COMPARISON OF MICROSCOPY AND REAL-TIME PCR IN DIAGNOSIS OF SNAIL *SCHISTOSOMA* INFECTION IN RIVER ASAO, KISUMU COUNTY, WESTERN KENYA

BY

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DECLARATION

By student

I hereby declare that this thesis is my original work and has not been presented for the award of degree in any other university or institution.

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ABSTRACT

Schistosomiasis is the third most devastating parasitic infection of the genus *Schistosoma.* It causes about 200 million deaths globally per annum which is passed by infected intermediate snail hosts of the genus *Biomphalaria* and *Bulinus*. Western Kenya is among the endemic regions in Kenya with a high prevalence of the disease. The disease control is mainly based on mass drug administration of praziquantel to the final host, with little attention paid to the role of snail intermediate host on the prevalence and transmission of schistosomiasis. Specifically, there is limited data on the species diversity of snails, distribution and parasite infection rates of snails, in inland waters flowing to Lake Victoria. Additionally, less sensitive approaches such as microscopy have mostly been used in studies related to the intermediate hosts, *Bulinus* and *Biomphalaria* snails. Therefore, this study aimed to determine: the distribution of the intermediate host snails along River Asao; prevalence of infection in intermediate host snails among the sampling sites; the correlation between the effectiveness of microscopy and advanced molecular tools such as the real-time PCR (RT PCR) in detection and quantification of the parasitic load among infected snails and finally, to quantify infection in snails as the parasite multiplies within the snails and off-host miracidia quantification. The methods involved sampling of snails from sampling sites along river Asao. A GPS visualizer tool was used to map the location of the intermediate snail hosts and focal points of infection. The locations of the snails collected was recorded and used to map the distribution of both infected and uninfected snails following screening in the lab. The snails were then identified morphologically using identification keys as well and standard Polymerase Chain Reaction technique. The parasite load of infected snails was determined by use of microscopy through counting of cercariae shed and by real-time PCR through quantification of the parasite DNA present in the snail host. Only *Biomphalaria pfeifferi* and *Bulinus globosus* were present in river Asao. *Biomphalaria* snails were evenly distributed, while *Bulinus* snails were focally distributed. Microscopy detected 16% infected *Bulinus* and 2% infected *Biomphalaria* snails while real-time PCR detected 28% and 17% infected snails of the *Bulinus* and *Biomphalaria* species, respectively. Microscopy-positive snails also tested positive for real-time PCR. There was no correlation between the cercariae number by microscopy and RT-PCR ($r= -0.261$, $P= 0.438$) and also between miracidia number by microscopy and RT-PCR value ($r = -0.312$, $P = 0.192$). However, an increase in number of *Schistosoma* sporocysts inside infected snails was significantly shown by real-time PCR (($\chi^2 = 14.18$, $df = 4$, $P = 0.0067$). Microscopy is suitable for quantification of *Schistosoma* cercariae and miracidia by counting but is not as sensitive as real-time PCR in the detection of infected snails. Real-time PCR is a useful tool for quick results when identifying and quantifying infected snails in a certain region and thus easy to carry out proper control measures to prevent disease transmission and snail breeding. The infection distribution map can be used to raise awareness of active transmission sites to lower chances of the disease transmission.

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LIST OF ABBREVIATIONS AND ACRONYMS

CDC	:	Center of Disease Control
Cox 1	:	Cytochrome oxidase subunit 1 gene
CT	:	Cyclic threshold
GC	:	Guanine Cytosine
GPS	:	Global positioning system
HRM	:	High Resolution Melting analysis
ITOC	:	International center of insect physiology and ecology, Thomas Odhiambo Campus
MDA	:	Mass Drug Administration
NACOSTI	:	National Commission for Science, Technology and Innovation
NTDs	:	Neglected tropical diseases

RT PCR/ REAL-TIME: Real-time Polymerase chain reaction/ Quantitative PCR

SERU	:	Scientific Ethics Review Unit
TAE	:	Tr is base, Acetic Acid, EDTA
UV	:	Ultraviolet
WHO	:	Water Sanitation and Hygiene
WASH	:	World Health Organization

