SCHISTOSOMA MANSONI SUSCEPTIBILITY TO PRAZIQUANTEL IN ENDEMIC LOCALITIES IN KENYA

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JUNE 2017
DECLARATIONS

‘I duly declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award’

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DEDICATION

I dedicate this thesis to my wife Margaret and our three sons, Bradley, Joel and Gabriel.

Thanks a lot for bringing so much joy in my life
ACKNOWLEDGEMENTS

First and foremost, I thank the Almighty God for making it possible for me to successfully carry out this research project.

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>ACUC</td>
<td>Animal Care and Use Committee</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ions</td>
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<tr>
<td>Cav</td>
<td>Voltage gated Ca²⁺</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DYF</td>
<td>Dye filling defective</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>Effective dose required to kill 50%</td>
</tr>
<tr>
<td>IVM</td>
<td>Ivermectin</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>KU</td>
<td>Kenyatta University</td>
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<tr>
<td>LE</td>
<td>Egyptian</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per Kilogram</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance associated protein</td>
</tr>
<tr>
<td>MSc</td>
<td>Masters of Science</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NTD</td>
<td>Neglected tropical diseases</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PgP</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Principal investigator</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>PZQ</td>
<td>Praziquantel</td>
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<tr>
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<td>Ribonucleic acid</td>
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<td>RT-PCR</td>
<td>Realtime-Polymerase chain reaction</td>
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<tr>
<td>SCOX1</td>
<td>Mitochondrial cytochrome C-Oxidase</td>
</tr>
<tr>
<td>SMDR2</td>
<td><em>Schistosoma mansoni</em> drug resistant transporter</td>
</tr>
<tr>
<td>TCBZ</td>
<td>Benzimidazole triclabendazole</td>
</tr>
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<td>WHO</td>
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ABSTRACT

Schistosomiasis is a neglected tropical disease (NTD) caused by blood-dwelling flukes of the genus *Schistosoma* transmitted through freshwater snails. While the disease affects as many as 249 million people worldwide, treatment largely relies on a single drug, praziquantel (PZQ), which is extremely effective against all schistosome species known to infect humans, it is well-tolerated in the body, making it suitable for mass treatment campaigns. The near exclusive use of this drug for such a prevalent disease has led to concerns regarding the potential for drug resistance to arise and the effect this would have on affected populations. The aim of this study was to investigate if *S. mansoni* susceptibility to PZQ is altered following repeated treatments.

In this study, a modified *in vitro* technique by Liang et al., was used to determine the effect of PZQ on miracidia. This was achieved by hatching of miracidia from eggs obtained from fecal samples of infected individuals, subjecting the miracidia to varying concentrations of PZQ and observation of viability (dead or alive) of miracidia under a dissection microscope. Similarly, the generation of a lab strain with reduced susceptibility was achieved *in vivo* whereby the parasite was subjected to sub-lethal doses of PZQ and gradually increased over five generations until the parasites could withstand a normally lethal dose.

A total of 72 isolates of *S. mansoni* from infected individuals with a history of multiple treatments with PZQ were analysed for PZQ sensitivity in the *in-vitro*. Similarly, 38 isolates of *S. mansoni* from field-collected *Biomphalaria* snails were analysed.

The overall miracidial mortality of *S. mansoni* at a concentration of $10^{-5}$ M PZQ for the entire study population was between 82.1-84.6 %, with the lowest observed value being 72.7% ($P >0.05\%$). In general, results of the present study found no evidence for the presence of such strains in the cohort of adults and children living in endemic areas of western and central Kenya who have undergone recent or historical treatment with PZQ and did not harbor *S. mansoni* with reduced PZQ susceptibility. However, under experimental conditions, a strain with reduced susceptibility to PZQ was produced within only 5 generations ($P < 0.05\%$).

The likelihood of this happening in the field is real, if sufficient PZQ pressure is applied. The threat of PZQ resistance remains a great public health concern in Kenya especially with intensified use of PZQ for schistosomiasis control and elimination. Regular monitoring of PZQ sensitivity in schistosomes is therefore needed in control programs for early detection of changes in parasite sensitivity to the drug.
CHAPTER ONE
INTRODUCTION

1.1 Human schistosomiasis and its public health significance

Schistosomiasis is a water-borne parasitic disease that affects more than 249 million people (World Health Organization, 2014) 97% of whom are on the African continent (Savioli et al., 1997; Steinmann et al., 2006) with a global disease burden calculated at 24-56 million disability-adjusted life-years lost (King, 2010).

The chronic and debilitating nature of the disease results in high costs in public health and economic productivity in developing countries, and has prompted the initiation of large scale control programs (Crompton et al., 2003; Fenwick et al., 2003).

Although there is no vaccine, the disease can be treated and controlled with praziquantel (PZQ), a drug developed in the 1970s (Magnussen, 2003) and shortly thereafter identified as the treatment of choice by the World Health Organization (Andrews et al., 1983). Praziquantel is the current anti-schistosomal drug and is the least expensive, easiest to use and most readily available of all (Hagan et al., 2004). It is extremely effective against all schistosome species that are known to infect humans and is well-tolerated, making it suitable for mass treatment campaigns. These campaigns are particularly targeted at school-age children who represent a high-risk group for infection, and the most heavily infected segment of the population (Cioli et al., 1995; Sudtida et al., 2006). Even though such programs have immediate and important beneficial effects, the concerns are that they inevitably leave some individuals untreated, and they do not interrupt transmission, making re-infections a certainty (Danso-Appiah and De Vlas 2002).

Praziquantel use has increased over the years, not only in intensity but also, in frequency. In 2006, approximately 12 million people were treated with PZQ and by 2012 this number had
reached approximately 42 million (World Health Organization, 2014). There is concern that with increase in PZQ use, drug resistance is likely to emerge (King et al., 2000; Cioli and Pica2003; Hagan et al., 2004; Botros et al., 2006). While the drug is highly effective against sexually mature forms of the parasite, it may not be effective against the juvenile schistosomes less than 2 weeks old (Pica-Mattoccia and Cioli, 2004; Aragon et al., 2009).

In 2012, the WHO announced a ‘roadmap’ for the elimination of 17 neglected tropical diseases (NTD) (World Health Organization, 2012), one of which was schistosomiasis. It was proposed that the disease could be eliminated as a public health problem in multiple African countries by 2020 and globally by 2025. This in turn inspired a global alliance of 22 partners including the WHO, The Bill and Melinda Gates Foundation, World Bank and major pharmaceutical companies to announce through the 2012 ‘London Declaration’ a sustained program to ‘control’ schistosomiasis by 2020 (http://unitingtocombatntds.org). While 42 million PZQ tablets were dispensed in 2012 (World Health Organization, 2014) this number is likely to increase greatly in the near future to meet the immediate goal of disease control. There are significant grounds for concern that drug pressure will increase significantly in the years ahead.

As PZQ is often administered with a significant time lapse measured in months or years between treatments, it can leave a significant reservoir of schistosomes infecting people that are unaffected by the drug. This, combined with continuing exposure of PZQ to the parasite, means the drug can often only provide short-term relief from infection. Despite this drawback, PZQ remains the only readily available treatment for schistosomiasis amid concern that as it becomes more widely dispensed, drug resistance traits may emerge thus removing the most effective, albeit flawed drug from the limited treatment options available.
There have been a number of *in vivo* and *in vitro* studies documenting differential sensitivity of *S. mansoni* isolates to PZQ. For example, a relatively low cure rate was reported during a study of PZQ efficacy and side effects in Senegal in 1991 (Stelma et al., 1995). A subsequent study of a field isolate derived from snails in the same geographical area suggested that, when compared with two isolates from Puerto Rico and Kenya, the Senegal isolate matured in mice at a significantly slower rate thus likely rendering it less susceptible to the drug at the times tested (Fallon et al., 1997).

Ismail et al., (1999) generated 12 *S. mansoni* isolates from patients who had failed to be cured by 3 doses of PZQ that would normally prove effective. These isolates were maintained in mice and 8 were found to have a significantly higher ED$_{50}$ than controls as well as a significantly diminished contractile response *in vitro*.

In a related study, 3 of 6 isolates retained their decreased response to PZQ after several passages through the life cycle in the absence of PZQ (William et al., 2001a). In addition, these isolates had an associated diminished reproductive fitness suggesting reduced PZQ sensitivity may have come with a significant biological cost. In a study using miracidial assays to determine PZQ sensitivity, Lamberton et al., (2010) noted that *S. mansoni* hatched from eggs obtained from the feces of children after two or more PZQ treatments were more likely to survive *in vitro* exposure to the drug compared with those from newly infected children.

Although this may point to a variation in susceptibility of adult *S. mansoni* worm survival to repeated drug treatment, it may also reflect differential PZQ susceptibility to miracidia (the larval schistosome that hatches from eggs) at different stages of their maturity as they pass through the host.
While treatment failure has been reported in travelers returning from areas endemic for *S. haematobium* (Mendonca da Silva *et al.*, 2005; Alonso *et al.*, 2006), a study of individuals infected with *S. haematobium* on Pemba Island, Tanzania found no indication of PZQ resistance as determined by egg counts and miracidial viability after 20 years of mass drug administration (Guidi *et al.*, 2010).

Fallon and Doenhoff (1994) were able to induce PZQ resistance in the laboratory by exposing a pool of *S. mansoni* isolates from Puerto Rico, Brazil, Kenya and Egypt that had been maintained in the laboratory for up to 10 years to increasing sub-curative doses of PZQ. Mice infected with the 6th generation of selected cercariae were then treated with 3 x 300 mg/kg doses at days 28, 35 and 37 after infection, and showed only a 7% reduction in worm burden compared with an 88% reduction in mice infected with non-selected worms.

Cioli *et al.*, (2013) infected mice with *S. mansoni* (LE strain) and treated with PZQ following a protocol based on Fallon and Doenhoff (1994) to generate worms with similarly reduced drug sensitivity. This led to a decrease in genetic heterogeneity suggesting that multi-generational PZQ exposure resulted in reduced population diversity. When schistosomes of the drug-selected strain were bred with those of an unselected strain, the F1 offspring were found to have intermediate PZQ sensitivity suggesting the reduced susceptibility trait was co-dominant (Pica-Mattoccia *et al.*, 2009, Couto *et al.*, 2011) and were able to generate *S. mansoni* with reduced PZQ susceptibility within a single life cycle by feeding PZQ to snails harboring the parasite.

