USE OF A PCR-BASED PROCEDURE FOR DIFFERENTIATING BETWEEN SCHISTOSOMA MANSONI AND SCHISTOSOMA RODHAINI

BY

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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Master of Science in Infectious Disease Diagnosis in the School of Pure and Applied Sciences at Kenyatta University
ABSTRACT

Schistosoma mansoni, the parasite responsible for causing human intestinal schistosomiasis, is closely related to Schistosoma rodhaini, a parasite of rodents and carnivorous mammals. S. rodhaini was discovered in Kenya for the first time in the early 1960’s in the wild rodents and domestic dogs obtained from a locality near the town of Kisumu on the shores of Lake Victoria, western Kenya. Schistosoma mansoni and schistosoma rodhaini at cercariae level are closely related morphologically. As a result it is difficult to differentiate them at cercariae level which may lead to misdiagnosing where the two parasites are endemic underscoring the need for an alternative diagnostic tool. The presence of S. rodhaini in an area known to be endemic for S. mansoni such as western Kenya could potentially complicate the epidemiological picture or control of human intestinal schistosomiasis in the area. Consequently, sampling of snail populations collected from field locations to gather basic epidemiological information on human schistosomiasis for a particular area may not provide reliable results under such circumstances. In the present study, a polymerase chain reaction (PCR) based technique was developed to help the identification of cercariae or adults of S. mansoni and S. rodhaini towards improved diagnosis. Three isolates of S. rodhaini were recovered from naturally infected Biomphalaria sudanica collected from habitats present along the Lake Victoria shore in western Kenya. From the three isolates a portion of the 16S-12S region of the mitochondrial DNA was amplified by PCR in schistosome genomic DNA, and the resulting product (750bp in size) was incubated with restriction enzyme BamHI or AccI for 4 hr at 37°C. Species-specific restriction fragment length polymorphism (RFLP) band patterns revealed on agarose gel by electrophoresis and ethidium bromide staining differentiated between the two related schistosome species. The enzyme BamHI cut the amplified product in S. rodhaini DNA into 2 smaller fragments (153bp and 604bp in size) but not S. mansoni DNA. Similarly, the enzyme AccI cut the S. mansoni PCR product into 2 fragments of 110bp and 649bp in size but not that of S. rodhaini, producing species-specific RFLP band patterns on agarose gel. These results demonstrate technique reliably identified both the adults’ worms and cercariae of S. mansoni or S. rodhaini, and it may be useful in accurately identifying and monitoring S. mansoni transmission sites, and other epidemiological studies on S. mansoni or S. rodhaini. The technique requires further evaluation to determine its usefulness in identifying S.mansoni group species as it has the potential for providing new information. This technique may be useful in accurately identifying and monitoring S. mansoni transmission sites and also the technique is useful in other epidemiological studies on S. mansoni or S. rodhaini where both parasites are present sympatrically.
DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other University or Institution.

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DEDICATION

This thesis is dedicated to my wife Eunice Njuguna, son Mungai, daughters, Wangui, Njoki and Wanjiku, and to my dear mother and my late father who in one way or another have been an inspiration to my life.
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I humbly acknowledge all forms of assistance I received from different people. However contributions from a few need special mention.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td><strong>CHAPTER ONE: INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Schistosomes and Schistosomiasis</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Biology and life-cycle of human schistosomes</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Clinical manifestations and immunopathology of human schistosomias</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Control of human schistosomias</td>
<td>8</td>
</tr>
<tr>
<td>1.5 Diagnosis of human schistosomias</td>
<td>9</td>
</tr>
<tr>
<td>1.6 Human schistosomias in Kenya</td>
<td>9</td>
</tr>
<tr>
<td>1.7 Animal schistosomias in Africa</td>
<td>10</td>
</tr>
<tr>
<td>1.8 Significance of animal schistosomes in the epidemiology of</td>
<td>13</td>
</tr>
<tr>
<td>schistosomias</td>
<td></td>
</tr>
<tr>
<td>1.9 Molecular approaches for identifying schistosome species</td>
<td>15</td>
</tr>
<tr>
<td>1.10 Problem statement and justification of the present study</td>
<td>16</td>
</tr>
<tr>
<td>1.11 Hypothesis</td>
<td>16</td>
</tr>
<tr>
<td>1.11.1 Objectives</td>
<td>16</td>
</tr>
<tr>
<td>1.11.2 Overall objective</td>
<td>16</td>
</tr>
<tr>
<td>1.11.3 Specific Objectives</td>
<td>17</td>
</tr>
<tr>
<td><strong>CHAPTER TWO: LITERATURE REVIEW</strong></td>
<td>18</td>
</tr>
<tr>
<td>2.1 <em>Schistosoma mansoni</em> group species</td>
<td>20</td>
</tr>
<tr>
<td>2.2 <em>Schistosoma mansoni</em></td>
<td>21</td>
</tr>
</tbody>
</table>
2.3 *Schistosoma rodhaini* ...................................................... 26
2.4 *S.mansonii-S.rodhaini* interactions ........................................ 28
2.5 *S.rodhaini* and the epidemiology and control of *S.mansonii* ........... 31
2.6 Methods for identifying cercariae of mammalian schistosome species .... 33
2.7 PCR as a tool for identifying and characterizing schistosomes .............. 38
2.8 Genomic DNA targets useful for species identification and population
 characterization ........................................................................... 39

CHAPTER THREE: MATERIAL AND METHODS .................................... 45
3.1 Study area ................................................................................. 45
3.2 Snail collection .......................................................................... 46
3.2.1 Screening of snails for schistosomes ...................................... 47
3.2.2 Infection of mice with cercariae and schistosome isolation ............ 48
3.2.3 Schistosome recovery and isolation from mice ......................... 49
3.2.4 Isolation of schistosomes in mice ........................................... 51
3.2.5 Perfusion .............................................................................. 52
3.2.6 Confirmation of *S.rodhaini* Identity by DNA sequencing ............. 55
3.2.7 Establishment of *S.rodhaini*(w2018-01) in the laboratory ............ 56
3.2.8 Characterization of the cercarie shedding pattern of *S.rodhaini* .... 58
3.2.9 Development of a PCR-based assay for differentiating between *S.rodhaini*
 and *S.mansonii* ....................................................................... 59
3.3.0 Parasite material ...................................................................... 59
3.3.1 The 16S-12S region of the mtDNA as a target for *S.mansonii* and
 *S.rodhaini* identification .......................................................... 60
3.3.2 Genomic DNA ........................................................................ 61
3.3.3 Primers ............................................................................... 62
3.3.4 PCR Amplification of 16S-12S region of the schistosome mitochondrial DNA ................................................................. 62

3.3.5 Restriction Fragment Length Polymorphism Analysis of the 16S-12S mt-DNA ......................................................................... 63

3.3.6 Optimization of the PCR-RFLP assay for cercariae identification ................................................................. 63

3.3.7 Analysis of Adult Schistosomes derived from Cercariae Obtained from naturally infected, field-collected Biomphalaria snails ................................................................. 64

3.3.8 PCR-RFLP Analysis of mammalian schistosome cercariae obtained from field-collected Biomphalaria snails ................................................................................................. 65

CHAPTER FOUR: RESULTS ........................................................................................................ 66

4.1.0 Snail collections and results of screening for schistosome infections ................................................................. 66

4.1.1 Laboratory mice infections with schistosome cercariae ......................................................................................... 66

4.1.2 Maintenance of S.rodhaini lifecycle under laboratory conditions ................................................................ 68

4.1.3 Characteristics of the cercariae shedding pattern of S.rodhaini (W2018-01) ......................................................................................... 73

4.2 Sequence analysis of the 16S-12S region of the mtDNA of S.mansoni ........................................................................ 73

4.2.1 PCR Amplification of the 16S-12S mtDNA of the adult S.mansoni and S.rodhaini ........................................................................ 75

4.2.2 RFLP Analysis of PCR products of adult S.mansoni and S.rodhaini ........................................................................ 78

4.2.3 Optimal conditions of the PCR-RFLP assay of cercarial DNA from S.mansoni or S.rodhaini ........................................................................ 78

4.3.0 Analysis of adult worms derived from cercariae shed by field collected Biomphalaria snails ........................................................................ 82
CHAPTER FIVE: DISCUSSION AND CONCLUSIONS ........................................... 86

5.1 *S. rodhaini* in western Kenya ......................................................... 86

5.2 The PCR test for identification of *S. mansoni* or *S. rodhaini* ........ 89

5.3 Evaluation of the PCR assay using field-obtained cercariae or adults of
*S. mansoni* group species ................................................................. 91

5.4 CONCLUSIONS .................................................................................. 92

5.5 RECOMMENDATIONS ......................................................................... 93

REFERENCES ......................................................................................... 94
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taxonomic classification of mammalian schistosomes</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Classification of snail hosts involved in the transmission of human schistosomes in Kenya</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Non-human mammalian schistosomes occurring in Sub-saharan Africa</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Species of the <em>S. mansoni</em> group, their snail intermediate hosts, continental distribution, and their mammalian hosts</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td><em>Biomphalaria</em> species occurring in Africa and the neighbouring regions</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Localities in western Kenya where <em>Biomphalaria</em> snails were collected</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>Sequences of the primer pair used to PCR amplify a portion of the 16S-12S region of the mtDNA in <em>S. mansoni</em> or <em>S. rodhaini</em></td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>A list of the audit schistosome isolates derived from field collected <em>Biomphalaria</em> snails grown in laboratory mice and analyzed by the developed PCR-RFLP assay</td>
<td>65</td>
</tr>
<tr>
<td>9</td>
<td><em>S. rodhaini</em> isolates from naturally infected <em>B. sudanica</em> snails collected in Western Kenya from sites along the shores of L. Victoria, and details of their collection sites and dates</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td><em>S. rodhaini</em> (W2018-01) infection in laboratory-raised <em>B. sudanica</em> and <em>B. pfeifferi</em></td>
<td>70</td>
</tr>
<tr>
<td>11</td>
<td>Identity of mammalian schistosomes isolates recovered from naturally infected <em>Biomphalaria</em> snails collected from various locations in western Kenya in or around Lake Victoria</td>
<td>70</td>
</tr>
<tr>
<td>12</td>
<td>Identity of unknown samples of cercariae analyzed using the PCR-RFLP technique</td>
<td>80</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Global distribution of human schistosomiasis</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Life cycle of mammalian schistosomes</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Map of Kenya showing schistosome distribution</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>A typical <em>S. mansoni</em> egg</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Egg of <em>Schistosoma rodhaini</em></td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Schematic representation of the ribosomal gene complex</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>A schematic representation of the mitochondrial genome of a eukaryote</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>A scoop, for collecting freshwater snails from field</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>Typical mammalian schistosome cercariae</td>
<td>51</td>
</tr>
<tr>
<td>10</td>
<td>The apparatus used to expose lab mice to schistosome cercariae</td>
<td>53</td>
</tr>
<tr>
<td>11</td>
<td>The apparatus for perfusing mice to recover adult schistosomes</td>
<td>54</td>
</tr>
<tr>
<td>12</td>
<td>A 24-well culture plate used for screening snails cercariae</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>Various morphological forms of <em>S. rodhaini</em> eggs</td>
<td>71</td>
</tr>
<tr>
<td>14</td>
<td>Typical <em>S. rodhaini</em> cercariae emergence rhythm observed for w2018-01</td>
<td>72</td>
</tr>
<tr>
<td>15</td>
<td>Predicted RFLP patterns with enzymes <em>BamHI</em> on <em>S. rodhaini</em> and <em>Accl</em> on <em>S. mansoni</em></td>
<td>76</td>
</tr>
<tr>
<td>16</td>
<td>Agarose gel showing PCR products of schistosome adult worms</td>
<td>77</td>
</tr>
<tr>
<td>17</td>
<td>Agarose gel showing restriction digest types of the 750bp product</td>
<td>81</td>
</tr>
<tr>
<td>18</td>
<td>RFLP- PCR band patterns generated when enzyme is used</td>
<td>84</td>
</tr>
<tr>
<td>19</td>
<td>Agarose gel stained with ethidium bromide showing the species specific</td>
<td>85</td>
</tr>
</tbody>
</table>

**APPENDIX**

Sequences of *S. mansoni* and *S. rodhaini* ................................................................. 112
CHAPTER ONE

INTRODUCTION

1.1 Schistosomes and Schistosomiasis

Schistosomes (also known as blood flukes) are digenean trematodes belonging to the family Schistosomatidae. They are parasites in the blood-vascular system of vertebrates, primarily birds and mammals. A characteristic feature of members in this family is that mature female worms are more slender than the male worms and are normally carried by the males in a ventral groove known as the gynaecophoric canal, which is formed by ventrally flexed lateral outgrowths of the male body (Rollinson and Southgate, 1987). Also, schistosomes utilize aquatic snails as intermediate hosts for the completion of their life cycles. Mammalian schistosomes are placed in the subfamily Schistosomatinae, and 5 genera are currently recognized viz. *Schistosoma*, *Schistosomatium*, *Bivetellobilharzia*, *Heterobilharzia* and *Orientobilharzia* (Rollinson and Southgate, 1987). The genus *Schistosoma* is important from the medical and economic viewpoint, as it comprises species that parasitize and cause disease in humans and domestic animals.

Table 1 provides a detailed taxonomic classification of mammalian schistosomes. *Schistosoma* species are responsible for causing schistosomiasis (also known as bilharzia) in humans, a major public health problem worldwide. Human schistosomiasis afflicts at least 200 million people worldwide, mostly children, in 76 tropical countries with another 500-600 million people being at risk of contracting the disease (WHO, 1993; Chitsulo *et al.*, 2000). The number of people estimated to be
infected or at risk continues to increase as population growth increases. Africa is home to about 85% of the total number of infected people worldwide.

Table 1: Taxonomic classification of mammalian schistosomes

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Platyhelminthes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Trematoda</td>
</tr>
<tr>
<td>Subclass</td>
<td>Digenee</td>
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<tr>
<td>Order</td>
<td>Strigeata</td>
</tr>
<tr>
<td>Superfamily</td>
<td>Schistosomatoidea</td>
</tr>
<tr>
<td>Family</td>
<td>Schistosoma</td>
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<tr>
<td>Species groups</td>
<td><em>S. mansoni, S. haematobium, S. japonicum, S. nasales, S. hippopotami</em></td>
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</tbody>
</table>
Seven species are responsible for causing human schistosomiasis: *S. geineensis* *S. haematobium*, *S. intercalatum*, *S. mansoni*, *S. japonicum*, *S. mekongi*, and *S. malayesiensis*. *S. haematobium* is confined to the African continent and the neighbouring regions, and causes urinary schistosomiasis. *S. mansoni* occurs in Africa, Madagascar, the Arabian Peninsula, South America and the Caribbean region (Rollinson and Southgate, 1987). *Schistosoma intercalatum*, a causal agent of human intestinal schistosomiasis is confined to equatorial Africa. *Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosoma malayensis* cause intestinal schistosomiasis, and are endemic in China and Southeast Asia.
Figure 1 Global distribution of human schistosomiasis. (Source: Centers for Disease Control)
1.2 Biology and Life Cycle of Human Schistosomes

Schistosomes, like other digenean trematodes have a complex life cycle (Figure 2) involving parasitic forms in a snail intermediate host, free-living larval forms and parasitic stages in a definitive host (Rollinson and Southgate, 1987). The adult male and female worms live paired in the blood-vascular system of humans or other susceptible mammalian hosts, where they mature, mate and reproduce. The female worms produce eggs in the blood stream, and depending on the infecting species, these work their way into the host tissue and eventually into the lumen of the intestine or the urinary bladder, from where they are passed out into the environment via urine or faeces. The schistosome eggs are fully developed and contain ciliated larval forms (miracidia) when they leave the body of the definitive host. On coming into contact with freshwater, the eggs hatch and the miracidia, which are free-swimming forms, are released into the water, and immediately embark on the search for a suitable snail host. In a suitable snail host the miracidia enter, and transform into mother sporocysts then into daughter sporocysts in which cercariae (another free-swimming larval form that is infective to the definitive host) develops.
The schistosomula incorporate host proteins, and blood group antigens, in their interguments. Their metabolism shifts to glycolysis. The schistosomula migrate through the pulmonary capillaries to the systemic circulation, which carries them to the portal veins where they mature. The male and female adults pair off, with the thin female entering and remaining in the gynaecophoric canal of the male worm. The pair now migrates together along the endothelium, against portal blood flow to the mesenteric (S. mansoni, S. japonicum) or vesicular (S. haematobium) veins where they begin to produce eggs.

