 2014).

The laboratory aspect of the work was conducted at the University of Zimbabwe in the Infectious Diseases Immunology Laboratory. The participants filled in questionnaires that required information such as name, date of birth, water contact behaviour and treatment history. The information was also cross checked with school register data to ensure accuracy of gender, age and name.

2.6 Parasitology methods
2.6.1 The Identification of *S. haematobium* eggs using the Urine Filtration Method

The participants provided a urine sample for 3 consecutive days which was analysed using the urine filtration method and light microscopy (Mott, 1983). The mean egg count was calculated and used to categorize *S. haematobium* negative and positive participants. Those who had 0 eggs were classified as negative while those who had greater than 0 eggs were positive.

Ten millilitres of urine was collected using a syringe, mixed thoroughly 3 times and filtered through a nitrile membrane (13 mm diameter, 12-14 µm pore size). The nitrile membrane was removed by opening the filter holder (13 mm diameter swinnex type) and placed on a clean slide using forceps. Iodine (10%) was used to stain the nitrile membrane. The stained nitrile membrane was then viewed under a microscope using x10 objective lense. The number of eggs were counted and recorded per 10 ml of urine.

2.6.2 Identification of *S. mansoni* Eggs Using the Kato Katz Method

Participants provided one stool sample which was used to enumerate and check for the presence of *S. mansoni* eggs. This was done for identifying the participants who were infected with both *S. mansoni* and *S. haematobium* in order to exclude them from this study.

A wooden spatula was used to press the stool sample through a sieve in order to separate out debris and large particles from stool. The smooth stool was taken from the underside of the sieve and was used to fill a 41.7mg hole on the template which was placed on a slide. The template was then removed from the slide carefully making sure that the stool remained on the slide. Fifty percent malachite green glycerine soaked cellophane was
used to stain the stool on the slide. A second slide was placed and pressed on top of the cellophane to evenly distribute the faecal material. The slides were then wrapped with tissue paper for 24 hours and used for viewing *S. mansoni* eggs.

### 2.7 Molecular methodology

#### 2.7.1 Blood collection

Approximately 5 ml of blood was collected from each child before and after praziquantel mass drug administration for DNA extraction and serological assays. The blood was stored at 4°C overnight after clotting at room temperature. The blood was then centrifuged at 3000 rpm for 10 minutes and serum was extracted. The serum was then stored at -20°C.

#### 2.7.2 DNA Extraction

DNA extraction from whole blood was done using the Qiagen extraction kit (Inqaba Biotech) which also supplied the DNA extraction protocol:

The frozen blood was thawed quickly with mild agitation at 37°C in a water bath and stored on ice before beginning the extraction procedure. The volume of blood used for the extraction process was 300µl. All centrifugation steps were conducted at room temperatures. Qiagen protease was dissolved in hydration buffer (buffer FG3) and stored in aliquots at 2-8°C. Seven hundred and fifty microlitres of FG1 buffer was pipetted into a 1.5ml centrifuge tube. Three hundred microlitres of whole blood was then added and mixed by inversion of the tube 5 times. These tubes were then centrifuged at 10.000g for 3 minutes in a fixed- angle rotor. The supernatant was discarded and the centrifuge tube was inverted on paper towels for 2 minutes to remove any liquid phase at the same time.
taking caution of retaining the pellet in the tubes. One hundred and fifty microlitres of FG2/ Qiagen protease was then pipetted into the tubes. The tubes were immediately vortexed at 4 pulses of high speed for 5 seconds until the pellet was homogenized. The tubes were then centrifuged for 3 seconds at 10,000g and placed in a water bath at 65°C. Protein digestion was indicated by a colour change of the solution from red to olive green. One hundred and fifty microlitres of 100% isopropanol was pipetted into the tubes, mixed thoroughly by inversion until threads or clumps of the DNA were visible. The tubes were then centrifuged at 10,000g for 5 minutes at room temperature. The supernatant was then gently poured off leaving the DNA pellet. Ten millilitres of 70% ethanol was added to the eppendorf tube containing the pellet and inverted several times. This was then centrifuged at 2000g for 3 minutes at room temperature. The ethanol was poured off and aspirated. The pellet was air dried for 10 minutes and 500µl of buffer FG3 was added to the tube. The tube was vortexed gently for 5 seconds and left overnight at room temperature to dissolve the DNA pellet. This DNA was then used for gel electrophoresis and polymerase chain reactions for this research.

2.7.3 Identification of IFN γ +874A/T genotypes in school children using ARMS PCR method

The ARMS PCR technique was used to identify the IFN γ +874A/T mutations in participants’ DNA by using a thermo cycler (Thermo Electron Corporation thermocycler). Two types of master mixes were made for the reaction. The differences were in the set of primers used. The first master mix contained the primer for the wild type allele and A allele mutation while the other master mix contained the non-mutant primer and the primer for the T mutation. The master mix prepared contained, 2.5µl of
PCR Buffer, 2.5µl of MgCl₂ (25mM), 1.5µl of dNTPs (2.5mM), 1.0µl of Generic and the mutated primer (2.5mM), 0.5µl of HGH forward and reverse primer (control), 5.0µl of Taq enzyme (5U/µl) and 5.5µl of water.