Clearly, while studies of PZQ resistance have to take into account confounding factors such as the rate of schistosome maturity as well as individual variations in drug metabolism and immune competency, the development of reduced susceptibility to PZQ is a real possibility. In the present study the susceptibility of miracidia hatched from eggs derived from fecal
samples of patients living in two regions of Kenya endemic for schistosomiasis was examined \textit{in vitro}.

\textit{Schistosoma mansoni} cercaria obtained from naturally infected \textit{Biomphalaria} snails (the intermediate hosts for \textit{S. mansoni}) was collected from several endemic localities in Kenya to examine \textit{in vitro} susceptibility of \textit{S. mansoni} to PZQ was also done. In addition, an \textit{S. mansoni} isolate maintained through snails and mice was used to generate a laboratory isolate with significantly reduced susceptibility to PZQ using a protocol described by Fallon and Doenhoff (1994)

\subsection{Problem Statement and Justification}

There have been raised concerns that patients infected with schistosomiasis who have been under multiple treatment of the drug praziquantel may have developed reduced susceptibility to the drug and consequent development of resistant worms. The existence of schistosomiasis disease in Kenya has led to heightened efforts to treatment and control measures through the administration of a readily available drug, praziquantel.

The continued widespread and repetitive use PZQ for over 20 years in some African countries and Kenya has caused worries on its efficacy measures to the parasite and selection of drug resistant worms (King \textit{et al.}, 2000; Cioli andPica-Mattoccia, 2003; Hagan \textit{et al.}, 2004; Botros \textit{et al.}, 2006). The determination of whether or not there is a reduction of the susceptibility of \textit{S. mansoni} to Praziquantel is of great significance. These findings will be of great value particularly in future prospects of development drugs targeting the new resistance strain (if any) of \textit{S. mansoni}

\subsection{Alternative hypothesis}

\textit{Schistosoma mansoni} susceptibility to PZQ in infected individuals is altered following repeated treatments
1.4 Null Hypotheses

No reduced susceptibility of *S. mansoni* to PZQ in patients even after multiple treatments with PZQ for isolates derived from infected humans

No development of reduced sensitivity of *S. mansoni* to PZQ in specimens derived from snails from natural infection habitats

No generation of an *S. mansoni* lab strain with reduced susceptibility to PZQ even after exposure to sub-lethal doses of PZQ

1.5 Objectives

1.5.1 General objective

The aim of this research is to investigate if *S. mansoni* susceptibility to PZQ is altered following repeated treatments

1.4.2 Specific Objectives

1. To determine *S. mansoni* sensitivity to PZQ using *in-vitro* assays with parasites obtained from naturally infected individuals

2. To establish *S. mansoni* sensitivity to PZQ from isolates derived from naturally infected *Biomphalaria* snails collected from diverse endemic localities in Kenya.

3. To establish an *S. mansoni* laboratory strain with reduced susceptibility to praziquantel
CHAPTER TWO
LITERATURE REVIEW

2.1 Schistosomiasis

Schistosomiasis is a water-borne parasitic disease that affects more than 249 million people (World Health Organization, 2014) 97% of which are on the African continent (Steinmann et al., 2006) with a global disease burden calculated at 24-56 million disability-adjusted life-years lost (King, 2010). A high proportion of infected populations occur in endemic areas in Kenya as well. The chronic and debilitating nature of the disease results in high costs in public health and economic productivity in developing countries, and prompted the initiation of large scale control programs (Crompton et al., 2003; Fenwick et al., 2003).

Parasitic flatworms of the genus *schistosoma* cause schistosomiasis. Schistosome infections can result in permanent damage to various organs, major morbidity, devastating effects on childhood development and adult productivity and, in some cases, death. The global health burden of schistosomiasis is now considered, in some analyses, to be similar to that of malaria or tuberculosis (King, 2010; Hotez and Fenwick, 2009,). The two major schistosome species that are responsible for causing human schistosomiasis in Kenya are *S. haematobium*, and *S. mansoni*.

Contamination of open water with human excreta containing the parasite's eggs initiates human to-snail transmission when miracidia released from hatching eggs penetrate into the appropriate fresh water snails species, which serve as intermediate hosts. The complete life cycle of schistosomiasis is highlighted in figure 2.1
Figure 1.1: The schistosome life cycle, (1) Egg (2) larval stage-miracidia (3) Intermediate host -snail (4) Sporocyst in snail (5) Larval stage-cercaria (6) definitive host-human (7) Schistosomulae (10) Adult stage-worms (http://www.dpd.cdc.gov/dpdx).

2.2 Schistosomiasis control

Schistosomiasis control has been attempted in several ways: chemotherapy, vector elimination, improved sanitation and health education (WHO, 2010). Chemotherapy has become the key tool in the global strategy against schistosomiasis (Doenhoff et al., 2008).

Although there is no vaccine, the disease can be treated and controlled with praziquantel (PZQ), a drug developed in the 1970s (Magnussen, 2003) and shortly thereafter identified as the treatment of choice by the World Health Organization (Andrews et al., 1983). PZQ is the least expensive, easiest to use and most readily available of all anti-schistosomal drugs (Hagan et al., 2004).

Though new lead anti-schistosomal compounds have been identified (Sayed et al., 2008), no new drugs other than repositioned anti-malarials such as artemisinins (Keiser and Utzinger, 2012) have entered the market since the development of PZQ. Furthermore, due to the success of PZQ, other anti-schistosomal drugs are no longer available in most parts of
the world. Thus, treatment and control of this hugely prevalent disease relies almost entirely on a single drug.

The reasons why PZQ has displaced other older drugs that have been used in the past is because of being effective against all other human schistosome species, while oxamnique (which is still in limited use against S. mansoni infections) and the related drug hycanthone are not (Cioli et al., 1995).

Praziquantel also has relatively mild side effects, is inexpensive, and has proven its value in large-scale schistosomiasis control efforts in a variety of countries (Vennervald et al., 2005; Xianyi et al., 2005; Toure et al., 2008). Efforts to develop a vaccine against schistosomiasis have not been successful and chemotherapy will continue to be the most practical for controlling Schistosomiasis.

2.3 Drug resistance in schistosomes

Reliance on a single drug for any disease of this magnitude is dangerous, as there are few if any alternatives should resistance arise. Thus, even though PZQ has overall proved successful in treatment and control programs, reported failure rates in the field nonetheless may reach as high as 30% (Day and Botros, 2006; Mutapi et al., 2011), and this value could be implicit, as the standard-used Kato-Katz technique for measuring egg counts can underestimate levels of infection and has problems with reliability (Kongs et al., 2008; Lin et al., 2008). Additionally, liver-stage juvenile schistosomes (21 days post infection) are refractory to PZQ, a major concern in regions with high re-infection rates. Worms become fully susceptible only when egg production begins approximately 6 weeks following infection of the mammalian host (Pica-Mottochia et al., 2004; Aragon et al., 2009).
Furthermore, the molecular target of PZQ has not been rigorously defined. Thus, though substantial evidence suggests that PZQ interacts with schistosome voltage-gated Ca\textsuperscript{2+} channels, other molecular targets have also been proposed (Greenberg, 2005; Doenhoff \textit{et al.}, 2008).

Even if the molecular target of PZQ were known however, it is clear that other downstream factors contribute to PZQ action. For example, the juvenile worms that are refractory to PZQ still undergo a Ca\textsuperscript{2+}-dependent contraction and paralysis similar to that observed in adult worms (Pica-Mattoccia \textit{et al.}, 2008). Unlike adults, however, juveniles recover and survive, indicating that though the initial target is likely similar, adaptive responses that allow parasite survival come into play in the immature, but not mature worms (Hines \textit{et al.}, 2012).

Though there is as of yet no indication of widespread drug resistance, researchers have identified field and laboratory isolates that exhibit significantly reduced susceptibility to PZQ (Botros \textit{et al.}, 2006; Doenhoff and Pica-Mattoccia, 2006; Melman \textit{et al.}, 2009), possibly a precursor for emergence of more widespread drug resistance.

Other than resistance, there are other factors that can increase to failure rate which includes compromised health and immune-competency of the host (Day and Botros, 2006). One of the factors suggested to account for persistence of infections following PZQ treatment is the reduced susceptibility of juvenile parasites to the drug (Cioli and Pica-Mattoccia, 2003).

Recent infections will contain a significant portion of PZQ-refractory juvenile worms, leading to less than optimal cure rates. Thus, worms that persist following PZQ treatment were shown to have genotypes that do not differ significantly from susceptible worms, and therefore do not appear to represent a sub-population selected for PZQ resistance (Blanton
et al., 2011). Despite these caveats, there are nonetheless several reports of possible PZQ resistance in schistosomes, both in the laboratory and in the field (Cioli, 2000; Day and Botros, 2006; Doenhoff et al., 2008; Wang et al., 2012).

2.4 Experimentally-Induced PZQ Resistance

Fallon and Doenhoff (1994) exploited an approach to select for resistance to PZQ in *S. mansoni* whereby, a sub-curative, but increasing PZQ doses were administered to *S. mansoni*-infected mice over seven passages through the life cycle. By the seventh life cycle passage, this PZQ drug pressure produced a population of schistosomes in which 93% of the worms survived a PZQ dose that killed 89% of control, unselected worms.

Experimentally-induced PZQ resistance has been reported in *Schistosoma japonicum* using a similar approach (Liang et al., 2011). A notable recent advance in obtaining PZQ resistant schistosomes uses drug selection on the asexual stages of the life cycle in the snail host (Couto et al., 2011). The technique derives from observations showing that treating *S. mansoni* infected *Biomphalaria glabrata* snails with 1000 mg/kg PZQ interrupts almost 90% of cercarial shedding (Mattos et al., 2007).

Based on that finding, a study of successive treatments of *B. glabrata* infected with *S. mansoni* (LE strain) with the far lower dose of 100 mg/kg PZQ to select for cercariae that, upon infection of mice, developed into adult worms with a significantly reduced susceptibility to PZQ. The ED$_{50}$ of PZQ for these LE-PZQ worms in mice was approximately five-fold higher than that Robert M. Greenberg 2 for the parental LE strain (362 mg/kg for LE-PZQ versus 68 mg/kg for LE) (Couto et al., 2011).