1.3 Clinical Manifestations and Immunopathology of Human Schistosomiasis

The schistosome egg is responsible for most of the pathology associated with schistosomiasis (Lichtenberg, 1987). Eggs lodged in tissue elicit an inflammatory reaction resulting in the formation of granulomas. The organs mostly affected are liver and spleen, and a common feature of a schistosome infection is hepato-splenomegaly, a life threatening condition (Kibiki et al., 2004). Depending on the infecting species, symptoms of schistosomiasis may include abdominal pain, bloody diarrhea, haematuria and dysuria. In very severe cases, portal hypertension and oesophageal and gastric varices may occur. In general, schistosomiasis runs a chronic course, resulting in inflammatory lesions produced in and around blood vessels by the schistosome eggs or their products (Bica et al., 2000).
Schistosomiasis

Sporocysts in snail (successive generations)

Cercariae released by snail into water and free-swimming

Penetrate skin

Cercariae lose tails during penetration and become schistosomulae

Circulation

Migrate to portal blood in liver and mature into adults

Infective Stage

Diagnostic Stage

Eggs hatch releasing miracidia

S. mansoni

S. japonicum

S. haematobium

Paired adult worms migrate to:

mesenteric venules of bowel/rectum (laying eggs that circulate to the liver and shed in stools)

venous plexus of bladder

Figure 2: Life cycle of mammalian schistosome (source CDC)
Half the number of eggs produced by the adult worms become trapped in the tissue or are swept back into the peri-portal circulation and get trapped in the perisinusoidal capillary venules of the liver. A chronic granulomatous inflammatory response is induced by antigens secreted by miracidium through microscopic pores within the egg-shell. In general, human schistosomiasis is an immunologically complex infection. Research into the immune responses associated with severe morbidity of schistosomiasis is providing new insights into immune regulation mechanisms (Sturrock et al., 1997), the role of genetic predisposition to periportal fibrosis, and effects of co-infections with malaria or HIV/AIDS (Vennervald and Dunne, 2004).

1.4 Control of human schistosomiasis

Various strategies are recommended for schistosomiasis control, including chemotherapy, snail control, health education, community participation, and provision of clean water and improved sanitation. For chemotherapy, the antischistosomal drug praziquantel, is considered the most cost effective control approach to schistosomiasis (WHO, 2001). The cost of praziquantel has reduced significantly in the last several years, and the drug is now more readily available. New strategies involving chemotherapy targeting school-age children and/or integration with control of intestinal worms are also being advocated as the most effective ways of controlling the disease (WHO, 2001). The global strategy for control of schistosomiasis is aimed at reducing morbidity through effective chemotherapy (WHO, 1993). It has been shown that targeted chemotherapy can significantly reduce disease transmission (Muchiri et al., 1996).
1.5 Diagnosis of human schistosomiasis

Diagnosis is not only essential for the medical care of the individual patient but also for screening populations for mass treatment or epidemiological studies. Currently, diagnosis of urinary or intestinal schistosomiasis relies upon microscopy after concentration of parasite eggs in thick stool smears for *S. mansoni* (Parija *et al.*, 2003; Katz *et al.*, 1972; Peters *et al.*, 1980) or by filtration of urine through polyamide or nucleopore filters (Peters *et al.*, 1976; Mott *et al.*, 1982). However, several immunodiagnostic tests have also been developed or are under development (Makarova *et al.*, 2005; Attallah *et al.*, 1999) and immuno-chromatograph reagent strip assays for indirect diagnosis of urinary schistosomiasis and possibly intestinal schistosomiasis are widely used (Bosompem *et al.*, 2004; Van Dam *et al.*, 2004). Simple questionnaires are also currently being used for rapid screening of schistosomiasis (Lengeler *et al.*, 2000; Lengeler *et al.*, 2002). To complement conventional diagnostic tests, ultra-sonography has in recent years been used extensively to assess morbidity associated with schistosomiasis. Similarly, other procedures that rely on measuring serological markers to assess schistosomiasis morbidity are also being developed.

1.6 Human Schistosomiasis in Kenya

Schistosomiasis is endemic in Kenya, as shown in Figure 3. Currently, it is estimated that more than 3 million people are afflicted, with about 10 million more being at risk of infection (Division of Vector-Borne Diseases, Ministry of Health, Kenya, unpublished reports; Muchiri *et al.*, 1996). Both the urinary form of the disease caused by *S. haematobium* and the intestinal disease caused by *Schistosoma mansoni* exist in Kenya. *S. haematobium* is endemic in coastal Kenya, in Taveta, south-west
Schistosomiasis is particularly common in irrigation schemes present in several places in the country, notably, in Mwea and Taveta (Choudhry, 1975). The distribution of schistosomiasis is dictated by the distribution of its snail intermediate host. In Kenya, the freshwater planorbid snails in the genera *Bulinus (africanus)* and *Biomphalaria* serve as intermediate hosts of human schistosomes (Brown, 1994).

*Bulinus (africanus)* species are responsible for transmitting *S. haematobium*, the causal agent of urinary schistosomiasis. In this group, *Bulinus africanus*, *Bulinus globosus*, and *Bulinus nasutus* are the species involved in the transmission of urinary schistosomiasis in Kenya. *Schistosoma mansoni*, causal agent of intestinal schistosomiasis is transmitted by *Biomphalaria pfeifferi*, the most widespread, *B. sudanica* confined to the shores of Lake Victoria in western Kenya and Lake Jipe (in south-west Kenya), and *B. choanomphala* present in the deep waters of Lake Victoria up to a depth of 0.5-6.0m (Magendantz; 1972).

1.7 Animal schistosomiasis in Africa

In addition to causing disease in humans, several *Schistosoma* species also, parasitize both domestic and wild mammals (Rollinson and Southgate, 1987). As mentioned in table 3 among the most common animal schistosomes in Africa include *S. bovis* and *S. matheei*, *S. currassoni*, *S. magrebowiei*, and *S. leiperi* in both domestic and wild ungulates (Dinnik and Dinnik, 1965; DeBont and Vercruysee, 1998, *S. rodhaini* in rodents and carnivores (Nelson *et al.*, 1962), and *S. hippopotami* and *S. edwardienne* in
hippopotamus (Thurston, 1963, 1964, McCully et al., 1967; Pitchford and Visser, 1981; Morgan et al., 2003). Schistosoma mansoni and Schistosoma haematobium, the human infecting schistosome species in Africa, also infect non-human primates, such as baboons and monkeys (Rollinson and Southgate, 1987), table 2 provides a detailed classification of the snail hosts involved in the transmission of human schistosomiasis in Kenya.
Figure 3: Schistosomiasis distribution in Kenya (courtesy of KEMRI schistosomiasis team)
Table 2: Classification of snail hosts involved in the transmission of human schistosomes in Kenya.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Mollusca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Gastropoda</td>
</tr>
<tr>
<td>Order</td>
<td>Basommatophora</td>
</tr>
<tr>
<td>Family</td>
<td>Planorbidae</td>
</tr>
<tr>
<td>Genera</td>
<td><em>Bulinus; Biomphalaria</em></td>
</tr>
</tbody>
</table>

(Source: Brown 1994)

1.8 Significance of animal schistosomes in the epidemiology and control of human schistosomiasis

*S. rodhaini* was reported for the first time in Kenya during the early 1960’s in domestic dogs and wild rodents obtained in localities near the town of Kisumu, on the shores of Lake Victoria (Nelson et al., 1962). However, virtually nothing is known about its distribution or prevalence in the country. *S. mansoni* is also endemic in western Kenya around Lake Victoria. Although *S. rodhaini* has occasionally been found in humans (D’Haenens and Santele, 1955), it is not currently considered to be of serious medical significance. Nevertheless, the presence of *S. rodhaini* in an area where *S. mansoni* is also endemic such as western Kenya could complicate the epidemiological picture and control of the human parasite.
<table>
<thead>
<tr>
<th>Schistome species</th>
<th>Snail Host Genus</th>
<th>Mammalian Hosts</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mattheei</em></td>
<td><em>Bulinus</em></td>
<td>cattle, sheep, goats, antelopes, buffalo, wildebeast</td>
<td>humans and other primates are also infected; occurs in southern Africa</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td><em>Bulinus</em></td>
<td>cattle, sheep, goats, antelopes, horses, donkeys, camels, pigs, dromedaries</td>
<td>human infections extremely rare; occurs in northern, western and eastern Africa, and parts of central and southern Africa</td>
</tr>
<tr>
<td><em>S. curassoni</em></td>
<td><em>Bulinus</em></td>
<td>cattle, sheep</td>
<td>may occur in the same hosts with <em>S. bovis</em>; occurs in western Africa</td>
</tr>
<tr>
<td><em>S. margrebowiei</em></td>
<td><em>Bulinus</em></td>
<td>antelopes, cattle, goats,</td>
<td>occurs in western and southern Africa</td>
</tr>
<tr>
<td><em>S. leiperi</em></td>
<td><em>Bulinus</em></td>
<td>antelopes, cattle, buffalo, sheep, horses, zebra</td>
<td>occasionally occur in the same host with <em>S. bovis</em>; <em>S. mattheei</em>, and <em>S. margrebowiei</em>; occurs in eastern and southern Africa</td>
</tr>
<tr>
<td><em>S. rodhaini</em></td>
<td><em>Biomphalaria</em></td>
<td>rodents, carnivores</td>
<td>present in Nigeria, central Africa, Zimbabwe, and Lake Victoria</td>
</tr>
<tr>
<td><em>S. edwardiense</em></td>
<td><em>Biomphalaria</em></td>
<td>hippopotamus</td>
<td>present in Uganda, South Africa</td>
</tr>
<tr>
<td><em>S. hippopotami</em></td>
<td><em>Bulinus</em></td>
<td>hippopotamus</td>
<td>occurs in Uganda</td>
</tr>
</tbody>
</table>
S. rodhaini is a close relative of S. mansoni; they both utilize Biomphalaria snails as intermediate hosts, their cercariae (the free-swimming larval stages that develop in the snail host and are infective to mammalian hosts) are very similar morphologically, and therefore, very difficult to differentiate. If the two species are present in the same locality or habitat, then any epidemiological information obtained for the medically important S. mansoni by examining field collected snail populations will be unreliable. Similarly, if one was to rely on snail sampling to identify transmission sites for the human parasite, or to monitor transmission sites after a chemotherapeutic intervention for instance, then such information would be difficult to interpret if S. rodhaini is transmitted in the same locality or habitat as well.

Although various procedures are available for identifying and differentiating mammalian schistosome species at cercariae level, these tend to be tedious and cumbersome to use. They are also not suitable for routine use, and some of them may not necessarily provide reliable results. Simplified procedures for identifying cercariae or intra-molluscan stages of schistosomes are therefore, desirable.

1.9 Molecular approaches for identifying schistosome Species

It has been suggested that molecular approaches offer accurate, reliable, rapid, and simple ways for identification and characterization of parasitic organisms. In particular, approaches that rely on the amplification of genomic DNA by the polymerase chain reaction (PCR) (Saiki et al., 1988) are considered most suitable for this purpose.
1.10 Problem statement & justification of the Study

Very little is known on the prevalence and distribution of *S. rodhaini* in Kenya, and to what extent its distribution overlaps that of the human parasite, *S. mansoni*. Studies on the transmission ecology of *S. mansoni* or *S. rodhaini* are hampered by lack of simple and reliable techniques for telling the 2 species apart at cercariae level. A number of procedures have been used to identify schistosome cercariae, which are often tedious to perform, time consuming and do not necessarily identify the parasite reliably. Molecular approaches for species identification offer accuracy, reliability, rapidity and simplicity. A technique that reliably identifies and differentiates between *S. mansoni* and *S. rodhaini* will facilitate further studies on the biology and epidemiology of *S. mansoni* and *S. rodhaini* especially in localities where the 2 species occur sympatrically.

1.11 Hypothesis

Differences exist at molecular level between the closely related *S. mansoni* and *S. rodhaini* to allow their identification and differentiation.

1.11.1 Objectives

1.11.2 Overall Objective

To develop a molecular procedure for differentiating between *S. mansoni* and *S. rodhaini*, in order to facilitate studies of the epidemiology of *S. rodhaini*, especially in sympatric population.
1.11.3 Specific Objectives

I. Investigate the presence of the rodent parasite *Schistosoma rodhaini* in western Kenya, using a parasitological approach.

ii. Develop a PCR-based technique for differentiating between the human pathogen *S. mansoni* and its close relative *S. rodhaini*.

iii. Optimization of the PCR-RFLP assay for cercariae identification.

iv. Analyze adult schistosomes derived from cercariae obtained from field-collected *Biomphalaria* snails
CHAPTER TWO

LITERATURE REVIEW

In addition to causing disease in humans, several schistosoma species also, parasitize both domestic and wild mammals (Rollinson and Southgate, 1987). In Africa, among the most common animal schistosomes include *S. bovis* and *S. mattheei*, *S. currassoni*, *S. margrebowiei*, and *S. leiperi* in both domestic and wild ungulates (Dinnik and Dinnik, 1965; DeBont and Vercruysse, 1998, *S. rodhaini* in rodents and carnivores (Nelson *et al.*, 1962), and *S. hippopotami* and *S. edwardienne* in hippopotamus (Thurston, 1963, 1964; McCully *et al.*, 1967; Pitchford and Visser, 1981; Morgan *et al.*, 2003). *S. mansoni* and *S. haematobium*, the human infecting schistosome species in Africa, also infect non-human primates, such as baboons and monkeys (Rollinson and Southgate, 1987).