Twenty microlitres of the master mix was pipetted into 5µl of template DNA in PCR tubes. These were then placed in a thermocycler and amplified according to the cycles in Table 2.2. The PCR products were viewed under gel electrophoresis. All PCR reagents were purchased from New England bio labs including the PCR tubes. The primers used are indicated in Table 2.1 and were purchased at Inqaba Biotech Company South Africa.

### Table 2.1: Primer Positions and Expected Band Size.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Band Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ+874A</td>
<td>TTCTTACAACACAAAAATCAAATCA</td>
<td>288</td>
</tr>
<tr>
<td>IFN-γ+874T</td>
<td>TTCTTACAACACAAAAATCAAATCT</td>
<td>288</td>
</tr>
<tr>
<td>IFN-γ +874A/T Reverse</td>
<td>TCAACAAAGCTGATACTCCA</td>
<td>288</td>
</tr>
<tr>
<td>HGH Forward</td>
<td>GCCTTCCCAACCATTCCCTTA</td>
<td>425</td>
</tr>
<tr>
<td>HGH Reverse</td>
<td>TCACGGATTTTCTGTGTGTGTTTC</td>
<td>425</td>
</tr>
</tbody>
</table>

### Table 2.2: The cycles and temperatures used for the ARMS PCR reaction of IFN-γ +874A/T

<table>
<thead>
<tr>
<th>PCR profile</th>
<th>Temperature ( °C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Denaturation</td>
<td>95</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>45 min</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>50 sec</td>
<td>35</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>5 min</td>
<td></td>
</tr>
</tbody>
</table>
2.7.4 Identification of Genomic DNA an PCR products Using Gel Electrophoresis

A 1.5% agarose gel (multi-purpose agarose CSL-AG500) was made by measuring 1.5g of agarose powder into an Erlenmeyer flask containing 100ml of TAE buffer. The agarose powder was dissolved in the buffer using a microwave, constantly swirling the mixture at 30s intervals until all the agarose had dissolved. Ethidium bromide (0.5 μg/ml) was then added into the agarose/ buffer mixture. The agarose was allowed to cool at 40°C then poured into the gel mold containing combs to create wells. The agarose was allowed to set at room temperature until it could be used for the loading of samples. Loading dye was added to the DNA samples at a ratio of 1:5 and these DNA samples were then loaded into the wells gently. Lambda DNA HindIII ladder was used for genomic DNA whereas the 100kb ladder was used for PCR products to identify the base sizes and both ladders were purchased from New England Bio labs. TAE running buffer was added to cover the surface of the gel and the power supply was set at 100 V, 300amp and run for 1 hour. The gel was removed from the gel box and the excess buffer was drained off from the surface of the gel. The bands were then viewed under UV light and photographed.

2.8 Serological methods

2.8.1 Measurement of IFN-γ Using Elisa Technique

Baseline and follow up serum samples of individual identity numbers were used in the same microtiter plate in duplicate to avoid inter plate bias readings. All plates used were run with a blank well that was used to control for background noise readings. Positive controls used consisted of a serum sample of a known schistosomiasis positive individual and serially diluted IFN-γ standards of known concentration. The IFN-γ Elisa kit that was
used for this research was manufactured by Mabtech Company using the following protocol:

The coating solution which consists of 2 µg/ml of mAb-D1k capture monoclonal antibody was added to PBS buffer. One hundred microlitres of the coating solution was added into each well of Dynatech immulon microtiter plates and incubated at 4°C overnight. The plates were then washed 3 times with wash buffer (1 litre TBS and 400µl Tween 20) and blotted dry. The plates were then blocked by adding 200µl of TBS that contains 0.5% BSA in each well. The plates were incubated for 30 minutes on a shaker at room temperature. The plates were then washed three times and blotted dry. One hundred microlitres of serum samples that were diluted at a concentration factor of x10 with reagent diluent (0.5% BSA and 0.04% Tween 20 in TBS) were added into the wells in duplicates. These were then incubated overnight at 4°C. The plates were then washed 3 times with wash buffer and blotted dry. One hundred microlitres of mAb 7-B6 –biotin detection antibody was added in each well at 1µg/ml in TBS and incubated at room temperature for two hours. The plates were then washed and blotted dry. One hundred microlitres of conjugated antibody (Streptavidin-HRP) diluted at 1:1000 in TBS buffer solution was added in each well and incubated for an hour at room temperature. The plates were then washed 3 times and blotted dry. One hundred micro litres of substrate (OPD) was added in each well and incubated in the dark for 20 minutes. The optical densities were then measured immediately in a micro plate reader microplate reader supplied by Inqaba Biotech at 450nm.
2.8.2 ELISA of antibodies IgE, IgG4 and IgM