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Schistosomiasis is a disease caused by parasites in the form of flukes of the genus *Schistosoma* (WHO, 2022). Six species of schistosome are responsible for the disease: *Schistosoma haematobium, S. mansoni, S. japonicum, S. mekongi, S. intercalatum* and *S. guineensis*; and are endemic in 78 countries (Colley et al., 2014). schistosomiasis comes third after malaria and intestinal helminthiasis as the one of the most disastrous tropical disease in the world, with 218 cases annually and 700 million people at risk in endemic regions (WHO, 2022). An estimated six million individuals are infected in Kenya with schistosomiasis, while 15 million are at risk of getting infected (Chadeka et al., 2019). Some studies have shown very high prevalence rates of the disease among school going children living close to inland waters or along the lake region (Odiere et al., 2012; Sang et al., 2014; Sassa et al., 2020). The parasite exhibits a multistage life cycle, such that when the schistosome eggs get into contact with freshwater, they hatch into miracidia under optimal conditions and penetrate a suitable snail host. The miracidia develop into a sporocyst within the snail. Sporocysts transform into cercariae as they grow and multiply. The *Schistosoma* parasite grows into a mature worm, fertilizes and produces eggs (Mouahid et al., 2018; Viana et al., 2018). Human behavior leading to frequent contact with water poses major risk of schistosomiasis infection. Individuals acquire the disease when in contact with water that contains cercariae of the parasite (Ross et al., 2014). Diagnosis of the infection stages in snails and humans has relied on methods such as urine microscopy and Kato Katz, antigen, serum and DNA detection (Weerakoon & McManus, 2016).

Two snail genera, *Bulinus* and *Biomphalaria* serve as intermediate hosts of *S. haematobium* and *S. mansoni* respectively (Blanton, 2019). Within each genus are a number species: *Bulinus* with species such as *Bulinus truncatus, B. globosus, B. senegalensis, B. forskalii, B. camerunensis, B. africanus* and *B. tropicus* (Abe et al., 2018) while *Biomphalaria* has species such as *Biomphalaria pfeifferi, Bi. Choanomphala, Bi. alexandrina, and Bi. sudanica* (Abe et al., 2018). Among the physical factors, water velocity less than 0.3m/s, optimum water temperature (18°C to 28°C) (Kalinda et al., 2017), low water turbidity, water depth level, a slope below 20m/km, mud substrate that is firmly attached to the ground, small amount of organic pollution, and partial shade conditions favor the survival of both *Bulinus* and *Biomphalaria* snail species

(Chimbari et al., 2020). Studies have reported the focal nature of schistosomiasis, especially in the inland regions (Grimes et al., 2014), suggesting a non-uniform distribution of snail intermediate hosts and their intensity of infection by the parasite larval stages. Among the numerous factors contributing to the transmission of the disease to humans, lack of knowledge of the snail location and types of snails available in a location lead to poor prevention strategies. Studies done around River Asao showed the availability of infected children that indicate presence of infected snails (Mutuku et al., 2014a) and others showed a prevalence level of 36% of the individuals in informal settlements around the region (Odhiambo et al., 2014) Therefore, the river was chosen for the study due to the need to identify the numbers and species of snail vectors of *S. mansoni* and *S. haematobium* and to determine the distribution map along River Asao.