It has been suggested that non-human primates, primarily baboons may serve as reservoir hosts for human schistosomes (Fenwick, 1969; Ghandour *et al.*, 1995; Muchemi, 1992; Muller-Graf *et al.*, 1997; Munene *et al.*, 1998; Erko *et al.*, 2003). *S. mansoni* has also been found in rodents (Nelson *et al.*, 1962; Theron and Pointier, 1995; D’Andrea *et al.*, 2000) but the role rodents may be playing in the epidemiology of human intestinal schistosomiasis, particularly in Africa, is not clear. Table 3 provides a list of schistosome species parasitic in non-human mammalian hosts in Africa. Perhaps, of particular interest from the public health viewpoint is the fact that *S. mattheei*, a bovine schistosome endemic in southern Africa, and closely related to *S. haematobium* (Van Wyk, 1983; Pitchford, 1986) and *S. margrebowiei*, parasite of wild bovines may
occasionally infect humans (Christensen et al., 1983). Even more interesting, a number of non-human mammalian schistosome species present in Africa utilize, as intermediate hosts, the same snail host species used by the human schistosomes. For example, the *Bulinus (Africanus)* species that serve as intermediate host of *S. haematobium* also transmit *S. bovis* (Rollinson and Southgate, 1987; Brown, 1994). Of relevance to the present study is the fact that *Biomphalaria* species that transmit the human pathogen *S. mansoni* also transmit *S. rodhaini*, a parasite of rodents and carnivores. Not surprising, the schistosome species that share snail intermediate hosts are also very closely related phylogenetically (Rollinson and Southgate, 1987; Morgan et al., 2003b), and are even known to form hybrid species (Taylor, 1970; Kruger et al., 1988; Morgan et al., 2003b). For example, the bovine parasites *S. bovis* or *S. mattheei* are closely related to the human pathogen, *S. haematobium*; they are capable of forming hybrid species with *S. haematobium* and share with it *Bulinus (Africanus)* species as intermediate hosts (Chandiwana et al., 1987; Kruger et al., 1988). Similarly, the human parasite *S. mansoni* and *S. rodhaini* are close relatives as they are known to form hybrids and share *Biomphalaria* snail species as intermediate hosts (Taylor, 1970; Morgan et al., 2003b). This raises the possibility that such species could potentially form hybrid species in nature. Incidentally *S. rodhaini*, is endemic in tropical Africa and occurs in areas where *S. mansoni*, the causal agent of human intestinal schistosomiasis is also endemic. This certainly provides opportunity for the two species to interact. Another aspect of animal schistosomiasis often overlooked and which may be relevant to human health concerns the role non-human mammalian schistosome or avian schistosome cercariae may have as causal agents of what is often referred to as swimmer’s itch or cercarial dermatitis in
humans in Africa (Boros, 1989; Muller and Kimmig, 1994). This is an acute inflammatory reaction resulting when animal schistosome cercariae penetrate human skin, and is characterized by a skin rash consisting of round erythematous papules. Cercarial dermatitis is now recognized as an emerging disease (Horak et al., 2002). In general, however, severe dermatitis rarely follows exposure of human skin to cercariae of schistosome species that normally develop in humans.

2.1 Schistosoma mansoni Group Species

Mammalian schistosomes are traditionally categorized into 4 groups (S. haematobium, S. mansoni, S. indicum and S. japonicum) based on egg morphology, the species of the snail hosts involved as intermediate and continental distribution (Rollinson and Southgate, 1987). Species traditionally grouped under the S. mansoni group are listed in Table 4, which also shows the snail intermediate host involved, continental distribution, and the mammalian hosts.

Table 4: Species traditionally considered members of the S. mansoni group, their snail intermediate hosts, continental distribution, and their mammalian hosts

<table>
<thead>
<tr>
<th>Species</th>
<th>Snail Host</th>
<th>Continental Distribution</th>
<th>Mammalian Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mansoni</td>
<td>Biomphalaria spp</td>
<td>South America, Caribbean Africa, Madagascar</td>
<td>human, baboon monkeys, rodents</td>
</tr>
<tr>
<td>S. rodhaini</td>
<td>Biomphalaria spp</td>
<td>Africa</td>
<td>rodents, carnivores</td>
</tr>
<tr>
<td>S. edwardiense</td>
<td>Biomphalaria spp</td>
<td>Africa</td>
<td>hippopotamus</td>
</tr>
<tr>
<td>S. hippopotami</td>
<td>Bulinus sp</td>
<td>Africa</td>
<td></td>
</tr>
</tbody>
</table>
A recent phylogenetic study based on the analysis of 8350 bases of combined nuclear and mitochondrial DNA has shown that the long tail stem cercariae of *S. edwardiene*, *S. hippopotami* and the newly discovered mammalian schistosome cercariae clustered together in a well supported clade, and these did not associate with any of the recognized species groups, or with *S. mansoni* or *S. rodhaini* (Morgan *et al.*, 2003b). *S. mansoni* and *S. rodhaini* on the other hand, clustered together in this analysis. Based on these observations, only *S. mansoni* and *S. rodhaini* belong to the *S. mansoni* species group, and that *S. edwardiene*, *S. hippopotami* (probably transmitted through *Bulinus truncatus*), and the newly discovered mammalian schistosome species (utilizing the planorbid snail *Ceratophallus natalensis* as an intermediate host), belong to a new lineage (Morgan *et al.*, 2003b). Experimental studies have established that *S. mansoni* and *S. rodhaini* are capable of forming hybrid offspring (Taylor, 1970; Basch, 1991); a natural *S.mansoni-S.rodhaini* hybrid was discovered on the Ukerewe Island of L.Victoria, in Tanzania (Morgan *et al.*, 2003a)

### 2.2 Schistosoma mansoni

*Schistosoma mansoni* is the most widespread and probably the most important of the human schistosome species. Globally, the parasite is distributed throughout Africa, Madagascar, the Arabian Peninsula, parts of the Caribbean region and South America (Chitsulo *et al.*, 2000; Morgan *et al.*, 2001) and is responsible for causing human intestinal schistosomiasis. On the African continent, its distribution extends from the Nile delta, the middle and Upper Egypt spreading southwards through the Sudan, West Africa, East and Central Africa all the way to southern Africa.
Like *S. rodhaini*, the parasite is a member of the *S. mansoni* group species, and its eggs are characterized by a lateral spine (Figure 4), and these are normally excreted with the faeces of the definitive host. Occasionally, however, *S. mansoni* eggs may appear in the urine of infected individuals (Pope *et al.*, 1980; Ratard *et al.*, 1991; Mkoji *et al.*, 1998), and it has been suggested that this happens when a female *S. mansoni* pairs up with a *S. haematobium* male to produce parthenogenetically. Although humans are considered to be the natural definitive hosts of *S. mansoni*, several mammalian species are naturally infected by this parasite. Included among these are non-human primates such as chimpanzee, baboons, monkeys, several rodent species, carnivores such as domestic dog, insectivores (*Crocidura*), and artiodactyla such as cattle and sheep (Rollinson and Southgate, 1987). Of the mammalian species infected in Africa, baboons (*Papio* spp) are important reservoir hosts of *S. mansoni* (Nelson *et al.*, 1962; Fenwick, 1969; Muchemi, 1992; Ghandour *et al.*, 1995; Muller-Graf *et al.*, 1997; Munene *et al.*, 1998; Erko *et al.*, 2003).

Freshwater planorbid snails in the genus *Biomphalaria* serve as the intermediate hosts of *S. mansoni*, and the geographic distribution of the parasite is closely tied to that of the susceptible species of the snail intermediate hosts (Malek, 1985; Brown, 1994). Although 34 species of *Biomphalaria* are recognized, only 18 species are known or presumed to be susceptible to *S. mansoni* infection, 9 are known to be refractory to infection; and 7 have not yet been tested for their susceptibility to *S. mansoni* infection (Malek, 1985; Brown, 1994)
Figure 4 a typical *S. mansoni* egg showing the lateral spine and a fully developed miracidium inside. (Source CDC)

Of the susceptible species, 4 (*B. glabrata* in the Neotropical region, and *B. pfeifferi*, *B. alexandrina* and *B. sudanica* in Africa and the neighbouring regions) play the most significant role in the transmission of *S. mansoni* to humans. In addition, 3 African species, *B. choanomphala*, *B. camerunensis* and *B. stanleyi*, and 2 Neotropical species, *B. straminea* and *B. tenagophila* play lesser but important roles in specific foci (Greer et al., 1990; Brown, 1994). Interestingly, the 12 *Biomphalaria* species represented in Africa, (Table 5) appear to be susceptible to infection with *S. mansoni* (Brown, 1994). The snail species present in Kenya are *B. pfeifferi*, *B. sudanica*, and *B. choanomphala*. Phylogenetic studies of the *Biomphalaria* suggest that the genus may have an American
origin and the Neotropical *B. glabrata* and the African *Biomphalaria* species are close relatives, and are the most susceptible to *S. mansoni* infection (Campbell *et al.*, 2000; DeJong *et al.*, 2001).

Interestingly, several of the species that are susceptible to *S. mansoni* have relatives that are refractory. Also, *S. mansoni* has a remarkable ability to adapt to previously unsusceptible *Biomphalaria* species as hosts (DeJong *et al.*, 2001). According to De Jong *et al.* (2001), the African *Biomphalaria* species also exhibit interesting biogeographical patterns: *B. pfeifferi* the most important intermediate host of *S. mansoni* has widespread distribution and small genetic differences between populations. *B. stanleyi* from L. Albert in Uganda is derived from *B. pfeifferi* and is probably a lacustrine form of *B. pfeifferi*. Similarly, *B. choanomphala* may be a lacustrine form of *B. sudanica*. The *Biomphalaria* species that inhibit the Nile Basin (*B. alexandrina*, *B. choanomphala*, *B. smithi*, and *B. sudanica*) are poorly differentiated.
Table 5: *Biomphalaria* species occurring in Africa and the neighbouring regions

<table>
<thead>
<tr>
<th>Species</th>
<th>Geographic Distribution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pfeifferi</em></td>
<td>Tropical Africa, SW Arabia, Madagascar</td>
<td>most widespread species; most important host of <em>S. mansoni</em></td>
</tr>
<tr>
<td><em>B. rhodesiense</em></td>
<td>Southern Tanzania, Zambia</td>
<td>a <em>B. pfeifferi</em>, based on molecular evidence</td>
</tr>
<tr>
<td><em>B. choanomphala</em></td>
<td>L. Victoria, L. Kyoga, L. Albert In East Africa</td>
<td>a deep water lake species</td>
</tr>
<tr>
<td><em>B. smithi</em></td>
<td>L. Edward, Uganda</td>
<td></td>
</tr>
<tr>
<td><em>B. stanleyi</em></td>
<td>L. Albert, Uganda, L. Chad, L. Chohoha (Burundi),</td>
<td></td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Sudan, Lake Victoria, N. Zambia, Ethiopia, L. Naivasha, L. Jipe, L. Tanganyika, L. Edward</td>
<td></td>
</tr>
<tr>
<td><em>B. barthi</em></td>
<td>Ethiopia</td>
<td></td>
</tr>
<tr>
<td><em>B. alexandrina</em></td>
<td>Egypt, NW Libya, N. Sudan</td>
<td></td>
</tr>
<tr>
<td><em>B. angulosa</em></td>
<td>Southern Tanzania, Zambia, Malawi</td>
<td></td>
</tr>
<tr>
<td><em>B. tchadiensis</em></td>
<td>L. Chad</td>
<td></td>
</tr>
<tr>
<td><em>B. camerunensis</em></td>
<td>Parts of west Africa, Central Africa</td>
<td></td>
</tr>
<tr>
<td><em>B. salinarum</em></td>
<td>Angola, Namibia in SW Africa</td>
<td></td>
</tr>
</tbody>
</table>

(Compiled from Freshwater snails of Africa and their medical importance by Brown)
2.3 *Schistosoma rodhaini*

*Schistosoma rodhaini* is a natural parasite of wild rodents and carnivores, and is phylogenetically related to the human pathogen, *S. mansoni* (Rollinson and Southgate, 1987; Morgan *et al.*, 2003b). Like, *S. mansoni*, *S. rodhaini* utilizes freshwater planorbid snails in the genus *Biomphalaria* as intermediate hosts (Rollinson and Southgate, 1987). The parasite is very rarely found in humans (Haenens and Santele, 1955), and is considered to be of no medical significance.

*Schistosoma rodhaini* was first isolated in mice experimentally exposed to mammalian schistosome cercariae and obtained from naturally infected *Biomphalaria* snails collected from a locality in the vicinity of the city of Lubumbashi in what is now known as the Democratic Republic of Congo (DRC) (Berrie and Goodman, 1962). Twenty years later, Schwetz collected from the same locality, *S. rodhaini* infected *B. pfeifferi*, and later discovered new foci and snails that harboured mixed infections of *S. rodhaini* and *S. mansoni* in DRC (Berrie and Goodman 1962). Since then, *S. rodhaini* has been reported from Rwanda-Burundi (Fain *et al.*, 1953), Uganda (Berrie and Goodman, 1962), Kenya (Nelson *et al.*, 1962) in East and Central Africa, Nigeria in West Africa (Akinbode *et al.*, 1981) and Zimbabwe in Southern Africa (Chandiwana and Taylor, 1985). The parasite was also discovered on the Ukerewe island in Lake Victoria, Tanzania, in East Africa from a naturally infected *B. sudanica* (Morgan *et al.*, 2003a).

*S. rodhaini* appears to be confined to tropical Africa but its distribution is patchy (Morgan *et al.*, 2003a). Within the East African region, the parasite is restricted to localities in and around Lake Victoria (Berrie and Goodman, 1962; Nelson *et al.*, 1962;
Fripp and Goodman, 1967; Morgan et al., 2003a). In Kenya, *S. rodhaini* was first reported in dogs and wild rodents examined during a survey conducted in a locality near the town of Kisumu on the shores of Lake Victoria, Western Kenya, in the early 1960's (Nelson et al., 1962). No other studies have been conducted on the parasite in Western Kenya since then, and virtually nothing is known on the distributional range of the parasite or its prevalence in the area.

Although considered a natural parasite of rodents, natural infections of *S. rodhaini* have been found in dogs and other carnivores (Pitchford, 1977; Deramee et al., 1953; Nelson, 1962; Schwetz, 1954). Laboratory mice and hamsters are susceptible to *S. rodhaini* infection but rabbits and guinea pigs are less susceptible (Fripp, 1968). Non-human primates (particularly, baboons and monkeys) are refractory to *S. rodhaini* infection (Nelson et al., 1962; Pitchford, 1977). Cases of humans infected with *S. rodhaini* are rare, and so far, only one case of a human infection has been reported (Haenens and Santele, 1955).

*S. rodhaini* has a distinctive egg characterized by a terminal or slightly sub terminal lateral spine on one end and a short blunt tubercle at the other end, bent in the direction opposite to the spine (Figure 6), and its eggs show variable morphological shapes (Fripp, 1967). The *S. rodhaini* egg is therefore different morphologically from that of *S. mansoni*. The ovary in the female lies in the anterior half of the body and only one egg at a time is found in the uterus. The male possesses spined tubercles on the tegument and has 5 testes.
Figure 5 Egg of *Schistosoma rodhaini* showing the terminal spine pointing upwards and the other end knob-like pointing in opposite direction (Kemri schistosomiasis group).

The *Biomphalaria* species naturally infected with *S. rodhaini* are *B. pfeifferi* and *B. sudanica*. However, *B. arabica* and *B. salinarum* are also susceptible under experimental conditions in the laboratory (Rollinson and Southgate, 1987).

2.4 *S. mansoni*-*S. rodhaini* interactions

Typically, *S. rodhaini* exists in areas where *S. mansoni* is also endemic. It is therefore, not unusual for the two parasite species to co-exist in the same locality or
habitat. Within the East African region for instance, *S. rodhaini* occurs in the Lake Victoria area where *S. mansoni* also commonly occurs. Given that these 2 species utilize *Biomphalaria* snails as intermediate hosts, and are also capable of infecting several species of wild rodents (Rollinson and Southgate, 1987), the possibility for interaction between the 2 species is high.

There are several ways through which *S. rodhaini* and *S. mansoni* could possibly interact. Such interactions could occur at the level of the snail intermediate host or in the definitive host. At the snail host level, inter-specific interactions could be competitive or non-competitive. Competitive interactions between larval digenetic trematodes frequently occur and are fairly common (Mouahid and Mone, 1990; Sousa, 1992). In such situations, one species tends to interfere with the establishment of the other species and in the process excludes the latter species. For instance, the echinostome parasite, *Echinoparyphium elegans* is known to suppress the development of *Schistosoma bovis* and inhibits cercariae generation in the snail *Bulinus truncatus* (Mouahid and Mone, 1990). In other situations, intra-molluscan larval trematode interactions may not only be non-competitive but one species will in fact aid the establishment of the second species in the snail host. For example, infection of the freshwater planorbid snail, *Bulinus tropicus* (normally refractory to the bovine schistosome *S. bovis*) with an amphistome, *Calicophoron microbothrium* renders *Bulinus tropicus* susceptible to infection with *S. bovis* (Southgate et al., 1989).