The antigen Sh13 was diluted in PBS buffer at a concentration of 5µg/ml. One hundred microlitres of this solution was used to coat each well of a microtiter plate overnight at 4°C. The plates were then aspirated, washed three times and blotted dry. The wash buffer consisted of 0.01% Tween 20 in PBS buffer. The plates were blocked by addition of 150µl of blocking solution for an hour at room temperature. The blocking solution consisted of 5% egg albumin in PBS buffer. The plates were then aspirated, washed three times with the wash buffer and blotted dry. Serum samples were diluted at the dilution ratio as indicated in the Table 2.3 for the different antibody assays. One hundred microlitres of the sample dilutions were then added into each well in duplicate and incubated for 2 hours at room temperature. The plates were then washed three times and blotted dry on paper towel. One hundred microlitres of peroxidise conjugated monoclonal antibody IgE, IgM and IgG4 all supplied by Southern Biotech were added into each well at a concentration of 1:1000 in the reagent diluent. These plates were incubated for 1 hour at room temperature then aspirated, washed six times and blotted dry. One hundred microlitres of OPD substrate was then added into each well and incubated in the dark for 20 minutes. Absorbance of each well was read immediately after the 20 minutes at 450nm using a microtiter plate reader.

Table 2.3: Antibody Elisa protocol

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sample dilution in reagent diluents (0.01% BSA and PBS buffer)</th>
<th>Substrate incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>1:200</td>
<td>20 minutes</td>
</tr>
<tr>
<td>IgG4</td>
<td>1:200</td>
<td>20 minutes</td>
</tr>
<tr>
<td>IgE</td>
<td>1:10</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>
2.9 Statistical Analysis

The D’Agostino and Pearson omnibus normality test was done first to designate the appropriate statistical analysis test on the variables and participants. Multivariate analysis was used to analyse protein antibody (IgE, IgM and IgG4) and cytokine (IFN-γ) profiles. The prevalence and intensity of *S. haematobium* infection was compared between the two areas by Pearson test and ANOVA. Mann Whitney tests were used to compare infection intensity between male and females. It was also used to determine any significant cytokine, antibody, protein and egg count reduction after treatment.

The mean egg counts were divided into 3 infection intensity categories: No infection # eggs = 0, Light infection for number of eggs/10ml of urine ≤ 50, Heavy infection for number of eggs/10ml of urine ≥ 51. The effect of variable IFN-γ genotypes on infection intensity was analysed using ANOVA (Kruskal-Wallis test). Graph pad prism version 5.0 (Graphpad software, San Diego, CA, USA) was used for the analysis of all data and the data considered as significant had values of p<0.05.

Specific IgE, IgG4 and IgM cut off points were calculated by addition of 2 SDs to the mean OD values of 10 schistosomiasis negative individuals. (IgE cut-off = 0.2836; IgG4 cut off = 0.8405; IgM cut off =2.784; INF-γ cut off =0.3073)
CHAPTER 3

Identifying *S. haematobium* infections and evaluating the efficacy of praziquantel treatment in school children

3.1 Introduction

According to Gryseels *et al.*, (2006) untreated *S. haematobium* infections can cause severe clinical morbidity. Praziquantel is the current treatment of choice that has been used to reduce morbidity by its mass drug administration to children (WHO, 2010). Most countries with schistosome endemic areas have reported efficacy praziquantel treatment rates of 75% but there have been population groups that have reported resistance to praziquantel treatment (Stelma *et al.*, 1995; Guisse *et al.* 1997; Liang *et al.*, 2001; Tshuente *et al.*, 2004). Those reports indicate the potential of developing schistosome resistant strains especially since praziquantel is being used in mass drug administration at frequent intervals as a strategy to eradicate schistosome infections in school children. There is therefore need to monitor its efficacy on different populations and timelines as they have varied exposure patterns to schistosomes and different genetic makeup which may also affect the efficacy of praziquantel treatment (Hu *et al.*, 2004).
3.2 Aims

This chapter determines and compares *S. haematobium* infection prevalence in the study participants at pre and post treatment timelines and the efficacy of mass drug treatment of children with praziquantel. Confounding factors such as the effect of gender and age on infection intensity are also addressed in this chapter.

3.3 Hypotheses


2. Gender and age affects susceptibility of *S. haematobium* infection intensity.

3.4 Materials and Methods

3.4.1 Study population

A total number of 232 school children aged between 7-13 years from highly *S. haematobium* endemic areas were initially present at base line for this study. At post treatment the number reduced to 161.
3.4.2 Diagnosis of infection status

At each survey timeline 3 urine samples and 1 stool sample was collected for S. haematobium and S. mansoni diagnosis. The full methodology of urine filtration and Kato-Katz is described in Chapter 2.