An important component in understanding the epidemiology of schistosomiasis involves the detection of infection within snail intermediate hosts. The most common approach is the induction of cercarial shedding by snails followed by microscopic examination (Haggerty et al., 2020). The use of microscopy methods in examining schistosome miracidia, cercariae or sporocysts needs enough time, expertise, and effort. Microscopy also detects only the cercariae shed by infected intermediate snail hosts, making it hard to diagnose the snails as positive during the sporocyst stage of the parasite inside the snails. In addition, microscopy misses light infection hence poor results in infection prevalence and quantification (Qin et al., 2018). Molecular techniques target the parasite DNA of an infected snail (Wong et al., 2014) and might be a suitable alternative for microscopy. For instance, PCR-high resolution melting (HRM) analysis can identify and analyze variation for a genome in nucleic acid sequences after real-time PCR run (Wittwer, 2009). The method is based on PCR melting (dissociation) curve techniques and is able to differentiate sequences of DNA on aspect of their make-up, length of the fragment, GC content, or strand complementarity (Reed et al., 2007). The technique was originally developed for qualitative detection of cercariae of the avian schistosome and *S. japonicum,* but have been advanced to test for *Schistosoma japonicum* in intermediate snail hosts(Driscoll et al., 2005) and also *S. mansoni* in water (Abbasi et al., 2010). Also previously polymerase chain reaction has been used to quantify *S. mansoni* eggs in animal and human stool and quantification of *S. mansoni* DNA (Guegan et al., 2019b). Previous studies show that a highly infected snail sheds a

higher number of cercariae, and a lowly infected one sheds a lower number of cercariae (Mutuku et al., 2014b). However, the PCR-HRM assay has never been used for the correlate of parasite DNA in host snails from their natural habitats with their cercariae shed as observed under the microscopy. The study therefore aimed to determine correlation between *Schistosoma mansoni* cercariae shed by snails (as observed by miscopy) and the quantity of sporocysts DNA in snails by the use of a real-time PCR technique.

The impact of dose-dependent *Schistosoma* exposure on infection establishment in the snails has been examined by few studies that have controversial outcomes (Mutuku et al., 2014b).. Snails get infected with varied amounts of miracidia under their natural habitat once in contact with or drawn to them by chemical cues (Mouahid et al., 2018). It is not clear that the extent to which sporocyst development and asexual expansion depends on initial dose of miracidium. At the same time, studies indicate that the infection rates of snails differ according to snail species in question (Kariuki et al., 2017). This suggest that there are differences between snails' intermediate hosts in their natural ability to control parasite establishment in their tissue. Whereas studies from snails collected from other parts of the world have shown that infection by one miracidium can produce hundreds and thousands of cercariae (Li et al., 2023), it is not certain whether this may be the case for snails collected from River Asao where *Schistosoma* infection is highly endemic. In laboratory-reared snails, daily *Schistosoma haematobium* sheds from 15 to about 160 and 250 to about 600 for the case of *S. mansoni*. The development period goes up to thirty days or slightly more for infected snails to release cercariae during which time the sporocyst numbers gradually increase (Braun et al., 2018). It is of interest to determine the dynamics of sporocyst development in field-collected snails from river Asao as a function of initial dose of miracidium. Therefore, the study used the real-time PCR to detect the quantities of sporocyst with the snail on varied number of weeks post infection with the same number of miracidia under laboratory conditions. The outcome will help to predict the time of snail infection by miracidia.

1.2 Statement of the problem

Schistosomiasis causes severe morbidity and chronic health problems to people in endemic areas. The shores of Lake Victoria and regions along rivers, such as river Asao, that drain into the lake, have been defined as endemic for schistosomiasis (Mwangi et al., 2014). There are two

clinical forms of the disease, transmitted by two different snail genera: *Bulinus* transmits urinary schistosomiasis and *Biomphalaria* transmits intestinal form (Blanton, 2019). Studies have reported the focal nature of schistosomiasis especially in the inland regions (Grimes et al., 2014), suggesting a non-uniform distribution of snail intermediate hosts and their intensity of infection by the parasite larval stages. However, there is limited information on distribution of the snail vectors and their role in transmitting trematode infections along River Asao in Nyakach Sub-County, Western Kenya.

An important component in understanding the epidemiology of schistosomiasis involves detection of infection within snail intermediate hosts. The most common approach is the induction of cercarial shedding by snails followed by microscopic examination (Haggerty et al., 2020). The method however suffers from the weakness that cercarial shedding is of low frequency and only a small proportion of snails get to shed cercariae. At the same time, studies indicate that the infection rates of snails differ according to snail species in question (Kariuki et al., 2017). The gold standard microscopy technique may therefore not reflect the actual *Schistosoma* parasite prevalence in the transmission site. A quick and sensitive real-time PCR technique has been developed as alternative but has only been applied in limited settings in Coastal Kenya (Kariuki et al., 2005). There is a need to measure the prevalence of *Schistosoma* infection with snail species collected in river Asao in Western Kenya by microscopy and PCR.