Dual infections of *S. rodhaini* and *S. mansoni* in the snail host are not uncommon (Schwetz, 1953; Brown, 1994; Morgan et al., 2003). However, it is not clear whether or not intra-molluscan interactions do occur between *S. rodhaini* and *S. mansoni*, and if they
do occur, what sort of interactions occur. The inability to easily identify and differentiate between the intra-molluscan stages of the 2 parasites has partly hindered studies on interspecific interactions of these 2 closely related schistosome species. The cercariae of *S. mansoni* and *S. rodhaini* are very similar morphologically, therefore, are not easily distinguishable by conventional techniques such as low power light microscopy. At the level of the definitive host, on the other hand, interactions between the adults of closely related mammalian schistosome species leading to species hybridization is a possible scenario even, in nature.

Hybrids of closely related schistosome species have successfully been produced experimentally in laboratory animals (Taylor, 1970; Theron, 1989; Basch, 1991). Also, natural hybrids have been reported for species within the *S. haematobium* group (Southgate *et al.*, 1976; Wright and Ross, 1980; Kruger, 1990). Although *S. rodhaini* rarely infects humans (Schwetz, 1953; Haenens and Santele, 1955), it is conceivable that its hybridization with *S. mansoni* could potentially occur in nature. Both *S. mansoni* and *S. rodhaini* naturally infect a variety of rodent species and carnivores (Nelson *et al.*, 1962; Rollinson and Southgate, 1987), raising the possibility that the 2 species could potentially interact in their natural definitive hosts to produce hybrid species whenever the 2 species occur sympatrically. Indeed, a collection of *B. sudanica* snails from a locality on the Ukerewe Island, Lake Victoria, Tanzania yielded an infection of *S. mansoni*-*S. rodhaini* hybrid males in mice which were revealed by DNA sequencing (Morgan *et al.*, 2003). While the exact consequences of species hybridization in nature may not be immediately very clear, such interactions could fundamentally alter the epidemiology of human schistosomiasis in a given locality or area. Therefore, it is
important to have reliable means of identifying closely related schistosome species such as *S. mansoni* and *S. rodhaini* so that hybrids could easily be spotted.

### 2.5 *S. rodhaini* and the epidemiology and control of *S. mansoni*

 Whereas the identification of *S. mansoni* and *S. rodhaini* at the egg level does not pose any problems identification of intra-molluscan stages of the parasites, in particular, the cercariae, is difficult and poses special challenges. Given that the cercariae of *S. mansoni* and *S. rodhaini* are morphologically very similar, and not easily distinguishable parasitologically, the presence of both species in a given area or locality could potentially complicate the epidemiology and control of human intestinal schistosomiasis in several different ways.

If the 2 species occur sympatrically in the same locality or habitat, identification of sites involved in the transmission of the human pathogen *S. mansoni* becomes difficult using parasitological procedures that involve screening of snails for schistosome infections and examination by microscopy.

Also, obtaining accurate information on the success or failure of the chemotherapeutic intervention against *S. mansoni* becomes difficult due to the fact that *S. rodhaini* cercariae may continue to be present in the snail populations even when *S. mansoni* has been eliminated after successful intervention with chemotherapy. Studies on the transmission ecology of any of the 2 parasite species also, become difficult to perform in localities where the 2 species co-exist. Even though *Biomphalaria* snails are capable of transmitting dual infections of *S. mansoni* and *S. rodhaini* studying the nature of
this dual transmission is hampered by the inability to rapidly identify the cercariae of these closely related species.

Similarly, investigations of any intra-molluscan interactions between *S. mansoni* and *S. rodhaini* are difficult to perform because it is not possible to accurately identify the species of the parasite occurring in the snail host. Also, because the 2 species are capable of forming hybrid species even under natural conditions, species hybridization could potentially complicate the epidemiology of *S. mansoni* in an endemic locality or area. Finally, can *S. rodhaini* break species barrier and infect immunocompromized individuals, for instance? This question cannot be answered without further investigations on this parasite. It is possible that species hybridization occurs in dioecious parasites present in immunocompromised individuals (Coombes and Jourdane, 1991).

Techniques that rapidly identify *S. mansoni* and *S. rodhaini* could also facilitate studies on immunological interactions between the two species. Exposure of humans to cercariae of animal schistosomes is believed to confer some level of protection against infection by human schistosomes (Nelson, 1962). Heterogonous immunity elicited by a different species does occur in trematode species including mammalian schistosomes (Navarrete *et al.*, 1994)

One wonders whether exposure of humans to *S. rodhaini* would not sensitize individuals against *S. mansoni* infection.

Consequently, accurate methods for identification of *S. mansoni* group species at cercarial level are needed. Molecular approaches are increasingly being used for parasite identification and especially for identification/diagnosis of closely related species (Scott *et al.*, 1993; Barber *et al.*, 2000).
Where closely related schistosome species occur together in one locality or habitat as in the case with *S. mansoni* and *S. rodhaini* in the Lake Victoria region, it is difficult to accurately identify habitats actively involved in the transmission of the human parasite for instance, or to evaluate success or failure of control efforts directed towards the human parasite by sampling natural snail populations. Examination of field-collected snails for schistosome-infections is a simple, rapid and inexpensive procedure for identifying disease transmission foci or for monitoring parasite transmission sites after chemotherapeutic intervention, for instance (Sturrock, 1986; Sturrock *et al.*, 1994). Snail sampling is also suitable for studying seasonal or diurnal patterns of schistosome transmission. However, the usefulness of this procedure as an epidemiological tool is hampered by the inability to rapidly identify and distinguish between intra-molluscan larval forms, in particular cercariae of closely related schistosome species. Procedures for identifying mammalian schistosome cercariae such as microscopic analysis of cercarial morphology (Bayssade-Dufour *et al.*, 1989; Touassem *et al.*, 1992; Bayssade-Dufour, 1977), deduction of cercariae identity from morphology of adult worms or the eggs they produce in laboratory animals (Prentice and Ouma, 1984; Kimura *et al.*, 1994; Yousif *et al.*, 1996), studying cercariae emergence patterns (Pitchford and Dutoit, 1976; Theron, 1984; Mouahid and Theron, 1986; Mouchet *et al.*, 1992), or analysis of cercariae isoenzyme patterns (Mahon and Shiff, 1978; Boissezon and Jelnes, 1982) tend to be long, cumbersome, and are considered impractical for routine use. In addition, some of the procedures available may not be of value in identifying cercariae of closely related species.
2.6 Methods for identifying cercariae of mammalian schistosome species

Although the eggs of both *S. mansoni* and *S. rodhaini* are characterized by a lateral spine, they are very distinct morphologically and easily identifiable by microscopy. At the cercariae level, however, the two parasite species are not easily distinguishable under a light microscope at low power. Mammalian schistosome cercariae can be distinguished by the pattern they exhibit as they are released by the snail intermediate host by examining eggs or adult worms recovered from lab rodents several weeks after cercariae exposure (Prentice and Ouma, 1984; Kimura et al., 1994; Yousif et al., 1996; Pitchford and Dutoit, 1976; Theron, 1984; Mouahid and Theron, 1986; Mouchet et al., 1992), by analysis of isoenzyme markers (Mahon and Shiff, 1978; Boissezon and Jelnes, 1982; Fletcher et al., 1981), by chromosomal analysis (Short and Grossman, 1981) or by examining patterns of morphological characters (Bayssade-Dufour et al., 1989; Touassem et al., 1992). Cercariae of *S. mansoni* can be differentiated from those of *S. rodhaini* by their patterns of cercariae shedding, with the former having a diurnal pattern while the latter is nocturnal (Pitchford et al., 1996; Pitchford and Dutoit, 1976; Theron, 1984; Mouahid and Theron, 1986). Analysis of isoenzyme markers has been applied to distinguish between the adults and cercariae of *S. mansoni* and *S. rodhaini* (Fletcher et al., 1981; Boissezon and Jenes, 1982; Rollinson et al., 1986). Differences between the cercariae of the two parasite species have also been revealed by chromosomal studies (Short and Grossman, 1981), and by examining patterns of sensory receptors and other morphological characters. Iso-enzyme analysis using the iso-electric focusing techniques has also been applied to characterize *S.*
mansoni-S. rodhaini hybrids (Bremond et al., 1989), and S. mansoni isolates (Sene et al., 1997), but this procedure is tedious and unsuitable for routine use.

A procedure commonly used to isolate and identify schistosomes involves exposure of laboratory rodents to cercariae shed from field-collected snails, and waiting several weeks for the parasites to grow into adults and reproduce. The identity of the parasite in this case is determined by examining the characteristic eggs produced by the adult worms or through examining the adult worms after staining for characteristic morphological feature. While such a procedure and others are useful for research purposes, they may not be suitable for routine application in epidemiological studies or to support schistosomiasis control efforts.

Molecular approaches such as those based on the polymerase chain reaction (PCR) technology (Saiki et al., 1988; 1997 Rollinson et al., 1997) can help elucidate the transmission ecology factors relevant to the epidemiology, prevention and control of intestinal schistosomiasis in Kenya. PCR is a primer-directed in vitro enzymatic reaction for the production of a specific DNA fragment. The present study explored the use of a PCR-based approach for identifying the cercariae of S. mansoni and S. rodhaini. PCR-based approaches for amplifying specific segments of the genomic DNA (Saiki et al, 1988) have been applied for species/strain diagnosis, detection, identification and characterization in a variety of organisms including parasitic protozoa or helminths (Barber et al., 2000; Morales-Hojas et al., 2001; Myjak et al., 2002; Szostakowska et al., 2002; Amar et al., 2002; Rubio et al., 2002; Itagaki et al., 2003; Strauss-Ayali et al., 2004; Amar et al., 2004; food borne pathogens Gonzalez et al., 2004. PCR is relatively simple, rapid, and can be performed using only small quantities of material. In addition to the conventional PCR
assay, several PCR-based procedures are available for use in species identification and population characterization.

They include PCR-RAPD, PCR-SSCP, PCR-AFLP, and PCR-RFLP.

The PCR-random amplified polymorphic DNA (RAPD) assay, also referred to, as Williams et al. (1990) first described arbitrary primed PCR and Welsh and McClelland (1990). The procedure is based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of an organism's genome. If two RAPD primers anneal in close proximity (up to a few kilo bases) to each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers results. The procedure has been applied for species/strain identification and genetic characterization of a variety of organisms including plants, fungus, protozoans, helminths, arthropods and molluscs (Ballinger-Crabtree et al., 1992; Landry et al., 1993; Cenis, 1993; Kaul et al., 2004; Gruijter et al., 2004; Daniel et al., 2004), and may also be useful in schistosome genetics, phylogenetics and taxonomy (Barral et al., 1993; Neto et al; 1993; Kaukas et al., 1994; Pillay and Pillay; 1994). The assay has also been shown to have potential in determining sex in schistosomes (Barral et al., 1993). A major advantage of the RAPD approach is that it is simple to perform, and complex and informative genomic DNA patterns can readily be generated without prior knowledge of nucleotide sequence in the organism of interest (Williams et al., 1990; Welsh and McClelland, 1990).

The PCR-single strand conformational polymorphism (SSCP) has been used for characterizing populations. The assay is based on the principle that changes in nucleic
acid composition affect the conformation of single stranded DNA and thereby the
mobility of the fragment when subjected to electrophoresis under non-denaturing
conditions (Hayashi, 1991; Nataraj et al., 1999). In other words, SSCP assays were
developed based on the fact that the mobility of a double-stranded DNA of a given length
on a gel is relatively independent of its nucleotide sequence. On the other hand, the
mobility of single strands of DNA can vary considerably as a result of only small changes
in nucleotide sequence. The SSCP procedure is designed to analyse genetic
polymorphism at single loci. A specific PCR primer pair flanking the target region is
used to amplify DNA from an individual. Single-stranded DNA is produced by
asymmetric PCR in which one primer is present in excess of the other. After the primer in
smaller quantities is used up, the reaction continues, producing only a single strand. The
mobilities of single strands are then compared by gel electrophoresis. This procedure has
potential for studying aspects of taxonomy, diagnosis, population genetics, drug
resistance and molecular evolution in parasitic organisms (Gasser and Chilton, 2001), and
has been applied to identify parasitic nematodes (Gasser and Monti, 1997). This assay
could potentially be used for differentiating and characterizing schistosomes. The PCR-
amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique
based on the selective amplification of a sub-set of DNA fragments generated by
restriction enzyme digestion (Vos et al., 1995). The technology comprises the following
basic steps: 1) digestion of the DNA with two restriction enzymes, preferably a 6-base
cutter and a 4-base cutter, 2) ligation of double-stranded adapters to the ends of the
restriction fragments, 3) the amplification of a sub-set of the restriction fragments using
two primers complementary to the adapter and restriction site sequences, 4) gel
electrophoresis of the amplified restriction fragment, and 5) the visualization of the DNA fingerprints by means of auto-radiography, phospho-imaging or other methods. Originally applied to the characterization of plant genomes, AFLP has in the recent past been used to characterize a variety of organisms including bacteria (Janssen et al., 1996; Gibson et al., 1998), fungi (Schmidt et al., 2004), protozoa (Martinelli et al., 2004), and arthropods (Paupy et al., 2004). The technique has potential use in characterizing helminthes including schistosomes. In the PCR-restriction fragment length polymorphism (RFLP), a specific gene locus or DNA target is amplified by PCR using gene or target specific primers. The resulting amplified product is then digested with a restriction enzyme that recognizes a cut site on the amplified DNA, the digestion fragments are separated on agarose or polyacrylamide gel by electrophoresis, and the RFLP patterns generated are visualized after ethidium bromide staining. PCR-RFLP has proved to be a popular tool for characterization of species and/or populations in a variety of organisms including microbial organisms (Park et al., 2004; Godoy et al., 2004), protozoa (Cuervo et al., 2004; Ranjit et al., 2004), helminths (Gasser and Hoste, 1995; Gasser et al., 1996; Maravilla et al., 2004; Traub et al., 2004), molluscs (Carvalho et al., 2004); arthropods (Nicolescu et al., 2004; Wagener et al., 2004), and vertebrates (Sell and Spirkovski, 2004; Pfeiffer et al., 2004). The PCR-RFLP procedure has been used successfully to distinguish between strains or species of parasitic worms (Gasser and Hoste, 1995; Gasser et al., 1996; Cunningham, 1997; Reno et al., 2000; Maravilla et al., 2004; Traub et al., 2004), including schistosomes (Despres et al., 1993; Barber et al., 2000).
In the present study, the PCR-RFLP assay was developed for use in identifying and differentiating between *Schistosoma mansoni* and *S. rodhaini*.

### 2.7 PCR as a tool for identifying and characterizing schistosomes

PCR-based procedures have been applied in a variety of situations for detection, identification and characterization of schistosomes. PCR assays are now available for detection of schistosome cercariae in waterbodies, and these have potential for use in monitoring human schistosome infestations of waterbodies in endemic localities (Hamburger *et al.*, 1998; Hamburger *et al.*, 2001). PCR has also been applied to detect and identify schistosome infections in the snail intermediate hosts (Hamburger *et al.*, 2004; Hanelt *et al.*, 1997).