3.5 Results

Table 3.1: Variables measured to obtain parasitological data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>7-13 year old</td>
</tr>
<tr>
<td>Gender</td>
<td>159 Females (68.5%) 73 Males (31.4%)</td>
</tr>
<tr>
<td>Infection status</td>
<td>Positive or negative according to the presence or absence of eggs in urine</td>
</tr>
<tr>
<td>Infection intensity</td>
<td>Grouped according to the number of eggs per 10ml of urine: None (0 eggs) Low (1-10 eggs) Medium (11-50 eggs) High (eggs &gt; 50)</td>
</tr>
<tr>
<td>Survey time line</td>
<td>Baseline (before any treatment) and at 6 weeks follow up (after praziquantel mass drug treatment)</td>
</tr>
</tbody>
</table>

3.5.1 Infection intensity status of the study participants at Baseline

The results did not meet the normality parametric tests (D’Agostino and Pearson omnibus normality test) hence, the non-parametric tests were used to analyse all data obtained in this study. It was observed that more females were uninfected than males. The number of infected males and females were the same. The Fishers test at p<0.05 indicated that there is a statistical difference between infection intensity between females and males.
Figure 3.1: The effect of gender on infection status at baseline. The number of infected children was less than the number of uninfected children. The females which were the majority of the study participants are seen to have a larger number of uninfected individuals than males. The data was analysed by Fisher’s exact test at $p<0.05$. The p value obtained was 0.0007, indicating that there was significantly more uninfected children than infected children in the study population.
Table 3.2: Summary of study population and their *S.haematobium* infection status at baseline

<table>
<thead>
<tr>
<th>Data analyzed</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>30</td>
<td>29</td>
<td>59</td>
</tr>
<tr>
<td>Negative</td>
<td>126</td>
<td>47</td>
<td>173</td>
</tr>
<tr>
<td>Total</td>
<td>156</td>
<td>76</td>
<td>232</td>
</tr>
</tbody>
</table>

3.5.2 Age-Infection intensity profiles in children at baseline.

The infection intensity distribution of *S.haematobium* exposed children is shown in Figure 3.2. The results indicate that in all the age groups the number of uninfected children was more than the infected children. The 7 year age group had the highest number of uninfected individuals. The 12 year age group had the highest number of infection in comparison to other age groups. The 13 year age group had the lowest number of infected individuals.
Figure 3.2: Baseline *S. haematobium* infection distribution by age. The bar graph indicates the comparison of infected and uninfected children per age group.

3.5.3 Prevalence of infection intensity in the study participants at Post treatment

Table 3.3: Summary of study population and their infection status at Post treatment

<table>
<thead>
<tr>
<th>Data analyzed</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>63</td>
<td>61</td>
<td>124</td>
</tr>
<tr>
<td>Excluded (<em>S. mansoni</em> positive at baseline)</td>
<td>45</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>Excluded (absent/insufficient data)</td>
<td>48</td>
<td>12</td>
<td>60</td>
</tr>
</tbody>
</table>

The results indicate that all the children who came at the follow up timeline did not have any eggs in their urine samples. All these children were diagnosed as negative. There
were a large number of children whose data was not accounted for as they were either absent or had insufficient data.

3.6 Discussion

The confounding factors in this study had no statistical significant effect on *S. haematobium* infection intensity. Studies in contrast have reported that males are more prone to infection than females and that the 11-12 year old age group usually has high infections compared to other age groups (Midzi *et al*., 2008). Adults have been also reported to have less infection rates than children. In this study the male population that came for the survey was lower than the female population; this could have probably affected statistical comparison.

The results from this study indicate that praziquantel is efficient at clearing the *S. haematobium* worms as the children had no eggs in their urine at post treatment. Midzi *et al*., (2008) also reported that praziquantel was efficient at treating schistosomiasis. They reported an 88.5% cure rate in other *S. haematobium* highly endemic areas in Zimbabwe. The efficacy results obtained in this study are in agreement with the results that Tshuente *et al* (2004) and Liu *et al* (2011) reported. Praziquantel damages the schistosomes tegument which results in the exposure of internal antigens that the hosts’ immune system could not detect. The availability of antigens then induces the immune system to clear the parasites. The schistosome mature worms are known to manipulate the host’s immune system in-order to survive. However the results from this research contradict with the results that Ishmael (1999), Cruiseee *et al* (1997) and Stelma (1999) reported. They found resistant schistosome strains to praziquantel treatment in Senegal and Egyptian populations. This has raised the fear that mass drug treatment using
praziquantel has the potential to develop resistant strains and frequent monitoring of its efficacy is required.

3.7 Conclusion
Age was found to have no statistical significant effect on acquiring *S.haematobium* infections in this study. It is difficult to conclude if males were more prone to infection than females because of the small population size that came for the survey in comparison to females. Praziquantel was efficient in clearing *S.haematobium* worms in the study participants from *S.haematobium* highly endemic areas in Zimbabwe.
CHAPTER 4

Determination of antibody profiles important in resistance and susceptibility to infection

4.1 Introduction

Antibodies are involved in the Th-2 inflammatory response and bind to the unwanted antigens so that immune cells such as macrophages can recognise and clear the unwanted antigens. IgE plays a key role in protective immunity while IgG4 and IgM block the IgE by binding onto the same sites that IgE bind to hence hindering the action of macrophage cells. The antigen used in this chapter is the recombinant Sh13 antigen that is specifically found on *S. haematobium* tegument.