Limited studies have examined the impact of dose-dependent *Schistosoma* exposure on infection establishment in the snails (Mutuku et al., 2014b). The studies have been limited as the outcome measure depended on the measurement of cercariae shedding. The extent to which infection rates in snails depend on initial exposure to miracidia has not been studied in Western Kenya. Previous studies show that a highly infected snail sheds a higher number of cercariae, and a lowly infected one sheds a lower number of cercariae (Mutuku et al., 2014b). The cercariae are transformed sporocysts within the snail that are shed by infected snails during conducive conditions. The relationship between sporocysts and cercariae is not exploited by the use of real-time PCR quantification and thus the study focused on the correlation of *Schistosoma mansoni* cercariae shed by snails and the quantity of its DNA (sporocysts DNA) in snails by the use of a real-time PCR technique. The real-time PCR can quantify the DNA components of target DNA but has not been used to determine the different amounts of the sporocyst in snails as they

asexually increase within the snail over a 5-week period as they multiply and transform into cercariae. Therefore, the study used the real-time PCR to detect the quantities of sporocyst with the snail on a varied number of weeks post-infection with the same number of miracidia under laboratory conditions.

1.3 Justification

The prevalence and intensity of *Schistosoma* infection depend on the number of snails in the environment and the number infected in the freshwater body (Haggerty et al., 2020). Precise knowledge of snail distribution is essential for facilitating possible prevention and intervention strategies for management of the infection transmission along river Asao. Accurate knowledge of snail-borne disease distribution and the prevalence of the infection would provide the required momentum to complement the existing control strategy in Kenya. Several countries, such as Nigeria and Kenya, forecast the spread of trematodiasis based on freshwater snail species present in a particular area so that preventive measures can be taken in advance (Abdulkadir et al., 2018; Choubisa & Jaroli, 2013; Opisa et al., 2011). The PCR technology is an excellent advancement compared to microscopy-based diagnostic methods, specifically real-time PCR, in identifying and differentiating *S. haematobium* and *S. mansoni*. The real-time PCR allows for the direct characterization of DNA amplicons when coupled with HRM (Ten Hove et al., 2008). Also, the inability to confirm infection levels among snails by a quicker technique may result to slow response towards tackling transmissions and thus more new infections to humans. Determining weekly changes in the quantity of the Schistosoma DNA shall enable infection history such as predicting the time at which snails got infection and thus explain how the real-time PCR is such reliable for studying Schistosoma- snail dynamics.

1.4 General Objective

To determine the types and distribution of schistosomiasis snails along River Asao in Western Kenya and compare their infection levels using microscopy and real-time PCR.

1.5 Specific Objectives

i. To determine the types of *Bulinus* and *Biomphalaria* genus snails and distribution pattern along river Asao.

ii. To compare the prevalence of *Schistosoma* infection within *Bulinus* and *Biomphalaria* snails collected along river Asao by using both molecular and microscopic approaches.

iii. To determine the correlation in *Schistosoma* parasite load in snails and cercariae through microscopic method and the molecular method (RT PCR HRM analysis).

iv. To determine correlation between miracidia number and its quantity of *Schistosoma* DNA; and sporocyst quantify in different week period by real-time PCR.

1.6 Null Hypothesis

i. There are no different types of *Bulinus* and *Biomphalaria* genus snails and the snail types are not equally distributed along river Asao.

ii. There is no significant difference in the prevalence of *Schistosoma* infection within *Bulinus* and *Biomphalaria* snails collected along river Asao by using both molecular and microscopic approaches.

iii. There is no relationship between *Schistosoma* parasite load in snails and cercariae through microscopic method and the molecular method (RT PCR HRM analysis).

iv. There is no relationship between miracidia number and its quantity of DNA and no significant difference between sporocyst quantity in different week period by real-time PCR.