Following PZQ, LE-PZQ worms were also less contracted than LE worms and, as assayed by fluorescent probes, showed less severe tegumental damage and, unlike LE worms,
appeared to retain a functional excretory system (Couto et al., 2010). The ability to use this approach to select for drug resistance at the snail stage is far less costly and labour intensive than previous strategies of applying drug pressure through multiple intra-mammalian stage passages. It holds the promise of much more readily providing new drug-resistant isolates that will be useful for studying the mechanisms of PZQ resistance.

2.5 Resistance in field isolates

There have been several past reports of schistosome field isolates exhibiting reduced PZQ susceptibility. In Northern Senegal, lower than expected cure rates were initially reported in the 1990s for S. mansoni infections treated with PZQ. Alarmingly, cure rates were reported to be as low as 18% (Gryseels et al., 1994; Stelma et al., 1995).

Subsequent follow-up studies and analysis of the data (Fallon, 1998; Danso-Appiah and De Blas, 2002) suggested that some portion (though not all) of this drug failure could be attributed to factors other than drug resistance, including high-intensity infection, rapid re-infection and transmission, presence of PZQ-refractory juvenile worms, variations in methodology for analysis of efficacy, and perhaps native tolerance of these schistosomes. Interestingly, in patients relocated to urban areas (which do not have ongoing transmission), cure rates rose to near-normal levels (William et al., 2001b).

Another site for intense study of potential PZQ resistance has been in Egypt, where schistosomes were isolated from several S. mansoni-infected patients (1·6% of those screened) who continued to pass viable eggs following three successive doses of PZQ (Ismail et al., 1996). The schistosomes isolated from these patients were subsequently propagated in mice, where they showed 3–5-fold lower sensitivity to PZQ, as measured by ED$_{50}$ (Ismail et al., 1996; Ismail et al., 1999).
Tests of known responses of worms to PZQ \textit{in-vitro} (i.e. in the absence of any confounding host factors) showed that at least some of these isolates were less susceptible to the drug (Ismail \textit{et al.}, 1999; Silva \textit{et al.}, 2005). Indeed, in vitro measures of PZQ susceptibility correlated well in some cases with ED$_{50}$ determinations in murine infections.

Further evidence for isolates showing PZQ insusceptibility has been found in Kenya. Researchers used an \textit{in vitro} assay on miracidia hatched from eggs excreted by \textit{S. mansoni}-infected Kenyan car washers to screen for \textit{S. mansoni} exhibiting decreased susceptibility to PZQ (Melman \textit{et al.}, 2009). Different patients produced eggs that hatched into miracidia with variable PZQ sensitivity (as measured by miracidial killing); miracidia from previously-treated patients showed significantly lower sensitivity to the drug.

Further characterization of an isolate from a patient who was never fully cured by PZQ (KCW) revealed that adult worms derived from these eggs were less sensitive to PZQ, both \textit{in-vivo}, in murine infections and \textit{in-vitro}, as assayed by schistosome length. Isolated incidents of failure of PZQ to cure \textit{S. haematobium} infections have been reported, including a notable case in which PZQ failed to cure Brazilian soldiers returning from Africa (Herwaldt \textit{et al.}, 1995), though there is currently no evidence for heritable resistance (Alonso \textit{et al.} 2006).

Evidence for drug failure has also been found in other trematodes, of particular interest is the liver fluke \textit{Fasciola hepatica}. Though not particularly susceptible to PZQ, \textit{F. hepatica} can be treated quite effectively with other compounds such as the benzimidazoles, which target β-tubulin, and are most frequently used as anti-nematodals. The benzimidazole triclabendazole (TCBZ) is effective against both immature and mature flukes (Moll \textit{et al.}, 2000) and has seen widespread use since its introduction. Recent reports have suggested the localized emergence of TCBZ-resistant fluke isolates (Fairweather, 2011) and work
described below has focused on defining the underlying source of resistance in one of these TCBZ-resistant isolates.

2.6 Mechanisms of resistance

As opposed to tolerance, which represents an innate lack of susceptibility that is not in response to prior drug exposure, drug resistance is a heritable increase in the frequency of individuals in a population able to endure doses of a compound following exposure of that population to the drug (Kwa et al., 1995; Coles and Kinoti, 1997). Drug resistance in schistosomes therefore depends on the selective pressure of drug exposure, and is heritable.

Resistance to a single class of drugs can arise via several mechanisms. The most obvious is target modification. For example, benzimidazoles such as albendazole act to inhibit microtubule polymerization; in nematodes and fungi, resistance has been mapped to a F200Y point mutation in β-tubulin (Dent et al., 2000). Similarly, simultaneous point mutations in three glutamate-gated chloride channel α-type subunits in Caenorhabditis elegans confer resistance (4000-fold) to the antiparasitic drug ivermectin (IVM) in these worms (Cioli and Pica-Mattoccia, 1984). Interestingly, mutations in the Dyf (dye filling defective) class of genes, which appear to affect cuticle permeability, produce moderate IVM resistance (2–5-fold) and act additively to increase resistance of the channel mutations. One of the more instructive cases of such non target-dependent development of resistance comes from studies on schistosome resistance to oxamniquine (Cioli and Pica-Mattoccia, 1984).

As noted above, oxamniquine is highly effective against S. mansoni, but lacks activity against other human schistosomes such as S. haematobium and S. japonicum (hyxanthone, a related antischistosomal compound, is active against S. mansoni and S. haematobium, but not S. japonicum). In a series of elegant and challenging experiments using genetic crosses
of drug-sensitive and drug-resistant schistosomes, Cioli Donato and colleagues showed that oxamniquine/hycanthone resistance in these worms was controlled by a single autosomal recessive gene. They also showed that the anti-schistosomal activity of the drug requires biotransformation to an active form by a parasite sulfotransferase. When activated, the drug is thought to act as an alkylating agent of schistosome DNA and other macromolecules, interfering with nucleic acid synthesis (Cioli et al., 1992; Pica-Mattoccia, 1992; Cioli et al., 1993;). The drug is inactive against schistosome species that lack this sulfotransferase activity and drug resistance can arise when this activity is lost in species that normally express it (Pica-Mattoccia et al., 1997; Nogi et al. 2009).

With regard to PZQ failure, the fact that the PZQ target has not been rigorously defined makes the search for differences more problematic. However, no clear changes in candidate targets have been found to date. Thus, voltage-gated Ca\(^{2+}\) (Cav) channel β subunits have been implicated in PZQ action (Valle et al., 2003; Greenberg, 2005), but an examination of Cav channel β subunits in different isolates showing reduced PZQ susceptibility revealed no meaningful sequence differences or changes in expression levels (Zhang et al., 2011). On the other hand, reducing Cav channel subunit levels in the planarian Dugesia japonica confers resistance to these free living platyhelminths against PZQ-elicited dramatic disruptions of normal regeneration patterns (Fallon et al., 1997; Valle et al., 2003).

There are also several non-target-based changes that could alter PZQ effectiveness. For example, as noted above, juvenile schistosomes are refractory to PZQ. Additionally, adult female schistosomes, though still PZQ-sensitive, are more tolerant of the drug than adult males (Pica-Mottiechia and Coili, 2004). Thus, changes in worm maturation rates (Vokřál et al., 2012) or sex ratios could influence the effectiveness of PZQ.
Interestingly, a study indicated that two other platyhelminths (the trematode *Dicrocoelium dendriticum* and the cestode *Hymenolepis nana*) are not capable of enzymatically metabolizing PZQ (Pereira *et al*., 1998). Acknowledging the caveat that schistosomes may differ from these other platyhelminths, these results nonetheless suggest that development of more efficient PZQ metabolism by the parasite is not a particularly likely scenario for acquisition of PZQ resistance.

Molecular differences associated with reduced PZQ susceptibility in schistosomes would provide useful markers to monitor emergence of resistance in drug administration programmes. They could also serve as entrées into understanding how resistance develops and provide insights into the mechanism of drug action. There have been a handful of attempts to define such molecular correlates of PZQ resistance. For example, subtractive PCR and cloning of differentially-expressed RNAs revealed higher levels of an RNA encoding subunit 1 of mitochondrial cytochrome c-oxidase (*SCOX1*) in schistosomes selected for reduced PZQ susceptibility (Pommier *et al*., 1999).

Analysis by semi-quantitative RT-PCR confirmed that the *SCOX1* RNA was expressed at 5–10-fold higher levels in the resistant worms than in a PZQ-sensitive strain. Interestingly, no differences were found in expression of RNAs encoding SMDR2, a schistosome multidrug transporter (see below), nor NADH dehydrogenase subunit 5, another mitochondrial gene. Surprisingly, however, the enzymatic activity of cytochrome c-oxidase showed an expression pattern opposite to that found for the *SCOX1* RNA.

Multidrug transporters underlie multidrug resistance (MDR), a phenomenon in which resistance to a single drug is accompanied by unexpected cross-resistance to several structurally unrelated compounds. Multidrug transporters have broad substrate specificity and actively remove xenobiotics and toxic compounds, including drugs, from cells and
tissues, though non-transport-related MDR can also occur (Blackmore et al., 2001; Pommeir et al., 2004). Genes for multidrug transporters are found in all living cells (Paulsen, 2003), and are classified into five basic families (Higgins, 2007; Van Veen, 2010). The crystal structure of at least one representative of each of these families has been solved (Borst and Elferink, 2002).

MDR is linked to gene amplification, overexpression or mutation of Pgp or other multidrug transporters, resulting in increased drug efflux. In addition to Pgp, known ABC proteins involved in MDR include the multidrug resistance associated proteins (MRPs; ABCCs), breast cancer resistance protein (BCRP; ABCG2), as well as others. (Ambudkar et al., 2003; Szakacs et al., 2006)
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area and Parasite Collection Sites

The study was primarily conducted in Western Kenya, specifically along the shores of Lake Victoria near Kisumu town (Plate 3.1), Mwea central Kenya, within the Mwea Rice Irrigation Scheme area (Plate 3.2) and Kibwezi area where S. mansoni, the causative agent of schistosomiasis is endemic in these areas.