4.2 Aims

The aim of this chapter was to investigate and compare profiles of the antibody inflammatory markers associated with resistance and susceptibility to infection in 7-13 year old children on praziquantel mass drug treatment against the sh13 antigen. The antibodies that were investigated were IgE, IgG4 and IgM.

4.3 Hypothesis

1. *S. haematobium* infected individuals produce higher levels of protective antibodies after treatment than before treatment
2. *S. haematobium* Sh13 antigen specific antibody profiles vary with age
3. Infected children produce higher levels of antibodies than uninfected children.
4. Resistant children have higher IgE than IgG4
4.4 Materials and Methods

4.4.1 Study Population

Serum samples of 124 children were used for the assays. The number of samples used for females and males were 63 and 61 respectively. These children were selected on the basis that they 1) had been present both at baseline and post treatment, 2) provided adequate urine, stool and blood samples and were negative for *S. mansoni*.

4.4.2 Immunological Assays

ELISA was used to measure the antibodies IgG4, IgE and IgM using the sh13 antigen. All serum samples were assayed in duplicate. The negative and positive controls used were known individuals who were negative and positive respectively. The full methodology has been described in chapter 2.

4.4.3 Statistical Analysis

Non parametric data analysis was used to analyse the data results from ELISA. Mann Witney test was used to compare all antibody production means between baseline and follow up time line. ANOVA Kruskal Wallis test was used to analyse all the antibody-infection intensity profiles and age-antibody production profiles.
4.5 Results

4.5.1 Comparison of Antibody profiles at baseline and follow up timeline

Figure 4.1: Comparison of antibody mean levels response against Sh13 antigen between baseline and 6 weeks follow up time line. a) IgG4 antibody levels, b) IgE antibody level and c) IgM antibody level. Results of Mann Whitney test at p< 0.05
Figure 4.1 represents graphs showing the antibody responses of IgG4, IgE and IgM in serum of 128 school children before taking praziquantel (baseline) and at 6 weeks after taking praziquantel (follow up). The mean levels for all the antibody types were found to have no significant difference at the two timelines when the serum was collected. The Optical density (OD) results from ELISA show that the IgE levels were lower than IgG4 levels which were also lower than IgM levels. An analysis of variance using the Kruskal Wallis test obtained statistically significant P values < 0.0001 upon comparing the means of the different types of antibodies at baseline line and follow up. This shows that the antibody means were different from each other.
4.5.2 Variation of antibody production profiles by age.

Figure 4.2: Comparison of age/antibody profile against Sh13 antigen a) IgE antibody levels, b) IgG4 antibody level and c) IgM antibody level. An analysis of variance using Kruskal-Wallis test for each antibody was done at p<0.05. IgE p =0.2413, IgG4 p =0.5370 and IgM p = 0.2582. The graph shows that in all the 7 age groups the levels of IgM production were higher than IgG4 and IgE. The levels of IgG4 were higher than IgE except for the 13 years age group were the levels of IgG4 and IgE were the same. The individual antibody production within the age groups was statistically similar.

4.5.3 Comparison of antibody profiles between infected and uninfected children.

The graphs in Figure 4.3 are a representation of the Mann Whitney test results done to compare the antibody mean values between S. haematobium infected and uninfected individuals. For all the antibodies that were tested, it is observed that the infected individuals have significantly higher IgM, IgG4 and IgE OD values in comparison to the
uninfected group. This means that the infected individuals produced statistically significant more antibodies tested than the uninfected children. IgM tested had p< 0.0001, while IgG4 had a p= 0.0443 and IgE had a p= 0.0242. It is also observed that the levels of IgM produced in both infected and uninfected children were greater than IgG4 levels which were also greater than the IgE levels. An analysis of variance using Kruskal Wallis test was done on the antibodies which resulted in p < 0.0001, indicating that the means of all the antibodies are statistically different.
Figure 4.2: Comparison of antibody mean OD levels response against Sh13 antigen between infected and uninfected individuals. a) IgE antibody levels, b) IgM antibody level and c) IgG4 antibody level.
4.6 Discussion
Antibody age profiles indicate how infection may differ amongst age groups. The results from this research indicated that each protective antibody investigated was not statistically different within the 7-13 age groups with the exception of the 13 yr old age group which had the same levels of IgG4 and IgE but notably lower level of IgG4 and higher levels of IgE. A study done by Naus et al., (1998) on Cameroonian children (4-13yrs) also reported that there was no significant difference in antibody response levels within the age groups but observed that the older children had higher levels of IgE and lower levels of IgG4 against adult worm antigens. Protective immunity has been suggested to develop with age and IgE plays a protective role of activating eosinophils, macrophages and platelets that are able to kill schistosomes (Hagan et al., 1991). Binding of IgE to the antigens on the worm can be blocked by IgM and IgG4 which block mast-cell degranulation (Hagan et al., 1991; Zhang and Mutapi, 2006).