1.7 Significance of the Study

This study will provide knowledge on the distribution, types of the snails, infected number of the intermediate host snails along River Asao that will help in locating areas that are infection transmission foci. The study will further ascertain whether the real-time PCR method has an advantage over microscopy in identifying and quantifying *Schistosoma* parasites in snails. The ability of real-time PCR to distinguish parasites levels within the snail could show the extent of transmissions within a certain locality, predict when snails got infected and thus allow warnings to prevent increase in new cases of the disease. The overall significance of the study is to provide information on proper and reliable techniques to be adopted in the detection and quantification *Schistosoma* parasite in snails thus having accurate data for focal points of *Schistosoma* transmission including predicting the time when intermediate snails got infected through quantification of sporocyst infected by a certain number of miracidia.

1.8 Study Limitation

This study had some limitations that include unpredictable moderate rainfall (5-20mm) (Environment, 2019) during some of the sampling days that could have interfered with snail abundance as it would have dislodged some snails. Presence of rainfall could have also affected the overall availability of snails. Also, the COVID 19 pandemic prevented field operations and thus data collection. Almost half of the snails, were lost during the microscopy rearing period and their DNA could not be recovered as most of their bodies had disintegrated. The reason for snail death could be due to change in environment and parasite virulence within snails that had not started shedding cercariae.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction

Blood flukes of the genus *Schistosoma* cause schistosomiasis (Sundaraneedi et al., 2017). schistosomiasis in human is caused by *Schistosoma* parasites which include *Schistosoma haematobium, S. mansoni, S. japonicum, S. mekongi, S. intercalatum* and *S. guineensis*. The disease is endemic in 78 countries (Colley et al., 2014; WHO, n.d.). There are two main types of schistosomiasis *S. haematobium* and S. *mansoni* which depend on the snail genera *Bulinus* and *Biomphalaria* respectively. Snails are the macro-invertebrates serving as the intermediate hosts of the disease and are mostly found in freshwater environments. (Gryseels et al., 2006).

2.2 Transmission Life Cycle of Schistosomiasis

The life cycle of a schistosome undergoes through a final mammalian host and an intermediate host snail from a freshwater body (*Figure 1*). Cercariae are the infective *Schistosoma* parasitic stage that is passed to humans when they get exposed with cercariae-infested water. The cercariae penetrate the human skin and get into circulation through lymphatic vessels and blood capillaries. During penetration, the fork-tailed larvae lose their tails as they change into *Schistosomula*, after which they move into blood circulation. The *Schistosomula* are carried to all body parts through blood flow, where they get dislodged into the liver via the hepatic portal vein after several days of circulation. Large numbers of the *Schistosomula* get to be held up in the capillaries within the lungs during migration (Verjee, 2019; Wilson, 2009). The female and male worms mature sexually then pair up inside the portal system and, where the two species of *Schistosoma* migrate to venous vesicle plexus and pelvic venous plexus for *Schistosoma mansoni* and *Schistosoma haematobium,* respectively. *Schistosoma mansoni* worms take 4 to 6 weeks to oviposit, while those of *S. haematobium* take about 90 days. In urinary schistosomiasis, schistosome eggs permeate walls of the vasculature and enter the bladder and are later shed in urine. For the case of intestinal schistosomiasis, the eggs enter intestinal lumen in intestinal and are later shed stool. When eggs get into fresh water, they hatch into ciliated miracidia that infect specific freshwater snails such as *Biomphalaria pfeifferi* in Africa, in the case of *S. mansoni.* Infection takes 8-12 hours, which is the life span of the miracidia that penetrates the snail's soft tissue with the influence of different variables such as temperature, turbulence, the velocity of the water source, and presence of light. Other variables include; miracidia time in contact with

snails, chemotaxis, and the number of miracidia relative to intermediate snails available. Successfully penetrated miracidia within the snail transform into sacculate mother sporocysts that undergo asexual reproduction in two rounds and where daughter sporocysts release free-swimming cercariae in 4 to 6 weeks. The cercariae penetrate a definitive mammalian host and continue with the life cycle. *Schistosoma haematobium* and *S. mansoni* cercariae only infect humans, while *S. Japonicum* cercariae infect humans and many other reservoir hosts, over 40 species (Carabin et al., 2005; Viana et al., 2018). In order to determine the percentage prevalence by microscopy the snails in the study were checked for cercariae shedding for a period of at least 5 weeks after collection.