The inhabitants of Kisumu, depend on Lake Victoria for their livelihood, those in Mwea are mainly subsistence farmers whereas those in Kibwezi depend on dam water for domestic use. The study areas were selected based on preliminary evidence of human water contact activities such as car washing, sand harvesting, rice farming, water collection for domestic use, bathing, and swimming.

Plate 3.1: Car Wash and Usoma along the shores of Lake Victoria in Kisumu County in Kenya where samples used in present study were collected (Google Earth 0°05'41.32" S, 34°44'19.28" E).
Plate 3.2: Mukou, Thiba and Mbui Njiru Primary schools in Mwea, Kirinyaga County, Kenya where samples used in present study were collected (Google Earth 0°41'20.40"S, 37°20'32.00" E).

3.2 Study Population

Participants in this study were school children (in Mwea) and adults (in Kisumu). Persons working as car washers along the shores of Lake Victoria near Kisumu (Plate. 3.3) are exposed to schistosomes as they stand ankle-to-knee-deep in the lake to wash cars that have been driven into the shallow water at the edge of the lake, while sand harvesters are exposed to schistosomes as they stand up to shoulder deep in the lake, shoveling sand from the bottom onto boats, and unloading it on the shore.

Both groups spend significant periods of time in the water of Lake Victoria or the surrounding streams as part of their occupation. All adults enrolled for this study came from one of these two groups and all had previously been treated with PZQ on several occasions, with the year of last treatment being between 2006 and 2013.
Men working at the Car wash site (00° 05' 44.75"S, 034° 44' 57.93"E) on the shores of Lake Victoria have been followed continuously since June 1995 as part of several longitudinal studies designed to study resistance to *S. mansoni* infection, and the sand harvesters have been followed continuously since February 2005 (Vennervald et al., 2005).

Plate 3.3: Lake Victoria, Kisumu where snails and fecal samples from car washers and sand harvesters were collect for the study (1) map of Kenya highlighting Kisumu-Study area (2) washing of vehicles on the shows of Lake Victoria in Kisumu (3) woman harvesting sand on the shows of Lake Victoria (4) men shoveling sand onto boats.

From 2004 to 2007, collaboration between the Kenya Medical Research Institute (KEMRI) and Japan International Cooperation Agency administered a school-based schistosomiasis and soil-transmitted helminth control project in Mwea, central Kenya (Plate. 3.4).

The project dispensed annual doses of de-wormers including PZQ to all school-aged children in the region regardless of their infection status. Thereafter, the National Deworming Program took over the control activities, and has continued with annual treatment of children since 2012 to date.
In this study, enrolled school children were between 5 and 16 years of age, and came from three primary schools: Mukou (00° 40' 54"S, 037° 20' 36"E, altitude 1098M), Thiba (00° 41' 12.42"S, 037° 20' 47.76"E) and Mbui Njeru (00° 42' 07"S, 037° 20' 42.89"E).

Plate 3.4: Mwea rice scheme (Central Kenya) where snails and fecal samples from school children were collected (1) pupils who formed part of study subjects (2) map of Kenya showing Location of Mwea-study area (3) packaged rice, the major cash crop product in Mwea (4) rice paddies.

3.3 Ethical Considerations

All ethical considerations were observed as described in the “informed consent explanation”: appendix VIII, while all adults and parents or guardians of the children involved in this study were provided with informed consent documents (Appendix IX, X & XI).

3.3.1 Inclusion criteria

Schistosoma infected individuals after diagnosis with Kato Katz technique. Written informed consent; able and willing to be examined on follow-up visits and to provide stool samples.
Those who satisfied the inclusion criteria were further tested for the following exclusion criteria.

### 3.3.2 Exclusion criteria

Not willing to be re-examined on follow-up visits and to provide stool samples, individuals with previous history of adverse reaction associated with praziquantel and children of less age 4 years. The World Health Organization (WHO) and pharmaceutical sector consider treatment with PZQ as being safe for children as young as four years of age, below this age limit is not yet fully endorsed for ‘off-label’ use of PZQ. (WHO, 2002)

### 3.4 Study Approvals

This study is a sub-study of a mother study By Dr. Charles Cunningham SSC#2227 entitle “Understanding the biology of schistosomes in response to praziquantel” with approvals from The KEMRI Scientific, Ethical and animal and use commitees (Appendix V, VI& VII), Kenyatta University (Appendix II) Institutional Review Boards as well obtained a research permit from National Council of Science Technology and Innovation-NACOSTI (Appendix III &IV) who approved this study.

### 3.5 Study Design

This was an experimental study in which mortality of *S. mansoni* miracidia and cercariae with a history of previous multiple exposures to PZQ (experimental group) in an *in vitro* assay was compared with that of a sample of the same parasite isolate not exposed to PZQ.

### 3.6 Parasite Isolates

Human faecal samples were collected from *S. mansoni* infected individuals (school children and adults) in Mwea and Kisumu and their immediate environs, or cercaria from naturally
infected *Biomphalaria* snails collected from the lakeshore, streams and other water bodies in the environs. Fecal samples were used to isolate *S. mansoni* miracidia, and the field-obtained snails were used as a source of cercariae for PZQ sensitivity testing in an *in vitro* assay.

### 3.7 Collection of fecal samples for *S. mansoni* infection

Fecal samples were collected and prepared for examination by microscopy for *S. mansoni* infection. The fecal sample was prepared on a microscope slide using the modified Kato-Katz procedure described by Katz and Pellegrino (1972), and examined under a compound microscope for the presence of schistosome eggs.

Briefly, fecal sample was passed through a fine sieve (110µm), than collected using a spatula. A template with a hole is then placed on a microscope glass slide where the sieved feces are carefully filled. The template is then removed and cellophane strip soaked in malachite green stain is then placed on the fecal sample and inverted onto a blotting paper where gentle pressure is applied to spread the fecal sample to a disk of about ½ inches in diameter. Schistosome eggs are then systematical searched under X10 magnification compound microscope. The number of eggs observed is then multiplied by 24 to get the number of eggs per gram of feces.

Fecal samples of individuals, who tested positive for *S. mansoni* infection, were then used to isolate miracidia for testing PZQ sensitivity *in vitro*. Hatching of miracidia was done by putting harvested schistosomes egg into a 1 litre of fresh water and allowing standing for 1 hour in a conical flask under natural light. Free swimming miracidia is observed by first drawing water from the top part of flask into a petridish and observing under a dissection microscope X10 magnification.
3.8 PZQ sensitivity assay of miracidia derived from fecal samples

Miracidial sensitivity to PZQ was tested *in vitro* using a modified version of the technique developed by Liang *et al.*, (2001). Freshly hatched miracidia derived from stools of sand harvesters were placed in each well (4-6 miracidia per well) of a 96-well micro-litre plate in 40μl tap water. Each row represented a single group of miracidia and received either 0, 10^{-6} M or 10^{-5} M PZQ. PZQ was prepared as a stock solution of 10^{-4} M in 1 % DMSO and the final concentration of DMSO was 0.1% in all wells including the control. Miracidia were observed with a dissecting microscope prior to (at start) 10 min, and 20 min after the addition of PZQ.

The number of dead miracidia was then recorded blind (without knowledge of the miracidial source or PZQ concentration used). Miracidia were assumed dead if they remained immobile. The percentage of miracidia mortality after treatment after 10 and 20 min with 10^{-6} and 10^{-5} M PZQ was calculated using Turn Math Jaxon formula as follows:

\[
\frac{(X \text{ live miracidia } CG) - (X \text{ Live miracidia } TG) \times 100}{X \text{ live miracidia } CG}
\]

Where; X = Sample mean  
  CG = Control Group  
  TG = Treatment group

3.9 PZQ sensitivity of miracidia set against number of patient treatments undergone

A new fecal sample was obtained from individuals who tested positive for *S. mansoni* infection, and used to isolate schistosome eggs. Miracidia was then hatched from schistosome eggs isolated from naturally infected individuals. Prazuquantel sensitivity testing was done using miracidia hatched from schistosome eggs.
3.10 PZQ sensitivity of *S. mansoni* cercaria derived from wild *Biomphalaria* snails

*Biomphalaria* spp. snails were collected from schistosome endemic localities where water habitats (streams, ponds, rice paddies, lakeshore, etc) known to be endemic for schistosomiasis. Collection of snail was done using standard snail scoop. Collected snails were then screened for *S. mansoni* by the “shedding procedure” whereby, Individual snails were placed in wells of a 24-well culture plates in 1 ml of distilled or mineral water and placed plates under light for 1-2 hours to allow the snails to shed cercariae. Snails in the culture plate wells were then examined under a dissecting microscope for presence of released cercariae.

Cercariae released were then used to infect lab mice. At 49 days post-infection, *S. mansoni* eggs were obtained from the mouse livers and hatched in tap water. The PZQ sensitivity of hatched miracidia was done using *in-vitro* assays as outlined above (section 3.8) by Liang *et al.*, (2001) technique. The percentage of miracidia mortality after treatment for 10 and 20 min with $10^{-6}$ and $10^{-5}$ M PZQ was calculated using Turn Math Jaxon formula like in section 3.8 above:

3.11 Generation of *S. mansoni* laboratory strain with reduced PZQ susceptibility

Miracidia were hatched from eggs retrieved from fecal samples obtained from 7 car washers and combined. This pool was used to infect *B. sudanica*. Thereafter, cercariae shed were subsequently used to infect out-bred mice.

An isolate with reduced sensitivity to PZQ was set out by exposure to sub-curative doses of PZQ which were increased gradually as described by Fallon and Doenhoff (1994) with the following differences. Briefly, 10 out-bred mice were infected with approximately 100 cercariae and randomly distributed into two groups and treated with either 100 mg/kg/day
PZQ in 2.5% Cremaphor EL (Sigma, USA) (n=5) or an equivalent volume of 2.5% Cremaphor EL vehicle alone (n=5) on each of days 28 and 35 post infection.

Three weeks after the final dose of PZQ or vehicle, mice were perfused with RPMI medium (Radke et al., 1971) and the number of worms counted. Statistical analysis of differences in worm yield from PZQ treated and vehicle treated mice was calculated using an unpaired Student’s t-test assuming equal variance.