The infection levels of children dropped significantly after praziquantel treatment but there was no significant increase in the protective antibody levels against Sh13 observed between the baseline (untreated) and follow up (6 weeks after PZQ treatment). These results are in agreement with the results that Rujeni et al., (2012) found in his research on people living in highly S.haematobium endemic areas and the research that Doctor et al.(1990) conducted on Egyptian males (12-56 yr old) exposes to S.mansoni. Caldus (2000) also reported a decrease in infection intensity of Brazilian individuals from 60% to 12% after praziquantel treatment. The PZQ drug kills the mature worms and induces the host’s immune cells to clear the worms (Joseph et al.,2004; Mutapi et al.,2005). The absence of worms means that there are no eggs produced thus, the children are then diagnosed as uninfected. The immune system alone is unable to wipe out the schistosome
worms because of the low number of antigens present or the unavailability of exposed antigens to induce an immune response against the schistosome worms. The presence of protective antibodies IgE, IgM and IgG4 before treatment could be because of the children’s prolonged exposure to heavy schistosome worm load and the slow acquisition of an immune response against the worms. IgE is produced by B cells and plays a role in opsonisation by inducing the production of cytokines from basophils and mast cells that activate eosinophils that in turn release eosinophil peroxidase to kill the worms. Macrophages are also stimulated and engulf the worms. Studies in Brazil report that children who were highly resistant to *S.mansoni* infection had six to eight fold higher IgE levels than less resistance children (Capron, 1994).

The presence of protective antibodies in uninfected children shown on **Figure 4.3** suggests that these children’s immune system had already been boosted against the sh13 antigen meaning that these children had been exposed and infected before by *S.haematobium*. Studies have shown that the antibodies levels drop after about a year which accounts for their presence observed in the serum of uninfected children. The infected children had higher levels of IgE, IgM and IgG4 than the uninfected children because of the presence and intensity of schistosome worms in their bodies.

**4.7 Conclusion**

Praziquantel effectively cleared the *S. haematobium* worms from the study participants. Production of IgE, IgG4 and IgM did not vary with age in the study population and the infected children had higher levels of the protective antibodies. The study participants showed antibody/ infection profile that is categorized under susceptible to re-infection due to the higher IgG4 in comparison to IgE levels.
CHAPTER 5

Evaluating IFN-γ profiles and the role of genetic variation in resistance and susceptibility to *S.haematobium* infection

5.1 Introduction

IFN-γ production has been reported to correlate to resistance to infection and Pravica *et al.* (2000) demonstrated that a mutation in the IFN-γ promoter site +874A/T influences IFN-γ production. A study done by Abbott *et al.*, (2007) indicated that individuals with the AA genotype predominated and this genotype is associated with natural immunity. Investigating the single nucleotide polymorphisms in the participants is essential in understanding why some children are more susceptible to infections than other children.

5.2 Aims

The aim of this chapter was to identify if there is a genetic link in the production of IFN-γ that leads to resistance and susceptibility to infection. Confounding factors that may affect results such as the effect of age in IFN-γ production was also investigated in this chapter.

5.3 Hypothesis

1. *S.haematobium* infected individuals produce lower levels of IFN-γ than uninfected children
2. Age has an effect on the production of IFN-γ
3. There is a genetic link that affects production of IFN-γ and hence, predisposes children to resistance or susceptibility to re-infection
5.4 Materials and methods

5.4.1 Study Population

The serum samples from 124 children used to investigate antibodies in chapter 4 were used in this chapter.

5.4.2 Immunological Assays

ELISA was used to determine the IFN-γ levels in the serum samples. DNA was extracted from blood and genotyped using the amplification-refractory mutation system polymerase chain reaction (ARMS PCR) to identify mutations in the +874A/T IFN-γ promoter region.

5.4.3 Statistical analysis

Non parametric tests were used to analyse the results in this chapter. Mann Witney test was used to compare the means of IFN-γ production between baseline and post treatment (Figure 5.1a), between infected and uninfected children (Figure 5.1b). ANOVA was used to analyse the effect of IFN-γ production amongst the age groups (Figure 5.2), the effects of genotype on IFN-γ production (Figure 5.3) and the effect of genotype on infection intensity (Figure 5.4).

5.5 Results

The levels of IFN-γ measured by ELISA were plotted on graphs using Graph Pad and these levels were used to compare the effect of genotype on IFN-γ production.
5.5.1 IFN-γ profiles in school children under praziquantel treatment

![IFN-γ profiles](image)

**Figure 5.1: Comparison of IFN-γ profiles in**

**a) baseline and 6 weeks follow up time line, b) Infected And Uninfected Children.**

a) Indicates the mean level of IFN-γ produced at baseline and 6 weeks after treatment analysed using Mann Whitney at p< 0.05 are not significantly different meaning that the levels of IFN-γ in the children before praziquantel treatment did not change after taking the drug. The p value obtained was 0.4139. The comparison between infected and uninfected children was statistically significant at p< 0.05, meaning that the amounts of IFN-γ produced by uninfected and infected children were different. Infected children produced less IFN-γ than uninfected children. Using the Mann Whitney U test p =0.0214.

b) Indicates the comparison of IFN-γ produced between the infected and uninfected children. The uninfected children produced more levels of IFN-γ than the infected children and the results were analysed by the Mann Whitney at p< 0.05 obtaining a p =0.0214*. 