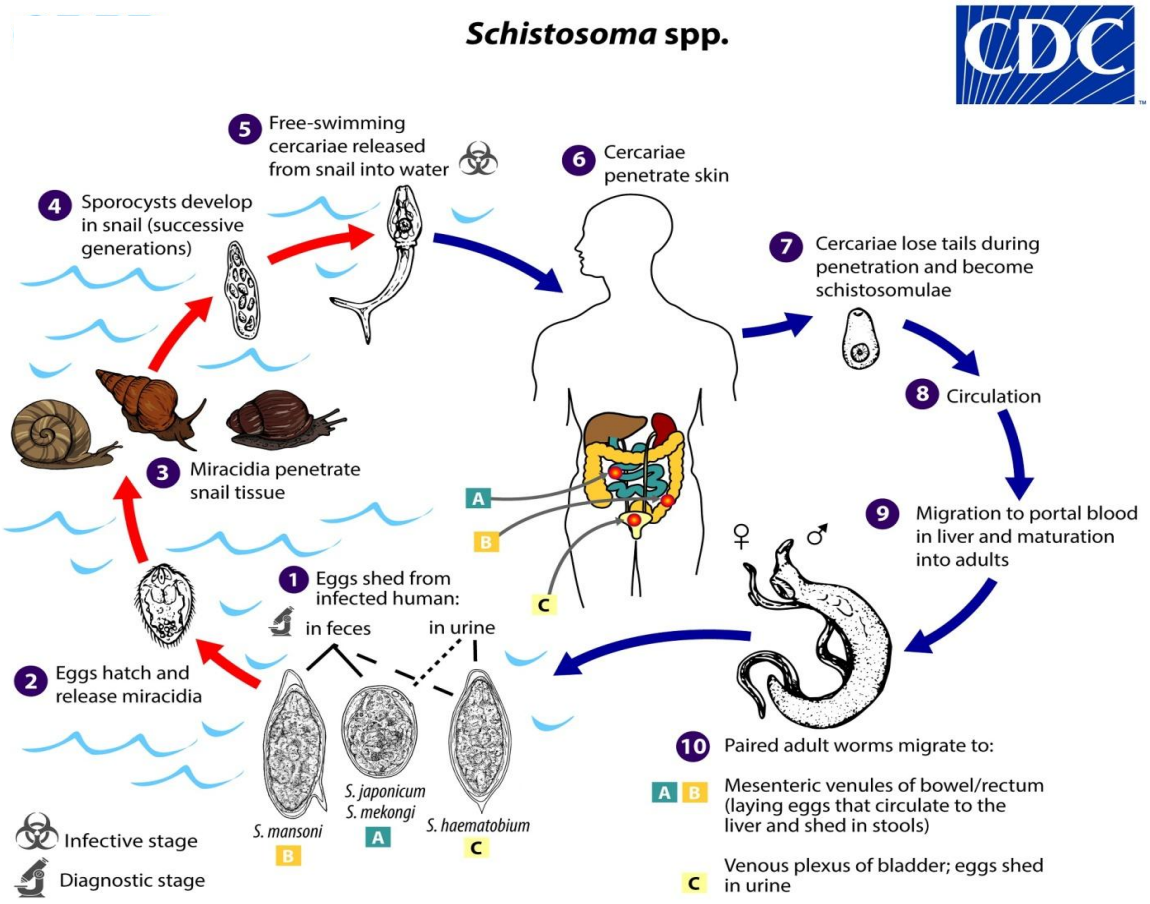

Figure 2.1: The transmission life cycle of Schistosomiasis (Intestinal and urinal) Content source: Global Health, Division of Parasitic Diseases and Malaria www.cdc.gov/parasites/