For each passage, eggs from the liver that survived PZQ treatment (selected) or vehicle treatment (non-selected) were used to infect B. sudanica nails and the cercariae subsequently used to infect the next generation of mice. This protocol was repeated for the second passage with the dose of PZQ being increased to 200 and 250 mg/kg/day for the third and fourth passages. Mice in the 5th passage received 300 mg/kg/day on days 28, 35 and 37 post-infection.

Praziquantel sensitivity of hatched miracidia obtained after each passage was assessed using the protocol described above. Approximately 6 drug-selected miracidia were placed in each of 48 wells of a 96 well micro titre plate. Twenty four wells were treated with 10^{-5} M PZQ and an additional 24 with the same volume of PZQ vehicle and observed blind after 0 (pre-treatment control), 10 and 20 min. Non-selected miracidia were treated identically.

The percentage of drug selected or non-selected miracidia surviving at each time point after treatment was calculated using Turn Math Jaxon formula in section 3.8

3.12 Statistical analysis

Data was collected and collated using a computer with Microsoft Excel and Word software.

Results were graphed to show the PZQ sensitivity of each isolate under test to PZQ.
The percentage of miracidia mortality after treatment for 10 and 20 min with $10^{-6}$ and $10^{-5}$ M PZQ was calculated using Turn Math Jaxon formula as follows:

$$\frac{(X \text{ live miracidia } CG) - (X \text{ Live miracidia } TG) \times 100}{X \text{ live miracidia } CG}$$

Where: $X$ = Sample mean  
  $CG$ = Control Group  
  $TG$ = Treatment group

Statistical analysis to compare miracidial survival/sensitivity based on number of PZQ treatments (low or high) of patients was performed using an unpaired Student’s $t$-test assuming unequal variance.

For miracidia derived from human subjects, the influence of previous treatment with PZQ on miracidial sensitivity was compared using regression analysis.

Analysis to compare wild miracidial survival/sensitivity compared with lab strain (untreated samples) was performed using an unpaired Student’s $t$-test assuming unequal variance.

The efficacy of $in$ $vivo$ treatment with PZQ was measured as the percent of reduction of worm burden based on the numbers recovered (Melman et al., 2009).

With respect to adult schistosome worms, the mean number of worms recovered by perfusion for each isolate after PZQ treatment was analyzed and graphed using Microsoft Excel.

Efficacy of $in$ $vivo$ treatment with PZQ as applied to the different isolates was measured as the percent of reduction of worm burdens based on the formula:

$$\% \text{ worm burden} = \frac{(X \text{ worm count } CG) - (X \text{ worm count } TG) \times 100}{X \text{ worm count } CG}$$

Where: $X$ = Sample mean  
  $CG$ = Control Group  
  $TG$ = Treatment group
Data analysis was conducted using Minitab version 17 statistical software.

95% Confidence Interval (CI) were used to estimate the strength of association between independent variables and each of the dependent variables. A $P$-value less than 0.05 were considered statistically significant.
CHAPTER FOUR

RESULTS

4.1 Study Parasite Isolates

A total of 72 isolates of *S. mansoni* from infected individuals with a history of multiple treatments with praziquantel were analysed for PZQ sensitivity in the *in-vitro* assay in the present study, 34 from school children in 3 primary schools in Mwea, and 38 from adults in 2 sites in Kisumu. Table 4.1 provides detailed results for each study school or location. Similarly, 38 isolates of *S. mansoni* from field-collected *Biomphalaria* snails were analysed for PZQ sensitivity in the assay, 2 from habitats in Mwea, 21 from habitats in Kisumu and 7 from habitats in Kibwezi. Table 4.2 provides details of the snail collection sites, habitat types, and number of isolates from each collection habitat.

4.2 PZQ susceptibility assay of miracidia derived from fecal samples

Fecal samples were obtained from individuals with a history of treatment with PZQ. For the purpose of a direct control, it was not possible to identify individuals within our patient groups who had not received PZQ previously. The number of patients sampled and history of treatment for schistosomiasis with praziquantel of all the subjects from 5 different locations are shown in table 4.1.

Of the 72 samples examined, there were no significant differences in cure rates among the 5 villages (P >0.05) and no differences between either age groups among different intensity of infection groups (all P values >0.05). Irrespective of the patient group the mean miracidial mortality of *S. mansoni* at $10^{-5}$ M PZQ was between 81.5 and 84.6 % with the lowest observed value of miracidia from a single patient being 72.7 % (Column 2 Table 4.1).
Table 4.1: *In vitro* susceptibility to PZQ of *S. mansoni* miracidia derived from eggs from patient fecal samples. Data shown as mean ± 1 SD. (*, p<0.05),

<table>
<thead>
<tr>
<th>Patient group and location</th>
<th>No. of patients sampled</th>
<th>Mean No. of PZQ treatments per patient (range)</th>
<th>Mean % miracidial mortality ± SD at 20 min</th>
<th>P value for 10⁻³M PZQ at 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults Car Wash-Kisumu</td>
<td>14</td>
<td>8.4 (3-20)</td>
<td>26.1±5.5 (17.7-33.3)</td>
<td>0.565</td>
</tr>
<tr>
<td>Adults Sand harvesters-Kisumu</td>
<td>24</td>
<td>4.7 (1-11)</td>
<td>21.4±3.1 (17.2-25.3)</td>
<td>0.354</td>
</tr>
<tr>
<td>Children Mwea-Mbui Njeru</td>
<td>11</td>
<td>3.8 (1-5)</td>
<td>27.4±5.0 (19.4-32.1)</td>
<td>0.683</td>
</tr>
<tr>
<td>Children Mwea-Mukou</td>
<td>18</td>
<td>3.3 (1-5)</td>
<td>27.0±5.4 (19.4-39.8)</td>
<td>0.643</td>
</tr>
<tr>
<td>Children Mwea-Thiba</td>
<td>5</td>
<td>3.8 (2-5)</td>
<td>25.4±4.7 (21.0-32.5)</td>
<td>0.403</td>
</tr>
<tr>
<td>Neg Control (PZQ sensitive isolate) Lab</td>
<td>0</td>
<td>23.7±3.3 (20.4-27.0)</td>
<td>82.7±2.4 (80.3-85.1)</td>
<td>0.5</td>
</tr>
<tr>
<td>Pos control (PZQ tolerant isolate) Lab</td>
<td>5</td>
<td>17.5±2.2 (15.3-19.7)</td>
<td>60.1±4 (56.1-64.1)</td>
<td>0.005*</td>
</tr>
</tbody>
</table>

4.3 **PZQ sensitivity versus number of previous treatments**

Determination of PZQ sensitivity of miracidia obtained from human fecal samples in relation to number of previous treatments undergone by sample donor was carried out. For the car washers and sand harvesters, the last treatment dates were between 1 and 9 years before the current study with a mean miracidial mortality of *S. mansoni* at 10⁻⁵M PZQ between 81.5 and 84.6% where $P>0.05$ (Table 4.1). In addition, there was no indication of reduced miracidial susceptibility to PZQ among samples obtained from the 34 school children that previously had between one and five PZQ treatments (one treatment per year) as part of the Kenyan National De-worming Program $P>0.05$ (Table 4.1). There was no indication of a correlation between the number of PZQ doses an individual has received and loss of PZQ sensitivity associated with miracidia (Figure 4.1).
Figure 4.1: Correlation between the number of PZQ treatment received versus PZQ sensitivity associated with miracidia among study participants (1) Car washers, (2) Sand harvesters, (3) Children and (4) all data sets) where P > 0.05.

4.4 PZQ sensitivity of *S. mansoni* cercaria derived from wild *Biomphalaria* snails

This study examined variation in PZQ sensitivity of *S. mansoni* miracidia obtained from mice infected with cercariae derived from naturally infected snail populations (schistosome endemic localities).

When miracidia derived from mice infected with cercariae from naturally infected snails collected in Kisumu, Mwea and Kibwezi areas were tested, the mean miracidial mortality at $10^{-5}$ M PZQ was between 75.8% and 90.3%. All the P > 0.05 where P= 0.45(mortality at $10^{-5}$ M PZQ = 83.05 ± 7.25 %; range = 75.8 – 90.3 %) data represented in table 4.2.
Table 4.2: *In vitro* susceptibility of wild *S. mansoni* miracidia to PZQ isolates derived from schistosome endemic localities in Kenya. Data shown as mean ± 1 SD. (*) p<0.05) Figure in bracket () represents the number of experiments done on each isolate.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Isolate Locality</th>
<th># of snail pool for cercaria</th>
<th>Miracidia mortality at 20 min</th>
<th>P value for 10^-6 MPZQ at 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(# of repeats)</td>
<td>10^6 MPZQ at 20 min (# of repeats)</td>
<td>10^-6 MPZQ at 20 min ( # of repeats)</td>
</tr>
<tr>
<td>CW1</td>
<td>Car wash-Kisumu</td>
<td>4</td>
<td>26.4% (24)</td>
<td>90.3% ± 3.3 (24)</td>
</tr>
<tr>
<td>CW2</td>
<td>Car wash-Kisumu</td>
<td>5</td>
<td>22.8% (24)</td>
<td>78.2% ± 4 (24)</td>
</tr>
<tr>
<td>MK1</td>
<td>Mukou stream-Mwea</td>
<td>3</td>
<td>28.8% (24)</td>
<td>87.2% ± 3.8 (24)</td>
</tr>
<tr>
<td>ASA1</td>
<td>Asao-Kisumu</td>
<td>6</td>
<td>22.3% (24)</td>
<td>76.3% ± 5 (24)</td>
</tr>
<tr>
<td>CW3</td>
<td>Car wash-Kisumu</td>
<td>3</td>
<td>25.0% (24)</td>
<td>81.0% ± 5 (24)</td>
</tr>
<tr>
<td>KB1</td>
<td>Kibwezi</td>
<td>7</td>
<td>24.9% (24)</td>
<td>83.1% ± 2 (24)</td>
</tr>
<tr>
<td>MK2</td>
<td>Mukou-Mwea</td>
<td>4</td>
<td>28.1 (24)</td>
<td>82.6% ± 3 (24)</td>
</tr>
<tr>
<td>CW4</td>
<td>Car wash-Kisumu</td>
<td>3</td>
<td>32.7 (24)</td>
<td>88.4% ± 2 (24)</td>
</tr>
<tr>
<td>ASA1</td>
<td>Asao-Kisumu</td>
<td>3</td>
<td>22.8% (24)</td>
<td>86.5% ± 1 (24)</td>
</tr>
<tr>
<td>NRS1</td>
<td>Nice R. stream-Mwea</td>
<td>3</td>
<td>27.2% (24)</td>
<td>83.4% ± 4 (24)</td>
</tr>
<tr>
<td>KZ2</td>
<td>Kibwezi</td>
<td>8</td>
<td>19.2% (24)</td>
<td>75.8% ± 4 (24)</td>
</tr>
<tr>
<td>Neg control</td>
<td>Lab isolate</td>
<td>10</td>
<td>23.7% (24)</td>
<td>82.7% ± 2.3 (24)</td>
</tr>
<tr>
<td>Pos control</td>
<td>Lab isolate</td>
<td>10</td>
<td>17.5% (24)</td>
<td>60.1% ± 4 (24)</td>
</tr>
</tbody>
</table>

4.5 Generation of *S. mansoni* laboratory strain with reduced PZQ susceptibility

In order to determine if this population still harbored the potential to generate *S. mansoni* with reduced PZQ sensitivity, a mouse infection model was used to study the impact of increasing amounts of PZQ on a population of parasites derived from 7 car washers.