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5.5.2 Effect of age on IFN-γ production

Figure 5.2: Comparison of Age/ IFN-γ profiles in children exposed to *S. haematobium*. The highest production of IFN-γ is observed in the 8 year old age group and the lowest IFN-γ production is observed in the 13 year old group. Analysis of variance using Kruskal Wallis at p<0.05 showed that there is no statistical difference between the IFN-γ levels produced (p= 0.8734)
5.5.3 Effect of genotype on IFN-\(\gamma\) production

Figure 5.3: Comparison of IFN-\(\gamma\) production in genotype groups of children exposed to *S. haematobium*. The mean levels of IFN-\(\gamma\) production were different for each genotype. The means were compared using the Kruskal-Wallis test and obtained a p value of 0.0090**. The genotype AA is seen to produce more IFN-\(\gamma\) than TT which also produced more IFN-\(\gamma\) than AT.
5.5.4 IFN-γ production in infected and uninfected genotypes

Figure 5.4: Comparison of IFN-γ genotype profiles in infected and uninfected children

Figure 5.3 indicates that there are 3 genotypes that were observed in the 124 school children exposed to *S. haematobium*. These genotypes are; AA, AT and TT. Analysis of variance using Kruskal Wallis test at *p* < 0.05 resulted in *P* < 0.0001 value which means that the IFN-γ levels produced by each genotype are statistically significant and different. Overall the infected children containing the homozygous A allele produced higher levels of IFN-γ than infected children containing the T allele. The AA genotype children who were negative for Schistosomiasis produced the highest levels of IFN-γ while the AT genotype produced the lowest IFN-γ levels.
5.5.5 Effect of age on IFN-γ production

The 8 year age group was observed to have the highest mean IFN-γ levels in comparison to the other age groups. The 13 year old age group produced the least mean OD value of IFN-γ while the other remaining age groups (10, 11 and 12) OD mean values were similar. Analysis of variance using Kruskal Wallis test at $p<0.05$ obtained a p value of 0.8734 which indicated that there was no significant difference in the mean production of IFN-γ within the age groups.
5.6 Discussion

Age was observed not to have a statistically significant effect on IFN-γ production in this study population. The small sample size of the population might have affected the results hence; it is difficult to conclude the effect of age on IFN-γ production. The results from this study indicate that the infected children were in their chronic phase of infection which has been reported to be characterised by low IFN-γ production. Similar results have been reported by Vianna et al (1994); Bahia-Oliveira et al (1996) and Brito et al (2000). High IFN-γ production has been observed in individuals who are in the acute phase of schistosomiasis infection (Correa-Oliveira et al., 1998). IFN-γ is a Th1 cytokine which is induced during early infection stage. When the mature worms lay eggs the host’ Th-2 inflammatory response is induced which down regulates IFN-γ production resulting in low IFN-γ levels.

Praziquantel did not have any effect on the IFN-γ levels at post treatment probably because praziquantel boosts the Th-2 immune response and not the Th-1 immune response hence, its presence or absence is expected to have no significant effect on IFN-γ production. According to Pearce et al (1991), egg production during chronic stage of schistosomiasis switches from a Th-1 to Th-2 immune response in murine models thus, since infection status is based on the presence or absence of eggs, IFN-γ being a Th-1 cytokine is expected to be lower in infected children than in uninfected children.

However, it should be noted that all cytokines are not specific, undiagnosed diseases in the participants can influence IFN-γ production (Wami et al., 2014). In this study the participants had been pre-screened of other helminth infections and malaria, it was important to monitor the IFN-γ levels to determine susceptibility to infection. The role of
IFN-γ in schistosome protection is not yet fully understood but has been suggested to activate effector cells such as macrophages which produce nitric oxide and superoxide (cytotoxic agents) that kill schistosomula (Wilson, 1998).

The children’s genotypes were investigated to identify if their genotypes relate to an increase or decrease in IFN-γ production which predisposes the children to be susceptible or resistant to infection (Gatlin, 2009). In this study it was observed that uninfected children with the +874 A allele produced more IFN-γ than uninfected children with the +874T allele whereas in infected children, those with the +874T genotype were the ones who produced higher IFN-γ than the other genotypes. Pravica et al., (2000) reported similar results that the +874T allele produced higher IFN-γ during infection. Gatlin, (2009) reported that Kenya males who had the T allele were more resistant to schistosome infection than the homozygous AA genotype men.

5.7 Conclusion

*S. haematobium* infected individuals produced lower levels of IFN-γ than uninfected children and age did not have an effect on the production of IFN-γ. A genetic link was found in the study participants that affects production of IFN-γ hence, predispose children to resistance or susceptibility to re-infection. The AA and TT genotypes are less susceptible to infection than the AT genotype children.
CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

6.1 Limitations of the study and Recommendations

In this study I have reported that the antibodies associated with susceptibility and resistance to infection against the Sh13 antigen are higher in infected than uninfected children but the effect of age on the results could not be conclusively made because of the small sample size. To reach more substantial conclusions the same studies should be done on larger populations and at different timelines to monitor praziquantel efficacy. It was also observed that since the children had greater IgG4 than IgE they are susceptible to re-infection.