Similarly, PCR assays involving RAPD analysis identify and discriminate schistosome species, strains or individuals, and genetically characterize schistosome populations (Barral *et al.*, 1993; Barral *et al.*, 1996; Dias Neto *et al.*, 1993). The PCR-RAPD assay has also been found to be useful for detecting differences between species or isolates of *Schistosoma* species. Also, PCR-RFLP techniques have been applied to identify and discriminate the closely related *S. haematobium* and *S. bovis* (Barber *et al.*, 2000), and to characterize geographic isolates of schistosome species (McManus and Hope, 1993). PCR has also provided a novel approach for determining the sex of *S. mansoni* miracidia and cercariae (Penschow *et al.*, 1993). Thus, PCR has great potential for schistosome identification and characterization. In the present study PCR was explored for its usefulness in identifying and discriminating between cercariae of *S. mansoni* and *S. rodhaini*. 
2.8 Genomic DNA targets useful for species identification and population characterization

Among the DNA targets commonly used to provide markers for species identification and population characterization include the ribosomal gene complex (rDNA), (Hills and Dixon, 1991) and the mitochondrial DNA (mtDNA) (Simon et al., 1994). Ribosomal genes are useful in providing diagnostic markers because they occur in relative abundance in eukaryotes, and contain spacer regions with sequences that are known to differ even between very closely related species (Hillis and Dixon, 1991). The value of the rDNA gene complex in this regard is based on locating a species-specific sequence in the gene complex, and designing flanking oligonucleotide primers that target that particular sequence for use in a PCR reaction. Analysis of the rDNA gene complex provides markers useful, not only for studying phylogenetic relationships in species, but also for species identification and characterization in a wide range of organisms (Blair and McManus, 1989; Knight et al, 1991; Allsopp et al. 1993; Kane and Rollinson, 1994; Goggin et al; 1994; Messner et al; 1995; Hillis and Dixon, 1991) including schistosomes (Walker et al; 1986; Kaje and Rollinson, 1994; Kaukas et al., 1994; Barber et al., 2000) marine parasites (Goggin, 1994). The rDNA gene complex consists of a 5' end non-transcribed spacer (NTS) region, followed by an external transcribed spacer (ETS) region, an 18S small subunit, an internal transcribed spacer (ITS) which is separated into two by a 5.8S gene; and downstream the ITS region is the 28S large subunit gene, which is followed by another NTS region (Long and Dawid, 1980) figure 7. This gene complex has been shown to be of value in parasite species identification, epidemiological studies or laboratory investigations (Blair and McManus, 1989; Waters and McCutchan, 1990; Hillis and Dixon, 1991; Johnston et al.,
1993). Of great value in taxonomic and phylogenetic analysis of species are the ITS and NTS regions of the gene complex (Waters and McCutchan, 1990; Hillis and Dixon, 1991; Bowles et al., 1995). Research focusing on the rDNA gene complex in mammalian schistosomes has indicated presence of species level variations in the sequences of the ITS region (Walker et al., 1986; Ali et al., 1991; Rollinson et al., 1993; De Gruijter et al., 2004; Kane and Rollinson, 1994; Barber et al., 2000). Walker et al., (1986) identified species-specific enzyme restriction sites within the ITS and NTS regions among members of the *S. haematobium* group species, and such species-specific restriction sites in the ITS2 have been exploited to differentiate between *S. haematobium* and *S. bovis* (Barber et al., 2000).

Mitochondria are subcellular organelles in which oxidative phosphorylation and other important biochemical functions take place within the cell. Within these organelles is a genome, called the mitochondrial genome, which is distinct from, but cooperates closely with the nuclear genome of the cell. In the majority of organisms the mitochondria genome consists of 2 ribosomal genes, 22 transfer genes and 13 protein- coding genes. The mitochondrial genome provides a rich source of markers for population genetic and systematic studies in a variety of organisms including parasitic helminthes (Feagin, 2000; Le et al., 2000).
Figure 7: A schematic representation of the ribosomal gene complex (Long and Dawid).
Mitochondrial DNA (mtDNA) sequences have been obtained for *Schistosoma* species (Blair *et al.*, 1999), and have been used for population characterization, phylogenetic and taxonomic studies among schistosome species (Depres *et al.*, 1993; Rollinson *et al.*, 1997; Morgan *et al.*, 2000). Figure 8 provides a schematic representation of the mitochondrial genome of a eukaryote. One of the regions of the mtDNA considered to be of value in species discrimination is 16S mitochondrial ribosomal gene. In the present study the 16S-12S region of the mtDNA gene was examined for markers for identifying and differentiating between *S. mansoni* and *S. rodhaini*. Molecular approaches based on amplifying specific targets of DNA in a PCR assay have been amplified in several identification studies in a variety of organisms, including parasite species (Scott *et al.*, 1993; Stich *et al.* 1993; Tanaka *et al.*, 1993), (Simpson, 1987; McCutchan *et al.*, 1984; Rollinson *et al.*, 1986).
Figure 8. A schematic representation of the mitochondrial genome of a eukaryote
(Source Sameer Z.Rainas in “Evolution of base substitution gradients in primate mitochondrial genomes”)
CHAPTER THREE
MATERIALS AND METHODS

3.1 The Study Area

The Nyanza area was chosen as an ideal site for the present study. *S. mansoni* is endemic in the area, and *S. rodhaini* was once isolated from the area (Nelson *et al.*, 1962). The study area lies within the equator between Latitude 00° 16N and 00° 58°S, and between Longitude 34° 14E and 34° 17E. The altitude of the area ranges between 1500-2000m above sea level. It experiences an equatorial type of climate with rainy seasons during the period March-May and September-December every year and dry seasons during January-February and June-August. Major occupational activities include farming, livestock keeping, and fishing. The economy of the area depends on agriculture, fishing and tourism. The area is known to be endemic for human schistosomiasis (Wijers and Munanga, 1971; Pamba, 1974; Kinoti, 1971a,b; Thiong’o *et al.*, 2001) with both *S. mansoni* and *S. haematobium* present, but *S. mansoni* is the most common. Schistosomiasis transmission takes place along the shores of Lake Victoria, and to some extent, in streams flowing into the lake and in habitats located near the lake. Also, present in western Kenya is the bovine parasite *Schistosoma bovis*, the causal agent of bovine schistosomiasis mainly in cattle (Southgate and Knowles, 1975; Jelnes, 1983). Freshwater snails in the genus *Biomphalaria* were collected from habitats and sites within this area and screened for the presence of mammalian schistosome cercariae.
3.2 Snail Collection

Between the year 2000 and 2002, *Biomphalaria* snails were collected from freshwater habitats present near or along the shores of Lake Victoria in western Kenya using a snail scoop (Figure 9). *Biomphalaria* collection sites were habitats located in and around the city of Kisumu and in the Homa Bay town-Mbita Point area, along the shores of Lake Victoria and habitats (streams, ponds, ditches, etc) located near the lake, in western Kenya. Each habitat was searched for snails along the edge or shoreline for 20-30 minutes. Latitude/longitude data was also recorded for each snail collection site using a hand-held global positioning system (GPS) apparatus (Magellan GPS2000 Satellite Navigator, Magellan Systems Corporation, California, USA). The snails collected from each habitat were sorted out into gastropod families and were identified to genera or species based on shell morphological characters, using identification keys and descriptions provided by the Danish Bilharziasis Laboratory (1987) and Brown (1994). Individuals in the genera or species were counted to determine abundance, and all the planorbid snails collected were routinely screened for presence of mammalian schistosome cercariae. For the purpose of this study, only data and information pertaining to the *Biomphalaria* snails is presented. Table 6 shows the localities and sites in western Kenya from which *Biomphalaria* snails were collected, and latitude/longitude data for the snail collection sites.
Table 6: Localities in western Kenya from where *Biomphalaria* snails were collected

<table>
<thead>
<tr>
<th>Snail species</th>
<th>Location</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. sudanica</em></td>
<td>Impala</td>
<td>00°02'55S</td>
<td>34°44'40E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Kimani farm</td>
<td>00°06'26S</td>
<td>34°44'46E</td>
</tr>
<tr>
<td><em>B. pfeifferi</em></td>
<td>ADC farm</td>
<td>00°05'34S</td>
<td>34°42'44E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>KFA</td>
<td>00°05'54S</td>
<td>34°44'55E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Homabay</td>
<td>00°31'34S</td>
<td>34°27'28E</td>
</tr>
<tr>
<td><em>B. pfeifferi</em></td>
<td>Rangwena river</td>
<td>00°31'59S</td>
<td>34°29'40E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Karachuonyo</td>
<td>00°26'58S</td>
<td>34°29'22E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Luanda market</td>
<td>00°28'59S</td>
<td>34°17'38E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Mbita point</td>
<td>00°25'05S</td>
<td>34°12'25E</td>
</tr>
<tr>
<td><em>B. sudanica</em></td>
<td>Tiengre</td>
<td>00°05'34S</td>
<td>34°42'25E</td>
</tr>
</tbody>
</table>

3.2.1 Screening of Snails for Schistosomes

Screening of *Biomphalaria* snails for mammalian schistosomes involved placing individual snails in wells of 24-well culture plates (Figure 13) in a volume of 1.5 ml of aerated tap water, and illuminating the wells with a light bulb for 1-2 hr to induce release of cercariae. After the light illumination period, each well was examined for the presence of cercariae under a dissecting microscope. Routinely, the snails were also left in the plate wells in darkness overnight to induce release of trematode cercariae that exhibit a nocturnal cercaria release pattern. Larval trematode cercariae, the infective stage of the parasite released by the snails swim freely in the water, searching for a suitable host to enter.
3.2.2 Infection of mice with cercariae and schistosome isolation

Cercariae from field-collected *Biomphalaria* snails were used to infect laboratory Swiss albino mice using the procedure described by Smithers and Terry (1965). Briefly mice were anaesthetized by an intraperitoneal injection of pentobarbitone sodium (Sagatal™) at dose of 0.12mg per kg body weight and abdominal area of each mouse was shaved using an electrical hair-shaving machine. A 12mm-diameter stainless ring (weighing 13.096gm) was placed onto the shaved abdominal area to prevent leakage of the cercariae suspension, and the cercariae placed onto the skin through the open end of the steel ring using a 1-ml micropipette (P-1000) (Gilson™) (Figure 11). The parasites were allowed 1 hour to penetrate the mice abdominal skin, after which water with remaining cercariae which had not penetrated or had died was removed using the micropipette. The mice were then placed in a cage lined with wood sawdust and the cage placed in a warm room to recover. After full recovery the mice were returned to their special room. The mice were ready for perfusion after eight weeks.
3.2.3 Schistosome recovery and isolation from mice

Eight weeks after exposure to cercariae, mice were killed by injecting each with an overdose of sodium pentobarbitone (0.24mg/kg body weight), and the adult worms were recovered by perfusion of the hepato-mesentric vascular system of the mouse gut using a solution of 20mm Na-Citrate essentially, as described by Smithers and Terry (1965). A 20-ml hypodermic syringe was used to pump water through the left heart of the mouse to perfuse the hepato-mesentric system.
Figure 9  The apparatus (snail scoop) used for collecting freshwater snails from field habitats
3.2.4 Isolation of Schistosomes in Mice

Mammalian schistosome cercariae shed by a field-collected snail were identified indirectly, by allowing them to infect laboratory mice and grow into adult
worms. By studying the morphology of the resulting adult worms after staining or the morphological characteristics of the eggs produced one deduces the identity of the infecting schistosome species, and indirectly, the cercariae. This procedure was used as an initial step in the identification of the mammalian schistosome cercariae obtained from field-obtained *Biomphalaria* by “shedding”.

### 3.2.5 Perfusion

A 20-ml hypodermic syringe was used to pump water through the left heart of the mouse to perfuse the hepato-mesenteric system. The recovered worms were cleaned in 3 washes of physiological saline solution (0.15M NaCl), preserved in 70% absolute ethanol, and stored at 4°C for use later in molecular analysis. Examining the schistosome eggs produced by the adult worms in the definitive host identified the infecting mammalian schistosome. To do this, fresh squashes of liver tissue from perfused mice previously exposed to cercariae from naturally infected snails were prepared between a pair of glass microscope slides, and were examined for presence of schistosome eggs under a compound microscope.
Figure 11 The apparatus used to expose lab mice to schistosome cercariae
Figure 12 The apparatus for perfusing mice to recover adult schitosomes
3.2.6 Confirmation of *S. rhodaini* Identity by DNA sequencing

Briefly, DNA of ethanol-preserved adult worm specimens was extracted in a total volume of 200 μl using the HotSHOT method described by Truett *et al.* (2000). The polymerase chain reaction (PCR) was used to amplify the 16S-12S of mtDNA. The primers used for PCR are provided in Morgan *et al.*, (2003). Each reaction contained 0.5μl of each primer, 13μl template DNA, 10x Taq buffer, 0.8μl mM dNTP, 3.75μl mM MgCl₂ and 0.05 U/μl of Taq polymerase (Promega). This mix was thermo cycled for 30 cycles, programmed to ramp between temperatures at 1°C per second. Cycle 1 was 95°C for 60 sec, 50°C for 45 sec and 72°C for 90 sec, followed by 29 shorter cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec. The reaction mix was then held at 72°C for 7 min to complete extension then dropped to 4°C. PCR products were viewed on an ethidium bromide stained 1% agarose and TAE gel. PCR products were concentrated and desalted using a Micron centrifugal filter device (Millipore), and were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems), and the products run on an ABI 377 automated sequencer. Forward and reverse sequences were aligned and edited using Sequencer™ version 4.0.5 (Gene Codes Corp, Ann Arbor, MI, USA, 1999).
The rDNA ITS2 and mtDNA 16S sequences obtained for the worms under investigation were then compared to published sequences of *S. mansoni* and *S. rodhaini* to confirm parasite identity.

### 3.2.7 Establishment of *S. rodhaini* (W2018-01) in the laboratory and its routine maintenance

One of the isolates of *S. rodhaini* (designated W2018-01) was established under laboratory conditions at the Centre for Biotechnology Research and Development (CBRD) of the Kenya Medical Research Institute (KEMRI), and is currently, routinely maintained through Swiss albino mice and laboratory-raised *Biomphalaria pfeifferi* snails. For routine maintenance of the parasite, 5-6 mice are infected at any given time, and each mouse receives between 100-150 cercariae percutaneously through shaved abdominal skin under pentobarbitone sodium anaesthesia using the procedure described by Smithers and Terry (1965). The mice are exposed to cercariae for 1-1.5 hr. Seven to 8 weeks after cercarial exposure, the mice are killed with an overdose of pentobarbitone sodium (0.24 mg/gm body weight), the animals are perfused to recover the worms essentially as described by Smithers and Terry (1965), and the perfused liver and intestinal tissue are transferred into a kitchen blender and homogenized in 0.15M NaCl (physiological saline). The homogenate is then passed through a series of 4 nested sieves (from top to bottom mesh sizes 710μm, 425μm, 212μm, 125μm, 45 μm) provided with a receiving dish. The eggs collect onto the last sieve, and are then transferred into one litre conical flask, cleaned in 3 washes of physiological saline by sedimentation and decanting, and finally, aerated tap water is added to the sediment containing eggs to allow egg hatching. The flask is covered with aluminium foil except a bit of the neck, and because the miracidia (the larval forms hatch from the eggs) are phototropic they will
move up the flask and collect in the neck area. Here they are harvested and used for exposing laboratory-bred *B. pfeifferi*. Snails are exposed to miracidia individually in 24-well culture plates in a volume of 1.5ml of aerated tap water. Each snail received 3 miracidia and the snails are exposed to the parasite for at least 1-2 hr. Four weeks after exposure to miracidia, the snails were screened for the presence of cercariae using the “shedding” method already described in section 3.1.3 above. Figure 13 shows a 24-well plate used for screening snails for cercariae or exposing snails to schistosome miracidia. If cercariae were present, they were used to initiate the cycle all over again by infecting mice (Section 3.1.4 above).
3.2.8 Characterization of the cercariae shedding pattern of *S. rodhaini* (W2018-01)

The establishment of the *S. rodhaini* isolate (W2018-01) in the laboratory, provided an opportunity to make observations on its course of infection in the *Biomphalaria* snail hosts, and study its pattern of cercariae release from the snails. Cercarial release patterns have been used to identify and distinguish between species or strains of mammalian schistosomes (Theron, 1984; Theron, 1989; Mouchet *et al.*, 1992). Laboratory-raised *B. pfeifferi* each infected with a single miracidium of *S. rodhaini* were placed in individual wells of a 24-well culture plate in a volume of 1.5 ml aerated tap water, and observed for cercariae release at one-hourly intervals over a period of 24 hr. At each interval, individual snails were rinsed briefly in aerated tap water, and transferred into wells of another culture plate. A drop of 0.1% solution of Lugol’s iodine (10g iodine and 20g of potassium iodide dissolved in a litre of water) was added into each well containing cercariae to immobilize and stain the parasites. The cercariae suspension was
then transferred into a 5.1 mm diameter plastic petri dish, gridded at the bottom to facilitate easy counting of cercariae. All the cercariae present in the suspension were counted and the numbers recorded for each well. Between 5-15 snails were used per experiment and a total of 4 experiments were performed. The percentage of cercariae released at each hourly interval (of the total shed over the 24-hr period), were plotted against time to present the pattern observed.