During the first and second passages, mice were treated with 100 mg/kg PZQ on days 28 and 35 after infection. After the first passage there was a small but significant fall in the number of worms recovered after treatment with 100 mg/kg PZQ, parasite drug interaction might have had a lethal effect causing reduction in worm numbers. This is compared to vehicle treated controls where an increase in the male to female ratio was from 1.8 to 3.0(Table 4.3).
During passage 5, 3 x 300 mg/kg PZQ was administered to mice on days 28, 35 and 37 (lethal dose of PZQ) after infection with no effect on worm numbers or sex ratio compared with vehicle treated controls. In contrast, treatment of mice infected with non-PZQ selected *S. mansoni* during passages 3, 4 and 5 with 2 x 200, 2 x 250 and 3 x 300 mg/kg PZQ respectively resulted in 36%, 66% and 86% reductions in worm numbers compared to untreated mice group.

**Table 4.3:** Worms recovered from infected mice after treatment with PZQ during 5 passages of *S. mansoni*. Data shown as mean ± standard deviation. * P< 0.05.

<table>
<thead>
<tr>
<th>Passage No.</th>
<th>PZQ treatment Mg/kg</th>
<th>No. of worms Mean ± SD</th>
<th>p value 10^5 MPZQ</th>
<th>Male: female ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 x Vehicle</td>
<td>48.0 ± 5.9*</td>
<td>≥0.005</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>2 x 100</td>
<td>37.2 ± 5.2</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>2 x Vehicle</td>
<td>43.8 ± 7.7</td>
<td>≥0.31</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>2 x 100</td>
<td>41.0 ± 7.6</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>2 x Vehicle</td>
<td>43.2 ± 4.6</td>
<td>≥0.11</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2 x 200</td>
<td>36.4 ± 8.6</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>2 x Vehicle</td>
<td>53.4 ± 4.2</td>
<td>≥0.17</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2 x 250</td>
<td>49.6 ± 4.6</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>3 x Vehicle</td>
<td>51.4 ± 1.7</td>
<td>≥0.61</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>3 x 300</td>
<td>50.2 ± 7.8</td>
<td></td>
<td>1.6</td>
</tr>
</tbody>
</table>

Miracidia hatched from livers of PZQ and vehicle treated mice during each passage were assayed for their ability to survive PZQ treatment *in vitro* (figure. 4.2) data suggests that miracidia produced by drug-selected *S. mansoni* are also less susceptible to the drug, especially during the 4th and 5th passage.

*In vitro* sensitivity of miracidia to PZQ at 10^-5^ M PZQ in killing PZQ selected and non-selected *S. mansoni* miracidia over 5 passages is described in figure 4.2 where the selected (parasite challenge with PZQ) is compared with negative control/non-selected (no drug used). The survival of the selected and non-selected miracidia is indicated at 10 min and 20 min time points and is calculated as a percentage of non-selected group at both time points.
Figure 4.2: *In vitro* efficacy of $10^{-5}$ M PZQ in killing of selected and non-selected *S. mansoni* miracidia. The survival of the selected (■) and non-selected (♢) miracidia treated with $10^{-5}$ M PZQ is indicated. Data shown as mean ±1 SD where; (*p < 0.05; **p < 0.01; ***p < 0.001)
5.1 DISCUSSION

This study investigated praziquantel sensitivity in *S. mansoni* isolates from infected individuals with a history of repeated treatments with PZQ, and field collected snails from 3 geographical locations in Kenya known to be endemic for schistosomiasis, Mwea (central Kenya), Kisumu (western Kenya) and Kibwezi (Eastern Kenya). These areas are particularly interesting for this study because PZQ has been used from time to time in these localities since 2002 or before (Karanja *et al.*, 2002). With concerns about the possible emergence of PZQ resistance or insusceptibility, this study was timely.

In a relatively recent study in which PZQ sensitivity of *S. mansoni* miracidia hatched from feces of adult Kenya car washer and sand harvesters was measured, it was observed that there was a 2.42-fold increase in the chance that miracidia would survive PZQ exposure if they were from individuals previously treated with PZQ compared to untreated (Melman *et al.*, 2009).

Miracidia derived from patients who had had between 4 and 20 PZQ treatments showed mortality that ranged from 30% to over 80% when exposed to $10^{-5}$ M PZQ *in vitro*, while the untreated cohort showed mortality ranging from 60% to 100%.

This present study returned to these occupational groups in Kisumu, western Kenya, and also, sampled a cohort of Kenyan schoolchildren undergoing PZQ therapy to determine if there is significant variability in PZQ sensitivity of the *S. mansoni* population infecting these individuals. In addition, the study examined variation in PZQ sensitivity of *S.*
*mansoni* miracidia obtained from mice infected with cercariae derived from naturally infected snail populations.

Irrespective of whether or not the study subjects had a history of PZQ treatment, the mean miracidial mortality of *S. mansoni* at $10^{-5}$ M PZQ observed in the present study was between 82.1% and 84.6%, with the lowest observed value for miracidia from a single patient being 72.7% (column 2 table 4.1). As these data are comparable with the PZQ sensitivity data of miracidia derived from eggs of the untreated cohort in the 2009 study of Melman *et al.*, (2009), it suggests that there is no evidence of diminished PZQ sensitivity in *S. mansoni* infecting these populations.

For the car washers and sand harvesters, the last treatment dates were between 1 and 9 years before the current study and thus, it is perhaps not surprising that, without sustained PZQ treatment, a population of *S. mansoni* with reduced responsiveness to PZQ has failed to materialize. Also, there was no indication of reduced miracidial susceptibility to PZQ among samples obtained from the 34 schoolchildren from Mwea that had previously received between 1 to 5 rounds of PZQ treatments, annually, as part of the Kenyan National De-worming Program.

Similarly, when miracidia derived from mice infected with cercariae from naturally infected snails collected in Kisumu, Mwea and Kibwezi areas were tested, no evidence of reduced susceptibility to PZQ was found (mortality at $10^{-5}$ MPZQ = 82.3 ± 4.9%; range = 75.8–90.3%). Thus, using miracidia as an indicator of *S. mansoni* sensitivity to PZQ in the definitive host the data implies that there is, as yet, no evidence to suggest that resistance or even reduced sensitivity is an immediate threat in the areas surveyed.
While results of the present study did not find PZQ insensitive *S. mansoni* isolates, concerns regarding emergence of PZQ resistance are real, given various reports of PZQ insensitive isolates from the field in some endemic localities (Day and Botros, 2006; Mutapi *et al.*, 2011). Efforts to regularly monitor PZQ susceptibility, especially in endemic localities where PZQ is frequently used is encouraged.

An interesting observation was recently made in Tanzania: administration of one round of PZQ treatment to school children in an endemic locality of Tanzania resulted in a significant reduction in the genetic diversity of *S. mansoni* populations within the children (Norton *et al.*, 2010; French *et al.*, 2013). While there are many reasons to account for such genetic ‘bottlenecking’ after PZQ treatment, one concern is that it may lead to a greater likelihood of development of PZQ resistant parasite strains. Clearly, at least in the case of *S. mansoni* infecting the school children who took part in this study this has not happened.

A recent analysis of genetic variability of *S. mansoni* in school children in the Mwea area suggests that there has been no reduction in schistosome worm burden but genetic diversity actually increased after 4 years of mass drug administration (Lelo *et al.*, 2014). This latter observation would be more in agreement with the observation of Huyse *et al.*, (2013), who reported that regular treatment with PZQ did not affect the genetic diversity of *S. mansoni* in Senegal.

In recent years, PZQ treatment of car washers and sand harvesters in Kisumu has become intermittent with the result that only a small proportion of the infected population is undergoing treatment at any one time. This would leave a significant reservoir of parasites unaffected by the drug and likely allow an *S. mansoni* strain with reduced susceptibility to be lost from patients, especially if, as has been reported, an ability to withstand PZQ
treatment also carries a cost to reproductive fitness (William et al., 2001a; Cioli et al., 2013).

In order to determine if this population still harbored the potential to generate *S. mansoni* with reduced PZQ sensitivity, a mouse infection model was used to study the impact of increasing amounts of PZQ on a population of parasites derived from 7 car washers. After the first passage there was a small but significant fall in the number of worms recovered after treatment with 100 mg/kg PZQ compared to vehicle treated controls and an increase in the male to female ratio from 1.8 to 3.0 (Table 4.3).

During passage 5, there was no effect on worm numbers or sex ratio compared with vehicle treated controls. In contrast, treatment of mice infected with non-PZQ selected *S. mansoni* during passages 3, 4 and 5 resulted in 36, 66 and 86% reduction in worm numbers compared to vehicle treated mice. This data suggests that the study generated a PZQ isolate with low susceptibility (P <0.05) to a normally effective dose of PZQ and is in close accordance with that of Fallon and Doenhoff (1994) who used a number of laboratory strains of *S. mansoni* from geographically diverse regions as the source of their genetic material.