Although this study has indicated a link between IFN-γ and mutations in the +874A/T promoter region: the children containing the AA genotype were the higher producers and less susceptible to infection, more studies will be profitable to explore the combined effect of the other IFN-γ single nucleotide polymorphisms on susceptibility and resistance to re-infection. The IFN-γ measured in this study was none specific hence to give a more conclusive result IFN-γ production in individuals should be further investigated by stimulating the study participants’ PBMC with the Sh13 antigen on a larger scale.

Due to time constraints the effects of other cytokines that regulate IFN-γ and are known to also play a role in protective immunity were not studied. The combined effect of these cytokines on the same population group would further help in understanding t the
correlations that take place in host’s immune system against the potential vaccine candidate Sh13. The results I have reported in this thesis will aid other researchers trying to understand the protective immune mechanisms that occur in schistosomiasis infected children and in finding a vaccine that will permanently eradicate schistosomiasis.
### Appendix

#### Buffers and Reagents

**PBS buffer pH 7.4**

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<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>8g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2g</td>
</tr>
<tr>
<td>Di-Sodium hydrogen orthophosphate anhydrous</td>
<td>2.9g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.2g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

**TBS buffer pH 9.6**

<table>
<thead>
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<th>Amount</th>
</tr>
</thead>
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<tr>
<td>Tris</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>8.76g</td>
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</tbody>
</table>

**Phosphate citrate buffer pH 5.0**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Di-sodium hydrogen orthophosphate anhydrous</td>
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</tr>
<tr>
<td>Citric acid</td>
<td>5.19 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
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</table>

**Ortho-phenyldiamine dihydrochloride (OPD)**

<table>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPD</td>
<td>8mg</td>
</tr>
<tr>
<td>Phosphate citrate buffer</td>
<td>20ml</td>
</tr>
</tbody>
</table>
Hydrogen peroxide $\text{H}_2\text{O}_2$ 30%

**Gel Electrophoresis results**

Photograph of 0.8% agarose gel stained with ethidium bromide showing DNA bands. M is the molecular weight marker (*HindIII* ladder) and the next wells contain the genomic DNA.

Photograph of 2.0% (w/v) agarose gel stained with ethidium bromide showing amplified 288 bp fragment of IFN-$\gamma$ gene. The 426bp band is the human growth hormone gene used as the internal control. M is the molecular weight marker (100bp ladder), A is arginine and T is thymine.
References


diagnosis of schistosome infection: can it be done with antibodies? Trends in
Parasitology, 20: 35-39
• Etard JF, Audibert M, Dabo A (1995). Age acquired resistance and
predisposition to reinfection with Schistosoma haemotobium after treatment
with praziquantel in Mali. Am J Trop Med Hyg. 52:549-558
Treatment for Schistosoma japonicum, reduction of intestinal parasite load, and
cognitive test score improvements in school-aged children. PLoS Negl
Trop Med. 6:e1634.
• Feldmeier H, Poggensee G (1993). Diagnostic techniques in schistosomiasis
• Fenwick A, Savioli L, Engels D, Bergquist NR, Todd MH (2003). Drugs for
the control of parasitic diseases: current status and development in
• Ferrari ML, Coelho PM, Antunes CM, Tavares CA, Da Cunha AS (2003).
Efficacy of oxamniquine and praziquantel in the treatment of Schistosoma
• Figueiredo JP, Oliveira RR, Cardoso LS, Barnes KC, Grant AV, Carvalho EM,
Araujo MI (2012). Adult worm-specific IgE/IgG4 balance is associated with
low infection levels of Schistosoma mansoni in an endemic area. Parasite
Immunology, 34:604-610
IFN-gamma production by antigen-presenting cells: mechanisms emerge.
approach to schistosome population dynamics and estimation of the life-span
• Gatlin MR (2002). Cytokine Gene Polymorphisms associated with resistance
vs susceptibility to reinfection with Schistosoma mansoni.
• Gatlin MR, Black CL, Mwinzi PN, Secor WE, Karanja DM, Colley DG
(2009). Association of the Gene Polymorphism IFN-γ +874, IL-13 −1055 and
IL-4 −590 with Patterns of Reinfection with Schistosoma mansoni. PLoS Negl
Trop Dis. 3(2):e375.doi:10.1371/journal.pntd.0000375.
• Gordon CA, Acosta LP, Gray DJ, Olveda RM, Jarilla B (2012). High
prevalence of Schistosoma japonicum infection in Carabao from Samar
Province, the Philippines: implications for transmission and control. PLoS Negl
Trop Dis. 6:e1778.


• Oguz G (2014). Cytokine Polymorphism Catalog (CytoCAT) for the Analysis of Phenotype Associations.


• Rujeni N, Nausch N, Midzi N, Graeme J (2012). Atopy is inversely related to schistosome infection intensity: a comparative study in Zimbabwean villages
with distinct levels of *Schistosoma haematobium* infection. Int Arch. Allergy Immunol. 158:288-298.