3.2.9 Development of a PCR-Based Assay for differentiating between *S. mansoni* and *S. rodhaini*

3.3.0 Parasite Material

A *S. mansoni* (isolate W2092-02) originally obtained from baboon fecal material collected from Kalimakoi, in the Kibwezi area, in southern Kenya and a *S. rodhaini* (W-2018-01) isolate originally obtained from a naturally infected *Biomphalaria sudanica* collected from the city of Kisumu on the Lake Victoria shore, western Kenya were used to optimize the assay. Both parasite species are routinely maintained under laboratory conditions as described in section 3.2.5 above at the Centre for Biotechnology Research and Development (CBRD) of the Kenya Medical Research Institute (KEMRI), Nairobi through out bred Swiss albino mice and laboratory-raised *Biomphalaria* snails. *S. mansoni* is maintained through *B. pfeifferi* originally collected from Thange River, in the Kibwezi area, and *S. rodhaini* is maintained in *B. sudanica* (isolate ID#W2018-01) originally collected from the ADC Farm, Kisumu town, western Kenya. The adult worms of cercariae of the *S. mansoni* or *S. rodhaini* isolates maintained in the laboratory were used as a source of DNA for optimizing the PCR assay. Section 3.1.6 provides detailed procedures for recovery of adult worms from infected mice and for collecting cercariae
from infected *Biomphalaria* snails. The parasite materials were used as soon as were collected or preserved in 70% ethanol in 2-ml cryo-preservation tubes, and stored at 4°C until used for DNA extraction.

### 3.3.1 The 16S-12S region of the mitochondrial DNA (mtDNA) as a target for *S. mansoni* and *S. rodhaini* identification

About the time the work described in this thesis began, partial sequences of the 16S-12S region of the mtDNA for both *S. mansoni* and *S. rodhaini* became available through the work of Morgan *et al.* (2003). These sequences each 762 bases long are available as GenBank Accession Numbers AF531311 and AF531309 for *S. mansoni* and *S. rodhaini* respectively (see appendix). Analysis of the sequences in both *S. mansoni* and *S. rodhaini* for restriction enzyme cut sites (using the program Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2) revealed inter-specific restriction sites for the enzymes *BamHI* and *AccI*. The predicted restriction fragment length polymorphism (RFLP) patterns observed on the sequences with the 2 enzymes are shown on Figure 16 based on these observations a PCR-RFLP assay was developed for identification of these 2 parasite species. Thus, restriction enzyme (RE) cut site variations within a portion of the 16S-12S region of the mtDNA between *S. mansoni* and *S. rodhaini* were exploited for species identification using RFLP analysis. The protocol described in the following sections was used to develop, optimize and evaluate the PCR-RFLP assay.
3.3.2 Genomic DNA Extraction

Adult schistosomes were used in the development and optimization of the PCR procedure. DNA for PCR amplification was extracted from ethanol-preserved, single adult worms of *S. mansoni* or *S. rodhaini*. The worms were placed in a 1.5 ml Eppendorf tube and soaked for 4hr in milli-Q filtered water; genomic DNA was extracted from the worms using the HotShot procedure described by Treutt *et al.* (2000). Briefly, 75μl of the lysis buffer (25mM NaOH, 0.2Mm EDTA) was added to the samples and heated to 95°C for 1 hr. After 1 hour the homogenate formed was cooled to 4°C, and 75μl of neutralizing reagent (40mM TrisHCl pH 4.0) was added to the sample, and 13μl of the sample was stored until used or was immediately used in a PCR assay. To obtain DNA from cercariae, ethanol-preserved cercariae in a 2ml plastic tube were allowed to settle to the bottom of the tube, the ethanol was carefully removed from the tube, and replaced with double-distilled water. To completely remove the ethanol, the cercariae were soaked in water over a 2-day period changing water every time cercariae have settled at the bottom after which the water was removed, and replaced with 30μl alkaline lysis reagent (25mM NaOH, 0.2Mm EDTA). The sample was then transferred into a 0.2 ml PCR tube, and heated at 60°C for 1hr on a heat block with finger vortexing every 15 min. Freshly shed cercariae in water (in a 2ml tube) were left on ice for 2 hours to allow the settling down. The water was removed and replaced with 30μl alkaline lysis reagent and heated at 60°C for 1 hr. A heated sample was cooled at 4°C, and 30μl of neutralizing reagent added to buffer the sample. A 13μl volume of the resulting preparation was then used in the PCR assay.
3.3.3 Primers

The primers used for DNA amplification have previously been described by Morgan et al (2003), and were designed for the amplification of 662 bases of a partial 16S region of the mitochondrial DNA (16S mtDNA) leading into the 12S region of the mtDNA. The forward primer is designated 16SF2 and the reverse primer 12SR2. The sequences of the forward and reverse primers are provided in Table 6.

Table 7 Sequences of the primer pair used to PCR amplify a portion of the 16S-12S region of the mtDNA in *S. mansoni* or *S. rodhaini*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 16SF2</td>
<td>5’GTG CTA AGG TAG CAT AAT AT 3’</td>
</tr>
<tr>
<td>Reverse 12SR2</td>
<td>5’AAC CGC GAC TGC TGG CAC TG 3’</td>
</tr>
</tbody>
</table>

3.3.4 PCR Amplification of the 16S-12S region of the schistosome mitochondrial DNA

The PCR amplifications targeting the 16S-12S region of schistosome mtDNA was performed in a 50-μl volume consisting of 22.5μl purified water, 5μl 10x buffer, 3μl 25mM MgCl₂, 1μl 10mM dNTPs, 2.5μl forward primer, 2.5μl reverse primer, 0.5μl Taq polymerase (Promega), and 13μl containing 10-100 ng template DNA. The PCR mixture was briefly spun at 13000 rpm for 10 seconds and amplified in the Perkin Elmer thermocycler model 2400 for 30 cycles using the following profile: Cycle 1 at 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 1 minute and 30 seconds then 29 cycles at 95°C, 48°C for 72°C for 1 minute and 30 seconds followed by 72°C for 7 minutes to complete extension and then held at 4°C. The resulting PCR products were confirmed on
a 2% agarose TAE gel by electrophoresis run at 80mV for one hour, and were visualized under UV light after staining with ethidium bromide. A 100bp DNA marker was used as a standard.

3.3.5 Restriction Fragment Length Polymorphism (RFLP) Analysis of the 16S-12S mtDNA

The 16S-12S mtDNA PCR products of both adult S. mansoni and S. rodhaini were concentrated and desalted using the Centricon 100 spin columns (Amicon, Beverly, MA, USA), and were digested with restriction enzymes BamHI, or AccI (Promega). Each RFLP reaction in a total volume of 20 µl included 1 µl of either RE, 2 µl of the appropriate 10X buffer, 10-12 µl of Centricon100-purified PCR products (~500 ng DNA), and 5-7 µl filtered water. The restriction digest was done using the following mix per sample in a Micro Amp Reaction Tubes: 1µl sterile deionized water, 1µl restriction, 2µl 10x restriction enzyme buffer, 1µl acetylated BSA 10mg/ml, 15µl PCR product to give a total volume of 20µl. The reaction was set in a water bath at 37°C for four hours and thereafter 1µl of the restriction enzyme was added for further four hours. The RFLP products were separated on a 2% agarose gel by electrophoresis and were stained with ethidium bromide, and were visualized and photographed for analysis of fragment sizes based on the anticipated species-specific restriction sites banding patterns.

3.3.6 Optimization of the PCR-RFLP assay for cercariae identification

The PCR-RFLP assay was initially developed using adult schistosomes as a source of DNA. To optimize it for identification schistosome cercariae, genomic DNA was extracted from freshly collected or ethanol-preserved, laboratory-obtained, authentic cercariae of S. mansoni or S. rodhaini as described in section 3.2.3 above. DNA from
varying numbers of cercariae (ranging from 1-100), were used to determine the optimal numbers of cercariae needed to perform the assay. Concentrations of MgCl₂ in the PCR reaction were also varied to obtain optimal amplification conditions for the assay. Other components of the PCR reactions optimized were concentrations of Taq Polymerase and primers.

3.3.7 Analysis of Adult Schistosomes Derived from Cercariae Obtained from Field-Collected *Biomphalaria* Snails

Using unidentified, ethanol preserved adult schistosomes derived from cercariae obtained from naturally infected *Biomphalaria* snails collected from field locations within Kenya, and grown in laboratory mice, the assay was evaluated for its usefulness in identifying unknown adult worms. DNA was extracted from individual worms for each isolate using the procedure already described in section 3.2.3 above. One to 2 individuals were analysed separately for each parasite isolate. The PCR protocol described in section 3.2.4 and the RFLP analysis described in section 3.2.5 were used. Table 7 shows details of parasite isolate, collection site, latitude/longitude data. For the purpose of the present study, an isolate was defined as an individual cercaria/adult worm or group of cercariae/adult worms derived from an individual snail collected from a specific habitat on a specific date. Schistosome DNA was PCR amplified and the resulting product was subjected to restriction enzyme digestion for RFLP analysis. A gel picture was obtained for documentation purpose.
3.3.8 PCR-RFLP Analysis of Mammalian Schistosome Cercariae Obtained from Field-Collected Biomphalaria Snails

Similarly, the PCR-RFLP assay was evaluated for its usefulness in identifying cercariae obtained from naturally infected, field-collected Biomphalaria snails. The ultimate goal of the present study was to develop a PCR-based assay that can be used for routine identification of cercariae of *S. mansoni* or *S. rodhaini*. Cercariae were obtained from naturally infected; field-obtained snails collected various habitats in Kenya. DNA was extracted, amplified and analysed for RFLP patterns as described in section 3.2.6 above.
CHAPTER FOUR

RESULTS

4.1 Snail Collections and Screening for Schistosome Infections

A total of 5690 *Biomphalaria* snails were collected from sites along the Lake Victoria shoreline in and around Kisumu and the Homa Bay town-Mbita Point area between December 2000 and April 2003; on examination for the presence of schistosome by the “shedding method” a total of 36 snails were found to shed mammalian schistosome cercariae that resembled human schistosome cercariae. The *Biomphalaria* snails that produced schistosome cercariae were identified as *B. sudanica* based on shell morphological characteristics.

4.1.1 Laboratory mice infections with Schistosome cercariae from field-collected snail

Eight weeks following exposure of laboratory mice to the cercariae shed by individual snails collected from the field, adult schistosomes were recovered by the portal perfusion technique from the various groups of infected mice. Table 11 shows the schistosome isolates, their collection sites, and latitude and longitude data for the collection sites. When the liver tissues of schistosome-infected mice were examined for the presence of schistosome eggs, mice exposed to cercariae shed by 3 different individual snails (out of the 36 snails that shed mammalian schistosome cercariae, 8.3%) showed presence of schistosome eggs that resembled those of *S. rodhaini*. One of the snails was collected from the KFA Kisumu town (on the lake shore), another from an irrigation canal on the ADC Farm Kisumu town, (near the lake shore), and the other one was collected from the Luanda creek, a swampy stream draining into the lake (and
located at the Luanda Market, approximately 45km west of Homa Bay town, on the Homa Bay-Mbita Point road). Detailed information on the *S. rodhaini* isolates obtained including the geo-reference data for the collection sites is provided in Table 8.

Table 9 *S. rodhaini* isolates from naturally infected *B. sudanica* snails collected in western Kenya from sites along shores of L. Victoria, and details of their collection sites and dates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Collection Site</th>
<th>Lat</th>
<th>Long</th>
<th>Alt (m)</th>
<th>Collection Date</th>
<th>Schistosome Sp</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6036-05</td>
<td>Lakeshore, KFA, Kisumu town</td>
<td>00°05'74&quot;S</td>
<td>034°44'95&quot;E</td>
<td>1129</td>
<td>November 1, 2000</td>
<td><em>S. mansoni</em>+ <em>S. rodhaini</em></td>
</tr>
<tr>
<td>W2018-01</td>
<td>Canal near lakeshore, ADC Farm, Kisumu town</td>
<td>00°05'07&quot;S</td>
<td>034°45'14&quot;E</td>
<td>1146</td>
<td>December 9, 2000</td>
<td><em>S. rodhaini</em></td>
</tr>
<tr>
<td>W2026-01</td>
<td>Swampy creek, Luanda market, ~45km west of Homa Bay town</td>
<td>00°28'65&quot;S</td>
<td>034°17'38&quot;E</td>
<td>1140</td>
<td>January 11, 2001</td>
<td><em>S. rodhaini</em></td>
</tr>
</tbody>
</table>

The parasite isolate collected from KFA in Kisumu town, is designated *S. rodhaini* (W6036-05). A total of 20 worms (14 males and 6 females) were recovered from 2 mice which had been exposed to mammalian schistosome cercariae from a single *Biomphalaria* snail (as routine procedure of any cercariae recovered from the wild). Examination of fresh liver tissue squashes of infected mice showed the presence of predominantly, *S. mansoni* eggs, with only a few *S. rodhaini*-like eggs. This *Biomphalaria* snail therefore had a mixture of *S. mansoni* and *S. rodhaini* parasites.

The isolate obtained from the ADC Farm, Kisumu, a site located adjacent to the KFA was designated W2018-01. A total of 60 worms (males and females) were recovered from infected mice. Examination of fresh liver tissue of mice infected with
cercariae from a single snail revealed the presence of only typical *S. rodhaini* eggs. The parasite eggs observed in the liver tissue of *S. rodhaini* infected mice exhibited various characteristic morphological forms.

4.1.2 Maintenance of *S. rodhaini* life cycle under laboratory conditions

Since its isolation, *S. rodhaini* W-2018-01 has been maintained routinely under laboratory conditions at the Centre for Biotechnology Research and Development (CBRD) Kenya Medical Research Institute (KEMRI) through laboratory mice and laboratory-raised *B. sudanica* ADC Farm, Kisumu). To date, this isolate has been in the laboratory for at least 18 generations.

The eggs of *S. rodhaini* W-2018-01 readily hatched into miracidia but hatchability was enhanced when liver tissue from infected mice was left at 4° C overnight prior to homogenizing the tissue to isolate parasite eggs. While laboratory-raised *B. sudanica* (ADC Farm, Kisumu) or *B. pfeifferi* (Kibwezi) were both susceptible to infection with *S. rodhaini* (mono-miracidial infections), the susceptibility of *B. pfeifferi* was about a half that of *B. sudanica*. Between 23-30% of the snails exposed to mono-miracidia (1 miracidium) per snail died before the infection became patent (i.e. before the snails started to release cercariae), but pre-patent mortality was greater for *B. pfeifferi*. Majority of the snails died within 3 weeks following exposure to the parasite miracidia. For both *B. sudanica* and *B. pfeifferi*, *S. rodhaini*-infected snails maintained at ambient temperatures in the range 24-26°C started shedding cercariae between 28-35 days after exposure to miracidia. At least 70% of the *S. rodhaini* infected snails survived to infection patency (characterized by cercariae release by the snails), and the snails
continued to shed cercariae over the next 4-6 weeks post-patency, with mortality of less than 20% surviving snails. Table 10 provides a summary of observations on snail susceptibility to *S. rodhaini* infection, pre-patent mortality, and time to infection patency for *B. sudanica* and *B. pfeifferi*.