Sexually mature female worms isolated from bisexual infections in mice have been shown to be less sensitive to PZQ *in vitro* than mature male worms (Pica-Mattoccia et al., 2004), while Delgado et al., (1992) showed a preferential killing of female worms *in vivo*. Despite some initial selection for males in the first round of PZQ treatment in the experiment reported here there was no subsequent evidence for the selection of either sex.

Interestingly, in a similar experiment using *S. mansoni* LE strain, Cioli et al., (2013) were able to generate an isolate that was able to withstand 3 X 300 mg/kg PZQ after 6
generations, but were unable to maintain the strain beyond the 11th generation under PZQ pressure due to a change in the male: female ratio from 2.5 in treated, non-selected worms to 8.7 in treated PZQ selected worms suggesting that female worms of the LE strain are more susceptible to PZQ after repeated exposure and selection.

Miracidia hatched from livers of PZQ and vehicle treated mice during each passage were assayed for their ability to survive PZQ treatment in vitro (Table 4.2). The data suggests that miracidia produced by drug-selected S. mansoni are also less susceptible to the drug, especially during the 4th and 5th passage suggesting that acquired resistance may be a heritable trait.

5.2 CONCLUSIONS

- The study has shown that a cohort of adults and children living in endemic areas of western and central Kenya who have undergone recent or historical treatment with PZQ do not harbor S. mansoni with reduced PZQ susceptibility. Schistosomes with a ‘resistant’ phenotype may not be problematic within these populations.
- This study has also shown there is a significant potential for the emergence of such a phenotype should sufficient PZQ pressure be applied.
- It is fortuitous that with perhaps only approximately 15% of people with schistosomiasis being treated with PZQ together with the intermittent nature of much of that treatment, a large refugium for drug sensitive parasites will continue to exist.
- Findings from this study conclude that, without sustained PZQ treatment, a population of S. mansoni with reduced responsiveness to PZQ has failed to materialize. Thus, using miracidia as an indicator of S. mansoni sensitivity to PZQ in the definitive host the data implies that there is, as yet, no evidence to suggest that resistance or even reduced sensitivity is an immediate threat in the areas surveyed.
5.3 RECOMMENDATIONS

- Continued monitoring of PZQ susceptibility using assays such as the ones employed here as control efforts are ramped up in the coming years.

- Improved assays are warranted as increased use of PZQ in control programs becomes a reality.

- *S. mansoni* parasites with reduced sensitivity can be generated in the lab using sub-lethal doses of PZQ and is: effective, fast, simple and cheap method and can therefore be used in similar studies.

5.4 FUTURE RESEARCH

- Trials to assess the safety and efficacy of higher doses for patients that fail to cure following exposure to standard PZQ dosages, or approval of more widespread availability of alternative treatments, such as oxamniquine, may both be prudent considerations when treatment failures occur.

- Because we lack a fundamental understanding of PZQ’s mode of action, a more explicit knowledge of PZQ’s targets will help us to devise much-needed improved assays for monitoring the emergence of resistance.

- Methods for genotyping individual eggs or miracidia need to be used to determine if the worm populations harbored by people before and after treatment show evidence of strong similarity, suggestive of the retention of fully adult worms that are not killed but merely temporarily silenced by PZQ treatment.

- Further studies should be continued by passaging the selected *S. mansoni* strain to determine if heightened female sensitivity to PZQ reported by Coeli *et al.* (2013) is due to the use of a laboratory strain as the founder population or whether a more genetically diverse founder population leads to a more stable long-term sex ratio.
REFERENCES


Schistosoma haematobium infection in Brazilians returning from Africa. Memorias do Instituto Oswaldo Cruz 100: 445-449.


Praziquantel sensitivity of Kenyan *Schistosoma mansoni* isolates and the generation of a laboratory strain with reduced susceptibility to the drug

Ibrahim N. Mwangi 1, Melissa C. Sanchez 2, Gerald M. Mkoji 3, Lelo E. Agola 3, Steven M. Runo 3, Pauline M. Cupit 4, Charles Cunningham 5,*

1 Center for Biotechnology and Research Development, Kenya Medical Research Institute, Nairobi, Kenya  
2 Dept. of Biology, University of New Mexico, Albuquerque, NM 87131, USA  
3 Dept. of Biochemistry and Biotechnology, Kenyatta University, Nairobi, Kenya

**ABSTRACT**

Schistosomiasis is a neglected tropical disease caused by blood-dwelling flukes of the genus *Schistosoma*. While the disease may affect as many as 240 million people, treatment largely relies on a single drug, praziquantel. The near exclusive use of this drug for such a prevalent disease has led to concerns regarding the potential for drug resistance and related effects. This study examines the potential for praziquantel resistance of *S. mansoni* isolates from human and animals in Kenya. Isolates were obtained from samples of eggs from *S. mansoni* and stored in a laboratory. The drug susceptibility of the isolates was assessed using the resistance assay. The study shows that the isolates have reduced susceptibility to praziquantel compared to the standard laboratory strain.

**1. Introduction**

Schistosomiasis is a water-borne parasitic disease that affects more than 249 million people (World Health Organization, 2014) with a global disease burden calculated at 24–56 million disability-adjusted life-years lost (King, 2010). Of the limited number of drugs available to treat schistosomiasis, praziquantel (PZQ) is the least expensive and easiest to use (Hagan et al., 2004) and, since PZQ is highly effective against all schistosome species that infect humans, its use in mass treatment campaigns has grown significantly. In 2006, approximately 12 million people were treated with PZQ and by 2012 this number reached approximately 42 million (World Health Organization, 2014). While the drug is highly effective against sexually mature forms of the parasite it is often unable to cure infections due to its inability to kill juvenile schistosomes at 2–4 weeks post-infection (Pica-Mattoccia and蜱, 2004; Aragon et al., 2009). As PZQ is often administered with a significant time lapse measured in months or years between treatments this can leave a significant reservoir of schistosomes infecting people that are unaffected by the drug. This, combined with continuing exposure to the parasite, means the drug can often only provide short-term relief from infection. Despite this drawback, PZQ remains the only readily available treatment for schistosomiasis amid concern that as it becomes more widely dispensed, drug resistance traits may emerge thus removing the most effective, albeit flawed drug from the limited treatment options available.

There have been a number of in vivo and in vitro studies documenting differential sensitivity of *Schistosoma mansoni* isolates to PZQ. For example, a relatively low cure rate was reported during a study of PZQ efficacy and side effects in Senegal in 1991 (Stolma et al., 1995). A subsequent study of a field isolate derived from snails in the same geographical area suggested that, when compared with two isolates from Puerto Rico and Kenya, the Senegal isolate matured in mice at a significantly slower rate than likely rendering it less susceptible to the drug at the times tested (Fallon et al., 1997). Rismail et al. (1999) generated 12 S. mansoni isolates
APPENDIX II: K.U. AUTHORIZATION FOR RESEARCH

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

Internal Memo

FROM: Dean, Graduate School
TO: Mwangi Ibrahim Ndungu
     C/o Department of Biochemistry & Biotechnology
     Kenyatta University

DATE: 25th October, 2014
REF: 156/CE/26257/11

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting of 22nd October 2014, approved your Research Proposal for the M.Sc. Degree Entitled, “Schistosoma mansoni Susceptibility to Praziquantel in Endemic Localities in Kenya”. You may now proceed with data collection, subject to clearance with the Permanent Secretary, Ministry of Higher Education, Science and Technology.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking forms per semester. The form has been developed to replace the progress report forms. The supervision Tracking Forms are available at the University’s website under Graduate School webpage downloads.

Thank you.

KEUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

C.c. Chairman, Department of Biochemistry & Biotechnology

Supervisors:

1. Dr. Steven Runo
   C/o Department of Biochemistry & Biotechnology
   Kenyatta University

2. Dr. Gerald M. Mkoji
   Centre for Biotechnology Research and Development
   Kenya Medical Research Institute
   C/o Department of Biochemistry & Biotechnology
   Kenyatta University
APPENDIX III: AUTHORIZATION FOR RESEARCH BY NACOSTI

NATIONAL COMMISSION FOR SCIENCE,
TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471,
2241349, 310571, 2219420
Fax: +254-20-318245; 318249
Email: secretary@nacosti.go.ke
Website: www.nacosti.go.ke
When replying please quote

Ref: No.

NACOSTI/P/15/6409/4973

Ibrahim Ndungu Mwangi
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on
“Schistosoma mansoni susceptibility to praziquantel in endemic localities in
Kenya,” I am pleased to inform you that you have been authorized to
undertake research in Kisumu County for a period ending 5th February,
2016.

You are advised to report to the County Commissioner and the County
Director of Education, Kisumu County before embarking on the research
project.

On completion of the research, you are required to submit two hard copies
and one soft copy in pdf of the research report/thesis to our office.

Said Hussein
FOR: DIRECTOR GENERAL/CEO

Copy to:

The County Commissioner
Kisumu County.

The County Director of Education
Kisumu County.
APPENDIX IV: RESEARCH PERMIT BY NACOSTI

THIS IS TO CERTIFY THAT:

MR. IBRAHIM NDUNGU MWANGI
of KENYATA UNIVERSITY, 54860-200
NAIROBI, has been permitted to conduct
research in Kisumu County
on the topic: SCHISTOSOMA MANSONI
Susceptibility to praziquantel in Endemic Localities in Kenya
for the period ending:
February 2, 2016

Applicant's Signature

Date of Issue: 6th March, 2015
Fee Received: Ksh 1,000

National Commission for Science, Technology and Innovation
RESEARCH CLEARANCE PERMIT

CONDITIONS: see back page
APPENDIX V: KEMRI SCIENTIFIC STEERING COMMITTEE APPROVAL

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00208, NAIROBI, Kenya
Tel (254) (020) 2722541, 2711349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/1000125

Charles Cunningham

15th February, 2012

Thro’
Director, CBRD
NAIROBI

REF: SSC No. 2227 (Revised) –Understanding the biology of schistosomes in response to praziquantel.