Table 10 *S. rodhaini* (W-2018-01) infection in laboratory-raised *B. sudanica* and *B. pfeifferi*

<table>
<thead>
<tr>
<th></th>
<th><em>B. sudanica</em></th>
<th><em>B. pfeifferi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Snails infected</td>
<td>60%</td>
<td>33%</td>
</tr>
<tr>
<td>Pre-patent snail mortality</td>
<td>23%</td>
<td>30%</td>
</tr>
<tr>
<td>Time to infection patency</td>
<td>28-35 days</td>
<td>28-35 days</td>
</tr>
</tbody>
</table>
Table 11 Identity of mammalian schistosomes isolates recovered from naturally infected *Biomphalaria* snails collected from various locations in western Kenya in or around Lake Victoria

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Collection Site</th>
<th>Lat</th>
<th>Long</th>
<th>Preliminary Identity of Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6000-03</td>
<td>Machakos</td>
<td>01° 31'54S</td>
<td>37° 17'04E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2001-04</td>
<td>Makueni</td>
<td>01° 54'51S</td>
<td>37° 31'22E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2006-01</td>
<td>Kalundu river</td>
<td>01° 21'52S</td>
<td>38° 00'36E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2018-04</td>
<td>ADC farm</td>
<td>00° 05'07S</td>
<td>34° 45'14E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2029-04</td>
<td>Rachuonyo</td>
<td>00° 26'50S</td>
<td>34° 29'22E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2030-03</td>
<td>Mkuyuni</td>
<td>02° 33'41S</td>
<td>32° 54'16E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2032-02</td>
<td>Mirongo</td>
<td>02° 34'45S</td>
<td>32° 56'17E</td>
<td><em>S. mansoni</em></td>
</tr>
<tr>
<td>W2045-01</td>
<td>KFA</td>
<td>00° 05'54S</td>
<td>34° 44'55E</td>
<td><em>S. mansoni</em></td>
</tr>
</tbody>
</table>
Figure 14: Various morphological forms of *S. rodhaini* eggs.

Figure 14 shows the various morphological forms of eggs observed for *S. rodhaini* W2018-01 in the present study. The *S. rodhaini* isolate (W2026-01) came from Luanda stream (a swampy creek full of papyrus reeds near the lakeshore) at Luanda Market, about 45km west of Homa Bay town on the Homa Bay-Mbita Point road. The isolate produced adult worms in cercariae-exposed mice, and a total of 61 worms (males and females) were recovered from the infected mice. On examination of fresh liver tissue of infected mice, only characteristic *S. rodhaini* eggs were observed.
Figure 15 Typical *S. rodhaini* cercariae emergence rhythm observed for W2018-01.
4.1.3 Characteristics of the cercariae shedding pattern of *S. rodhaini* (W2018-01)

A study of the cercariae shedding pattern of *S. rodhaini* W2018-01 indicated that the number of cercariae released by a single snail exposed to a single miracidium during a 24-hour period ranged between 275 and 766 cercariae per snail in 4 experiments. Figure 15 shows a typical *S. rodhaini* cercariae emergence rhythm observed for *S. rodhaini* W2018-01 in infected *B. sudanica*. *S. rodhaini*-infected snails continuously released cercariae during hourly observations over a 24 hour period, with at least 60% of the cercariae being released during daytime (between 06:00 hr and 18:00 hr). A distinct peak of cercarial output was observed in the early morning hours around 06:00-09:00 hr during which period 37% of the total cercariae shed during the entire 24hr period were produced. The cercariae shed during the 06:00-09:00 hr period represented 60% of the cercariae shed during daytime. Outside the peak cercarial release period (06:00-09:00 hr) infected snails continued to release cercariae in small numbers, throughout the 24 hr observation period.

4.2 Sequence analysis of the 16S-12S region of the mtDNA of *S. mansoni* and *S. rodhaini*

The nucleotide sequences of the amplified portion of the 16S-12S region of the mtDNA for the adult *S. mansoni* and *S. rodhaini* was similar to that described by Morgan *et al.*, (2003). The sequences of these fragments are stored in the GenBank™ with Accession Numbers AF531311 for *S. mansoni* and AF531309 for *S. rodhaini*. The amplified 16S-12S mtDNA fragment for *S. mansoni* was 759 base pairs long, whereas the fragment for *S. rodhaini* was 757bp. With the 2 bp differences observed between the DNA fragments in the two schistosome species it was unlikely the differences would be
resolved on an agarose gel by electrophoresis. In other words, amplification of the 16S-12S mtDNA fragment in a conventional PCR assay would not be expected to differentiate between *S. mansoni* and *S. rodhaini*.

Analysis of the 16S-12S mtDNA sequences of the 2 parasite species for restriction enzyme cut sites using program Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) revealed the presence of a cut site for the enzyme *BamHI* in the *S. rodhaini* sequence which was absent in the *S. mansoni* sequence. *BamHI* would cut the *S. rodhaini* sequence into 2 smaller fragments of 153bp and 604bp but the *S. mansoni* fragment would remain intact. On the other hand, a cut site for the enzyme *AccI* was observed in the 16S-12S mtDNA sequence of *S. mansoni* but a cut site for this enzyme was absent in the *S. rodhaini* nucleotide sequence. Figure 16 illustrates the predicted RFLP pattern expected with these enzymes. Digestion of the PCR product of *S. mansoni* would result in 2 smaller DNA fragments of 158bp and 601bp, whereas the PCR product of *S. rodhaini* would not be digested by the enzyme, and would remain Uncut. With the observed species-specific RE cut site differences on the 16S-12S mtDNA sequences in the 2 parasite species, it was envisaged that a PCR-RFLP assay could be developed to differentiate between *S. mansoni* and *S. rodhaini*. 
4.2.1 PCR amplification of the 16S-12S mtDNA of the adult *S. mansoni* and *S. rodhaini*

Using the primers 16SF2 and 12SR2 to amplify a portion of the 16S-12S region of the mtDNA in genomic DNA of adult *S. mansoni* and *S. rodhaini*, visible, identical bands of approximately 750bp were observed for each of the parasite on a 2 % agarose gel when the amplified genomic DNA was subjected to electrophoresis at 80mV for 1 hr, (Figure 17).
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>UNCUT PCR BAND</th>
<th>S. mansoni</th>
<th>S. rodhaini</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>759 bp</td>
<td>757 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S. mansoni)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>153 bp</td>
<td>604 bp</td>
<td></td>
</tr>
<tr>
<td>AccI</td>
<td>759 bp</td>
<td>601 bp</td>
<td>158 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>757 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 16:** Predicted RFLP patterns with enzymes *BamHI* on *S. rodhaini* and *AccI* on *S. mansoni*
Figure 17: Agarose gel showing PCR products of schistosome adult worms.
Lanes 1 and 4: 100bp marker; lane 2: *Schistosoma mansoni* adult worm; lane 3: *Schistosoma rodhaini* adult worm.
4.2.2 RFLP Analysis of PCR products of adult *S. mansoni* and *S. rodhaini*

When the PCR products for both *S. mansoni* and *S. rodhaini* were digested with the enzyme *BamHI* as described in section 3.3.5 and the resulting digest subsequently separated by electrophoresis on a 2% agarose gel, species-specific RFLP patterns were observed as predicted in section 4.1.1 above, and as shown in Figure 16. Incubation of the PCR products of *S. mansoni* or *S. rodhaini* with the enzyme *BamHI* produced 2 smaller bands, one 153bp and the other 604bp in size on agarose gel for *S. rodhaini* but the product for *S. mansoni* remained intact, and the expected band of 759bp was observed on the gel. This procedure reliably allowed differentiation between the 2 parasite species. Similarly, incubation of the PCR products of the 2 parasite species with the enzyme *AccI* produced on a 2% agarose gel 2 smaller bands, one of 158bp in size and the other 601bp in size for *S. mansoni* and as expected, *S. rodhaini* product remained intact and appeared as 757bp band in size on the agarose gel as shown in Figure 19. Again this second procedure also, reliably differentiated between the 2 parasite species.

4.2.3 Optimal conditions of the PCR-RFLP assay for analysis of cercarial DNA from *S. mansoni* or *S. rodhaini*

The ultimate goal of the present study was to develop an assay that can be used to reliably identify cercariae of *S. mansoni* or *S. rodhaini*. Unlike the adult worms, cercariae are smaller in size, and their analysis require optimization. Using DNA extracted from 1, 5, 10, 20, 50 and 100 cercariae of *S. mansoni* in the PCR-RFLP assay, it was observed that even with a single cercariae, PCR amplifiable genomic DNA could be obtained using the alkaline lysis extraction procedure.

The PCR amplification profile used for the adult worms and provided under section 3.2.4 was used for the cercariae. Figure 18 shows an ethidium bromide stained
agarose gel showing a PCR product of a partial 16S-12S mtDNA fragment of the expected size (769bp), amplified from various numbers of *S. mansoni* cercariae. The amount of PCR product present in a reaction increased in direct relation to the number of cercariae in the assay. While detectable RFLP band patterns were observable for *AccI* digested PCR product of a single cercaria of *S. mansoni*, on a 1.5% agarose gel under UV light, only faint bands were observed on a gel picture. On the other hand, easily detectable RFLP bands were observed when 5 schistosome cercariae or more were used. Routinely, DNA extracted from 20-50 schistosome cercariae was used in this assay. Using cercariae from *S. mansoni* or *S. rodhaini* routinely maintained in CBRD, KEMRI, RFLP band patterns similar to the ones observed with the adult worms of these species were observed when the restriction enzymes *BamHI* or *AccI* were used. In other words, *BamHI* cut the PCR product of *S. rodhaini* cercariae but not the product of *S. mansoni* cercariae, thereby distinguishing between cercariae of the schistosome species. Similarly, *AccI* digested the PCR product of *S. mansoni* cercariae but not the product of *S. rodhaini* cercariae, thus, distinguished the cercariae of the parasite species.
Table 12: Identity of unknown samples of cercariae analysed using the PCR-RFLP technique

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Location</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6035-03</td>
<td>ADC farm</td>
<td>00°05'34S</td>
<td>34°42'25E</td>
<td>1164m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2000-09</td>
<td>Makindu</td>
<td>00°28'46S</td>
<td>38°48'34E</td>
<td>1081m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W6036-04</td>
<td>KFA</td>
<td>00°44'95S</td>
<td>34°44.95E</td>
<td>1165m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2001-03</td>
<td>KFA</td>
<td>00°43'76S</td>
<td>34°44.78E</td>
<td>1049m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2006-01</td>
<td>Kalundu</td>
<td>01°21'51S</td>
<td>038°00'36E</td>
<td>1154m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2014-02</td>
<td>Kieumu</td>
<td>00°05'34S</td>
<td>34°42'25E</td>
<td>1164m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2018-04</td>
<td>A.D.C.</td>
<td>00°05'07S</td>
<td>34°45'14E</td>
<td>1146m</td>
<td>S.mansoni</td>
</tr>
<tr>
<td>W2030-02</td>
<td>Mikuyuni</td>
<td>02°33.41S</td>
<td>32°54'16E</td>
<td>1049m</td>
<td>S.mansoni</td>
</tr>
</tbody>
</table>
Figure 18 Agarose gel showing restriction digest types of the ~750bp PCR product using variable number of cercariae when S.rodhaini 16S-12S PCR products were cut with restriction enzyme BamHI. Both lanes 1 and 9 are the 100 bp marker; Lane 2: S.mansoni PCR product uncut with the enzyme. Lanes 3 and 4 digested S. rodhaini PCR product from(20) cercariae. Lanes 5 and 6:PCR product from 25 cercariae and from 40 cercariae digested with BamHI enzyme. Lane 7: PCR product of DNA extracted from 50 cercariae and digested with BamHI enzyme digested. Lane 8: PCR product DNA extracted from 50 cercariae of S.mansonii not digested by BamHI and remained as undigested product.
an ethidium bromide stained agarose gel showing a PCR product of a partial 16S-12S mtDNA fragment of the expected size (approximately 750 bp), amplified from various numbers of *S. mansoni* cercariae. The amount of PCR product present in a reaction increased in direct relation to the number of cercariae in the assay. While detectable RFLP band patterns were observable for *AccI* digested PCR product of a single cercariae of *S.mansoni*, on a 1.5% agarose gel under UV light, only faint bands were observed on a gel picture. On the other hand, easily detectable RRLP bands were observed when 5 schistosome cercariae or more were used. Routinely DNA extracted from 20-50 schistosome was used in this assay.

4.3 Analysis of adult worms derived from cercariae shed by field-collected *Biomphalaria* snails

Subsequently, the newly developed PCR-RFLP assay was evaluated for its usefulness in identifying or confirming identity of worm specimens recovered from laboratory mice previously exposed to cercariae shed by naturally infected, field-collected *Biomphalaria* snails. The cercariae used to infect laboratory mice were obtained from individual snails. In most cases, worm specimens derived from cercariae from individual snails usually turn out to be of the same sex, either male or females. Various schistosome isolates comprising adult worms derived from cercariae obtained from naturally infected *Biomphalaria* snails were analysed using the PCR-RFLP assay. Table 11 shows a list of the isolates, their collection sites, latitude/longitude data, and isolate identity. A total of 15 individual worms from 10 different isolates were analysed using
this assay. DNA of authentic *S. mansoni* and *S. rodhaini* was included in the analysis to serve as a reference for the unknown samples. All the schistosome specimens analysed and collected from different localities within Kenya including western Kenya were *S. mansoni*. The identity of schistosome specimens that had been earlier confirmed by egg morphology and DNA sequencing to be *S. rodhaini*, and which were collected from Luanda Market near Homa Bay and the ADC Farm in Kisumu city was re-confirmed using the PCR-RFLP assay as well. The PCR-RFLP assay was also evaluated for its usefulness in identifying mammalian schistosome cercariae obtained from field-collected *Biomphalaria* snails. Cercariae shed by individual naturally infected snails collected from various locations in Kenya including western Kenya, where *S. rodhaini* is known to be present were analysed. Amplifiable amounts of DNA were extracted from between 20-50 cercariae, and clearly visible RFLP band patterns were observed on 2 % agarose gel in restriction enzyme digested PCR products from these samples. A total of 8 cercarial isolates were analysed in this assay, and their identity after PCR-RFLP is shown on Table 12.
Figure 19: RFLP-PCR band patterns generated when restriction enzyme Accl was used on PCR products from the two parasites. Lanes 1 and 6 are the 100bp marker. Lane 2 undigested *S. mansoni* PCR product of the 16S-12S mtDNA fragment. Lane 3. *S. mansoni* PCR product of the fragment digested with Accl. Lane 4 undigested *S. rodhaini* PCR product of the fragment DNA. Lane 5 digested PCR product of the fragment DNA of *S. rodhaini*. 
Figure 20  Agarose gel stained with ethidium bromide showing the species-specific banding patterns from digestion of the ~750bp PCR product of 16S-12S mtDNA using enzyme *AccI*. Lanes 2 and 3 undigested PCR product of 16S-12S mtDNA of *S.mansoni* band. Lanes 4 and 8 digested PCR product showing a fragment of about 500bp *S.mansoni*. Lanes 5, 6 and 7 digested 16S-12S *S. rodhaini*. Lane 9: PCR product of 16S-12S undigested *S.rodhaini* using enzyme *AccI*. 1 and 10th lanes are 100bp marker ladder.
S. rodhaini was first discovered in western Kenya near Kisumu in the early 1960's in infected domestic dogs and wild rodents (Nelson et al., 1962). In the present study, S. rodhaini infected Biomphalaria snails were collected from sites within the Kisumu City area and also at Luanda Market some 45km west of Homa Bay town in South Nyanza, suggesting that the parasite is endemic in and around Lake Victoria. This confirms previous reports that have shown that S. rodhaini also occurs in several other localities in and around Lake Victoria, in Uganda and Tanzania (Berrie and Goodman, 1962; Morgan et al., 2003). The parasite has also been isolated from Rwanda-Burundi area, southwest of Lake Victoria (Fain et al., 1953). It is not clear from the present and previous studies, how common S. rodhaini is within the East African region, but the present study seems to suggest that in western Kenya the parasite occurs sporadically and tends to be focal in distribution. Further studies however, may be necessary to more accurately map its distribution in western Kenya, at least in snail populations. The PCR procedure developed in the present study to differentiate between S. rodhaini and the human pathogen S. mansoni will most certainly facilitate this process. Although the present study focused on searching for S. rodhaini, primarily in western Kenya, snail surveys conducted in other parts of Kenya where S. mansoni is known to be endemic such as the
area within the Athi river basin, have not shown any evidence of the presence of *S. rodhaini* in these localities.

In the surveys conducted in western Kenya during the 1960's *S. rodhaini* was recovered from wild rodents and domestic dogs (Nelson *et al.*, 1962). In the present study an examination of at least 41 wild caught rodents in the vicinity of the sites from which *S. rodhaini* (in Kisumu town and west Luanda Market west of Homabay town) were collected and comprising 8 genera and 8 species did not yield any schistosomes. (results not presented in this thesis). In the study by Nelson *et al.* (1962) more than 1100 rodents representing 13 genera and 15 species, only 3 rodents were found to harbour *S. rodhaini*. One of the rodent species found infected, *Lophurmys flavopunctatatus* is associated with aquatic habitats and was among the species caught in the present study.

Although dogs infected with *S. rodhaini* have been found in western Kenya (Nelson *et al.*, 1962), no attempts were made to examine dogs for the parasite in the present study. Out of the 36 *Biomphalaria* snails found to be shedding mammalian schistosome cercariae (the human type), only 3 were found to be carrying *S. rodhaini* based on the demonstration of typical *S. rodhaini* eggs in liver tissue of infected lab mice. In other words, *S. rodhaini* accounted for 8.3% of schistosome infections present in *Biomphalaria* snails collected from the study sites in western Kenya. The present and previous observations seem to suggest that *S. rodhaini* may not be common in nature in western Kenya. Interestingly, even though cercariae from individual snails were used to infect lab mice, an egg producing schistosome infection resulted in mice. Dual infections, in some cases consisting of both “male” and “female” cercariae are not uncommon. However, the fact that all the 3 snails that were found to harbour *S. rodhaini* yielded an egg-producing
S. rodhaini infection makes it interesting. One of the isolates (No.W6036-05) in fact yielded both S. mansoni and S. rodhaini as evidenced by the presence of eggs of both species in the liver tissue of mice infected with cercariae from an individual Biomphalaria snail. In the other 2 isolates (Nos. W2018-01 and W2026-01) on the other hand, only pure S. rodhaini were observed. In the present study, majority of the single snail cercariae infections producing adult worms in mice yielded only single sex worms, mostly males, and while the majority will be expected to yield S. mansoni, one cannot rule out the possibility that among these some of them may turn out to be S. rodhaini. Only a small number of the isolates were analysed using the newly developed PCR assay during the present study, but no S. rodhaini was detected. In laboratory studies both lab-raised B. pfeifferi and B. sudanica were found to be susceptible to infection with S. rodhaini (W2018-01) which is currently, routinely maintained in the laboratory at the Centre for Biotechnology Research and Development (CBRD) of KEMRI. However, B. sudanica was more susceptible and infected snails seemed to survive better than B. pfeifferi, suggesting that S. rodhaini is probably better adapted to B. sudanica than to B. pfeifferi. It should be noted that the S. rodhaini isolates obtained in the present study all came from B. sudanica. According to published literature, S. rodhaini exhibits a nocturnal cercariae shedding pattern. In other words, majority of the cercariae present in an infected snail will emerge at night (unlike S. mansoni cercarie which emerge during the day with a peak around mid-day). The cercariae emergence pattern exhibited by S. rodhaini (W2018-01) was unusual in that majority of the cercariae emerged during the early morning hours with a peak cercarial release occurring between 06:00 and 09:00h. Although this pattern is unusual, a similar pattern had previously been observed for a
Ugandan isolate of *S. rodhaini* (Fripp, 1967). Release patterns are synchronized with peak activity periods of the vertebrate species that serve as definitive hosts for the parasite species, in order to ensure successful transmission. Thus, a mid-day peak observed for the larva *S. mansoni* coincides with peak periods of activity for humans. The early morning peak cercariae release pattern observed for *S. rodhaini* (W2018-01) suggests that its definitive host maybe a species of animal most active during early morning. Known natural hosts of *S. rodhaini* are carnivores and rodents. Species from these 2 mammalian groups are the most likely definitive hosts of *S. rodhaini* (W2018-01) present in western Kenya even though no parasite was isolated in a few dozen rodent species examined during the present study. *S. rodhaini* (W2018-01) routinely being maintained will provide materials for further research on the biology of the parasite and especially its immunobiology, interactions and ecology.

5.2 The PCR Test for Identification of *S. mansoni* or *S. rodhaini*

In the present study, efforts were made to develop a PCR-based assay for identification of cercariae of *S. mansoni* or *S. rodhaini*. Sampling of the appropriate freshwater snail species for schistosomes is a simple and inexpensive way of identifying sites involved in the transmission of human schistosomes, or for monitoring schistosome transmission sites after instituting intervention measures such as chemotherapy (Sturrock, 1986; Sturrock *et al.*, 1994)

However, the current snail sampling procedures do not allow accurate identification of the parasite infecting the snails, and often, identification of the schistosome species being transmitted in a particular locality or area using parasitological methods is tedious and time consuming, and inaccurate. Given that *Biomphalaria* snails,
the intermediate hosts of the human pathogen, *S. mansoni*, also serve as hosts of *S. rodhaini*, a parasite of rodents and carnivores (a parasite considered to be of no medical significance), the development of a PCR assay to facilitate identification of cercariae of *S. mansoni* group species has provided a useful tool for epidemiological studies on human intestinal schistosomiasis caused by *S. mansoni* and could potentially, serve to support control of the disease. A similar procedure has been developed to differentiate between *S. haematobium*, the causal agent of human urinary schistosomiasis and its close relative *S. bovis* (Barber et al., 2000) and it is proving to be useful for identifying schistosome cercariae shed by naturally infected bulinid snails in Kenya. The new method is accurate, offers reliability and rapidity, unlike the parasitological procedures.

Although an assay based on conventional PCR is desirable because of its simplicity, a PCR-RFLP procedure targeting a portion of the 16S-12S mt DNA was developed. While the new test requires further evaluation to determine its usefulness in identifying *S. mansoni* group species obtained from the field, it has the potential for providing new information on the distribution and prevalence of mammalian schistosomes in this group in Kenya, and other endemic areas in tropical Africa where both *S. mansoni* and *S. rodhaini* may be present sympatrical. A potential drawback of the new procedure is the fact that it relies on the maternally inherited mtDNA (Simon et al., 1994) as a target. Consequently, it may not be useful for identifying potential hybrids of *S. mansoni* group species. *S. mansoni* and *S. rodhaini* are capable of forming hybrid both under laboratory conditions (Taylor, 1970) and in nature (Morgan et al., 2003). The ITS2 region of the nuclear ribosomal gene (rDNA) for instance would have been the most ideal
for this purpose. However, it did not provide sufficient markers to differentiate between
*S. mansoni* and *S. rodhaini* (results not presented in this thesis).

The other problem with the new procedure is that it requires the use of 2
restriction enzymes (the *BamHI* that cuts *S. rodhaini* DNA and *Acel* that cuts *S. mansoni*
DNA). It can be argued that this introduces too many steps in the assay, making it
tedious. In this study however, the results of the second enzyme provide a further
confirmation of the identity of the parasite, and rule out the possibility that the test failed.
In other words, one can more confidently make a statement about the identity of a
parasite if the two enzymes are used rather than when only one is used in the assay.

### 5.3 Evaluation of the PCR Assay using field-obtained cercariae or adults of *S. mansoni* group species

The PCR-RFLP assay was evaluated for its usefulness in identifying cercariae or
adult worms of the *S. mansoni* group schistosomes derived from field-collected
*Biomphalaria* snails, and it has proved to be a reliable assay. The assay accurately
identified DNA of *S. mansoni* and *S. rodhaini* and differentiated between the DNA of the
two species. The number of samples used in this evaluation, however, was limited and
further studies to evaluate the assay will be necessary in future. Compared to the
parasitological procedure for cercariae identification that involves exposing mice to
cercariae shed by field-obtained snails, and then waiting for at least 6 weeks to recover
the adult worms, and examine liver tissue for parasite eggs, the molecular procedure
offers rapidity, simplicity, and the identity of the cercariae (or even the adult worms) can
be known in a day or two from the time the parasite material is obtained from the field.
While the assay will be particularly useful for identifying cercariae, this assay will also be useful for identifying the adult worms derived from cercariae obtained from field collected snails. Very frequently, the adult worms obtained this way do not result in egg production infections (to allow easy identification of the parasite species), and unless one examines the morphology of the adult worm after staining, it will be difficult to make a definite identification of the collected parasite. The PCR procedure therefore, simplifies the identification of *S. mansoni* group schistosome species.

Although, identification of schistosome cercariae in the *S. mansoni* group species has also been made simple, it relies on the use of DNA from more than a single cercaria. The use of DNA from multiple individuals in the assay could potentially complicate accurate identification of a parasite. Dual schistosome infections in a snail host comprising both *S. mansoni* and *S. rodhaini* are not uncommon, and if these are to be encountered, the identification results could become ambiguous, where more than one species is present in a sample. Although it is desirable to have an assay that reliably identifies even single cercariae, consistent reliable results in the assay were obtained, whenever genomic DNA was used from individual cercariae. At least 10 cercariae were needed to run the PCR-RFLP assay optimally.

5.4. Conclusions

The present study revisited the status of *S. rodhaini* in western Kenya. While this parasite may be present in this region, further studies will be necessary to accurately determine its prevalence and map its distribution in the region, and in the rest of the country. Although the co-existence of *S. rodhaini*, a parasite of carnivore and rodents can potentially complicate the epidemiological picture of the human pathogen *S. mansoni*
(responsible for causing human intestinal schistosomiasis) in a locality or area, the
availability of a PCR-based assay to identify and differentiate between the two parasite
species, provides an opportunity to more reliably study the epidemiology of *S. mansoni* in
such an area or locality, and more accurately identify and monitor human schistosomiasis
transmission sites for disease control purposes. Further evaluation of the new assay is,
however, necessary especially in the hands of other investigators. Nevertheless, a simple
rapid and reliable procedure is now available for the first time for identification of
cercariae or adults of *S. mansoni* group schistosome species.

5.5 Recommendations

It would be important to know how common *S. rodhaini* is especially in the snail
population around Lake Victoria and further investigations of the interactions between
*S. mansoni* and *S. rodhaini* in the snail host around Lake Victoria may help understand the
biology of the parasite. It would also be necessary to look at the mammalian host range
that is maintaining *S. rodhaini* around that region, and also determine the extent of
*S. mansoni-S. rodhaini* hybrids in the Lake region
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105


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Sequence of Reverse S. \textit{rodhaini}

CGTAcTAGCCCTTTTATGATTAATAGCCAATCAAATCTctgatAtACTAACAGTCTTATCACCAGG

GTAATCTTTTGCTAATACCAGATCTATTAGACGGAAgGCCCAAGAGCTTTTTCTtATAGTT

GATTTGTTTGGGTAATGTATATTTTCTATTTTGGATCCCTTTAGGAT

AAAGGGTAAAGTTACCTCTTTGGGATAACTGAGTAAAGAGGAGGGAGAGGTCTATTGATCTGCTTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

Sequence of \textit{S. rodhaini} (Reverse Complement)

TTTtGTgtAGGGGaatATATATATAGTTTTATTTGTTttGCAGACTTGTaAaGGTTCAA

CGAGtGGGATTTAGGTAGTTTTTTATTTTGGATGGTTAAAGAGCTTTTTCTtATAGTT

GATTTGTTTGGGTAATGTATATTTTCTATTTTGGATCCCTTTAGGAT

AAAGGGTAAAGTTACCTCTTTGGGATAACTGAGTAAAGAGGAGGGAGAGGTCTATTGATCTGCTTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG

GTTAGAGGCTaGAAAGGCAATGCTGTGTTACTACCTCGATGTTTGGCTTGTTGAACCTTTCTGG
Reverse *S. mansoni*

CCtGCTTAgCCTTTTCTACGACTTAGCCAATAAAATCTCagaaATACTAACA
GTCCTAAtcACTACCAATTTTTAATTCTACTTAAATCAACCATATAAT
ATCCGTaTGAGCTCTCAATCAACTACATTCTACTTTTCTACCAAGATGCAAAATAG
AAAAAAAGTGGGACTAAACCTCTAGTCTAAACATTTTTGCAAAATATCTAT
AGTCACACTTCTCCTGTATATTTTCTTTCTCTCGATACGAGAAACTGT
ATCCTAATAATCTTTGCAGCTAATTAGAAAACCTATTAGATAGAAAAaCcAtCcT
GGCTTACCCGGCTTTAACTCAACTAGTCTCCTTCTCACCTTGGAGTCGAGAAGT
TAATAACAGATCACAATAGACCTCTTACTCTCCTCTCTCCTCTTTTCTAGTTCCCAAG
GAACTTTTATCTTTTACCTCTAATAGGATCCAAAATAAATGGATAATCTCA
TTACACCAAAACAATCcTTACACAAAAAAAGCTcCTTGGGGCCTTTCCGGCca
ATAATAtaAaTAGGGTTTCTCAAACC AccordtATTCTAAATTTCCAGAGATAGACTATC
ACCTATTCCACCACTCTCTGAAACCACCATTCAAAGCTCAGATCAGTTCAAAAGACCTAT

*S. mansoni* (Reverse Complement)

ATAGGTCTTTTATTGTAGACTTGTGAATGGTTTCAGAGGTGTGGAATAAGGTTG
ATAGTCTATTCTCTGAGTTAAGATGGTATAGGAACCCCTATAtTATATTATggG
CCGGGAAAGCCCAAGGCTTTTTTTTGTTAAgGaTTgTGGGATGGAATTG
AGATTATCCATTTTATTTTTGGAATCCTTTTATGGAGATAAAAGGATAAAGTTCCCTT
GGGGGAACTAGAGTAAAGGGACAGGGAGGGCTTATTGTACTGTGTTTATTACT
ACCTCGATGTTGCTGTTAAGAGCGTGGGAGGCTGAAAGGGAGGAGGAGG
TCTGTCGACTTTTAAATCTCTGTTAGTTAGTTAAGCCGGTGAAGCCAGGa
TgGtTCTTTATCATAATTGGCATATAGACATGTCGAGAAGGATTGTTACAG
TTGBTCTCTGATAGCATGGAATAAGGAAATAATACATAGGTAGGAGTGTGACTATA
GATATTGGTCAAAAATGTGTTTACAGAAGTTTTAGTCACACACTTTTCTAT
TTGACCTGTGAAGAATGTGAGATTAGAGAACATACGAGATATTAA
GATGTTGATTAAGTGAATTTAAAATTTTAACTTTGAGTAGCTaTAgGAGCTGT
TAGTAtttcGAGATTTTTATTGGCTACTAGTCGTAAGGAGAGGcTAAAGCaGG

Sequences of *S. rodhaini* and *S. mansoni* respectively that were used in the study

Source  (GenBank)