Thank you for your letter dated 14th February, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

FOR: Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health
KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713549, 0722-205591, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1

TO: DR. CHARLES CUNNINGHAM
DEPT OF BIOLOGY, UNIVERSITY OF NEW MEXICO, ALBUQUERQUE, USA

ATTN: DR. GERALD MKOJI (SITE PRINCIPAL INVESTIGATOR)

THROUGH: DR. KIMANI GACHUHI
THE DIRECTOR, CBRD, NAIROBI

April 27, 2012

Dear Sir,

RE: SSC PROTOCOL No. 2227- REVISED (RE-SUBMISSION):
UNDERSTANDING THE BIOLOGY OF SCHISTOSOMES IN RELATION TO
PRAZIQUANTEL (VERSION 1.3 DATED APRIL 4, 2012)

We acknowledge receipt of the following documents on 24th April 2012:
(b) Informed Consent Document (ICD) for Adults - English and Local Language Versions (Version 1.3 dated April 4, 2012).
(d) CVs for Dr. Charles Cunningham and Dr. Pauline M. Cupit from the University of New Mexico, USA.

This is to inform you that the Committee determines that the issues raised at the initial review of the application are adequately addressed. Consequently, the study is granted approval for implementation effective this 27th day of April 2012 for a period of one year. Please note that authorization to conduct this study will automatically expire on April 26, 2013.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by March 15, 2013. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

In Search of Better Health
APPENDIX VII: ANIMAL CARE AND USE COMMITTEE APPROVAL

KENYA MEDICAL RESEARCH INSTITUTE

KEMRI/ACUC/ 01.02.12

13TH February 2012,

Dr. Charles Cunningham
C/o Dr. Gerald M. Mkoji

Thro,
The Director
Centre for Biotechnology Research and Development,
Nairobi.

Dr. Charles Cunningham,

RE: Approval for the “Understanding the biology of schistosomes in response to praziquantel” Protocol

Following the receipt of the above mentioned proposal to the KEMRI ACUC, it has been established that all aspects of animal care and use have been addressed appropriately.

The committee grants you the approval to proceed with your study after obtaining all the other necessary approvals and expects you to adhere to all the animal handling procedures in KEMRI as described in your proposal.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Samson Limboso,
Chairperson, KEMRI ACUC

cc
Director CBRD
KEMRI Animal house
APPENDIX VIII: INFORMED CONSENT EXPLANATION

PROJECT TITLE: *Schistosoma mansoni* susceptibility to praziquantel in endemic localities in Kenya

INVESTIGATORS: Mwangi Ibrahim Ndungu, Department of Biochemistry and Biotechnology, Kenyatta University (KU), Nairobi Kenya

INTRODUCTION: The study being carried out is on bilharzia, a disease caused by worms that live in human beings, and which are transmitted through freshwater snails. The study aims to understand better how the bilharzia worms respond to praziquantel, the medication used to treat people infected with the bilharzia worms. A study like this will help us find ways of making better medications for treating the bilharzia disease. Bilharzia parasites will be collected from infected field snails and from bilharzia infected persons from Mwea, central Kenya and from various localities in Western Kenya in Kisumu County. Bilharzia causes sickness to millions of people all over the world including Kenya. In order to get the parasite samples we need to study, we will need to have people who have bilharzias give stool samples so that we can remove the bilharzia parasite eggs from the stool. Once we have separated the bilharzias eggs from the stool, we will get the eggs to hatch and then use the parasite larvae coming out of the egg in our research. I, Ibrahim N. Mwangi, the person carrying out this study, is requesting you to take part in this study by giving us stool samples from which to get the eggs of the bilharzia worms. Participating in this study is voluntary and no one will force you. However, if you accept to take part in the study, then you or your parent/guardian will have to give permission (that is, consent) for you to take part in this study. Even when you or your parent/guardian has given permission to participate in this study, it is still possible for you to leave the study, if you decide to do so, at any time in the future, without suffering any penalty or losing the benefits which you have been promised through participating in this study. Please take time to read this
information sheet about the study, and when you have read, feel free to ask questions or to seek clarification on any issues related to this study or your participation in it, both, now or even anytime, later. For your information, this study has already been approved by the KEMRI and the KU.

**PURPOSE OF THE STUDY:** The purpose of this study is to see how bilharzia parasites (which cause disease in people) respond when treated with praziquantel, the bilharzia medication. The aim is to have a better idea of how the medicine works to kill the parasite, and how sometimes the medicine fails. When it is clearly understood how the medication works to kill the parasite or why it may fail sometimes, then it may be possible to discover better ways to prevent or control the bilharzia disease. In this study, you will first be asked to give a small stool samples to see if you are infected with the bilharzia worms or other parasites, and if it is found that you are infected with the bilharzia worms, you will be asked to give a bit more stool so that we can isolate the bilharzia eggs.

**PROCEDURES TO BE USED:** you may be included in this study only if you or your parent/guardian signs a consent form, giving permission for you to take part in the study. Any person under 18 years old may also be included in the study only if they accept to take part, and if written consent has been given by a parent/ guardian.

**BENEFITS:** If you are found to have bilharzias or other parasites a qualified doctor will give you praziquantel, the medication for bilharzia free of charge. If you have other intestinal parasites, you will also receive medication for these infections as well, regardless of your bilharzia infection status, also, free of charge. If the doctor discovers that you have other medical conditions, he/she will refer you to a health clinic or hospital for further medical attention. However, you or your parent/guardian will be responsible for buying any other medications the doctor may prescribe for you for the other ailments.
RISKS, HAZARDS AND DISCOMFORTS ASSOCIATED WITH THE PROCEDURES: Giving stool samples will not cause any harm in you. The medications you will be given for treatment of bilharzia or other parasitic infections found in the stool you give, are known to be safe. However, in some people, they may cause some side effects which may include dizziness, headaches, stomach pain, but these are mild and last only for a brief period.

CONFIDENTIALITY: Your identity and test results will remain confidential. As a study participant, you will be assigned a number, and you or results of tests done on samples taken from you will be referred to by this number in all correspondence or publications arising from this study. All information and medical records will remain confidential, and will be kept in a lockable cabinet and only be accessible only to the people carrying out this study.

CONTACT OF SITE PRINCIPAL INVESTIGATOR: If you need more information about the study, please call: Mwangi Ibrahim Ndungu -Cell Phone: 0720-400 713.

CONTACT OF THE KEMRI's ETHICS REVIEW COMMITTEE: If you have questions about your rights as a research participant, please contact: The Secretary, KEMRI Ethics Review Committee, PO Box 54840-00200, Nairobi; Phone: 020-2722541, 0722-205901, 0733-400003; e-mail: erc@kemri.org
APPENDIX IX: INFORMED CONSENT AGREEMENT FOR ADULTS (>18)

I, Mr./Mrs./Miss ____________________________, being an adult aged 18 years and over, and able to decide on my own if I should take part in this study or not, give Mr. Ibrahim N. Mwangi permission to include me in the study being carried out in this area known as “schistosoma mansoni susceptibility to praziquantel in endemic localities in Kenya” which has been explained to me and I now, know what it is all about. I have been explained to about the tests to be done on me, the benefits for taking part in the study, and the medications I will receive if I am found to be sick with bilharzia or other intestinal illnesses caused by parasites, and the side effects I could suffer, which I understand are mild and temporary. I have been given opportunity to ask questions and to seek a clarification of the issues I had not understood clearly, and I am satisfied with the answers and the explanations I was given. I have also, been told that if later, I have additional questions or concerns about the study, I can contact the researcher in charge of the study, or the Ethics Review Committee (ERC) at KEMRI

I accept to take part in the study, and agree to give stool samples for the tests needed for this study. I have been told I can leave the study any time I wish, if I decide to do so, and I have been assured that I will not suffer any penalty or loss of benefits that I should get through this study.

__________________________________________  ________________
Signature (or Thumb Print of Participant)  Date
APPENDIX X: INFORMED CONSENT AGREEMENT FOR PARENTS

I, Mr./Mrs/Miss ____________________________, being an adult aged 18 years and over, and being the parent/guardian of: Master/Miss (Child’s Name) ________ Aged __, who attends ______________________School, do hereby give permission to Mr. Ibrahim Mwangi for my child to take part in the new study known as “schistosoma mansoni susceptibility to praziquantel in endemic localities in Kenya” which has been explained clearly to and now, know what the study is all about. I have been explained to about the tests to be done on me, the benefits for taking part in the study, and the medications I will receive if I am found to be sick with bilharzia or other intestinal illnesses caused by parasites, and the side effects I could suffer, which I understand are mild and temporary. I have been given opportunity to ask questions and to seek a clarification of the issues I had not understood clearly, and I am satisfied with the answers and the explanations I was given. I have also, been told that if later, I have additional questions or concerns about the study, I can contact the researcher in charge of the study, or the Ethics Review Committee (ERC) at KEMRI.

I accept that my child can take part in this study, and agree that he/she can give stool samples for the tests needed in this study. I have been told that my child can leave the study any time he/she decides to do so, and I have been assured that he/she will not suffer any penalty or loss of benefits that he/she should get through this study.

________________________     _________________
Signature (or Thumb Print) of Participant/Guardian         Date
APPENDIX XI: ASSENT FOR CHILDREN (13-17 YEAR OLDS)

You are being asked to take part in a study called *Schistosoma mansoni susceptibility to praziquantel in endemic localities in Kenya* which is being carried out by Ibrahim Mwangi. The study aims at finding out how the bilharzia worms (which cause disease in people, mainly children) is affected by the medication (praziquantel) used to treat the bilharzia disease. If we can get to know more about the effects of this medication on the bilharzia worms, then one day, researchers will be able to discover new ways of treating or controlling the disease. For this study to be carried out, you will be asked to give stool samples for checking if you have eggs of bilharzia worms in your body. If eggs of these worms are found in your stool sample, you will be asked to give a bit more sample so that we can isolate the eggs for doing analysis. If we find eggs of bilharzia worms or any other parasites in you, you will be given some medication by the doctor to get rid of the worms, so that you can get better.

You do not have to give a stool sample for this study, if you don’t want to, but there will be no harm if you gave a sample. That way, we can know if you have the worms that make people sick. Giving stool samples will not harm you in any way. Do you agree to take part in this study and give stool samples?

If you **agree to take part in this study and give stool samples, please** put a tick (√) next to the answer “YES”, in the space given below:

**YES** I agree to take part in this study and provide stool samples.

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Name of the Child  Signature or Thumb Print  Date