2.3 Risk Factors Associated with the Disease Transmission

Transmission of Schistosomiasis is affected by spatio-temporal variations (Walz et al., 2015). Humans become infected after direct skin touch to water containing cercariae shed by *Schistosoma*-infected snails during occupational, recreation, and domestic activities (Adenowo et al., 2015; Singh Saharan et al., 2016). These activities include fishing, swimming, and agriculture in open freshwater bodies (Colley et al., 2014; Gryseels et al., 2006). Previous studies suggest that open defecation raise the risks of spread of the infection (Sady et al., 2013).

Disease transmission risks occur due to people defecating and urinating while crossing or moving along the river as they conduct their daily activities. Also, traveling people from endemic to non-endemic regions can introduce infections if the specific snail's host is available in freshwater sources. Hence, the disease will be present in the area which did not have the disease (Assefa et al., 2021; Barakat, 2013). Lack of rain or poor rainfall leads to the construction of sustainable water development schemes to support agricultural and power needs, which involves people's movement from areas with different levels of the disease prevalence and hence further threats to the disease transmission (Adenowo et al., 2015). Pupils have a high prevalence of schistosomiasis (Balogun et al., 2022), with the disease mostly distributed in the age range between 10 and 15 years, who participate actively in activities that involve direct contact with infested water sources in endemic regions. After that, the number of infected individuals decreases as the children grow into adulthood as the water contact activities decrease (Assefa et al., 2021). Lack of knowledge about schistosomiasis, poor use of toilets, and irrigation of crops and plants with *Schistosoma* parasite-infested freshwater increases risk transmission of schistosomiasis (Nyati-Jokomo & Chimbari, 2017). Some studies have shown very high prevalence rates of the disease among school going children living close to inland waters or along the lake region (Odiere et al., 2012; Sang et al., 2014; Sassa et al., 2020). There is also lack of knowledge on transmission foci of the disease on River Asao and consequently the risk unto which the human might be at in contacting the infection. Studies have reported the focal nature of schistosomiasis especially in the inland regions (Grimes et al., 2014), suggesting a non-uniform distribution of snail intermediate hosts and their intensity of infection by the parasite larval stages. However, there is limited of information on distribution of the snail vectors and their role in transmitting trematode infections along river Asao in Nyakach Sub- County,

Western Kenya. The study therefore determined the prevalence of the disease per site in River Asao to Lake Victoria.

2.4 Bulinus and Biomphalaria Host Species and Schistosomiasis Distribution

Snails of the genus *Bulinus* and *Biomphalaria* belong to the subfamily Planorbidae. *Biomphalaria choanomphalla, Biomphalaria sudanica* and *Bi. Pfeifferi* are snail species that belong to the genus *Biomphalaria* but are located in different ecotypes since they are located and inhabit most small water bodies and stream along sub-Saharan Africa (Coulibaly & Idris, 2003). Intermediate snail host distribution is affected by ecological changes (Chimbari et al., 2020). Snails belonging to the *Biomphalaria* group are found in small numbers in Kano plain close to Lake Victoria and are rare in marshlands. *Biomphalaria pfeifferi* are located in permanent water sources and are absent in seasonal water bodies and marshes. *Biomphalaria sudanica* is mainly present in marshes and has spread schistosomiasis in Kano Plain. B*iomphalaria chaonomphala* is only found in Lake Victoria. Both *Schistosoma* intermediate hosts survive well in areas with rainfall ranges between 750 to 1,500mm. Survival is hindered when rainfall levels fall below 750mm. Transmission of *Schistosoma* is high during rainy seasons, that begin from March to May, and from October to December in Kenya and *Biomphalaria* populations are high during wet seasons (Hailegebriel et al., 2022; Mutinga & Ngoka, 1971).

All snails belonging to the genus *Bulinus* are found in four species groups; only those belonging to *Bulinus africanus* are responsible for transmitting *S. haematobium.* Only four subspecies out of 14 of the B. africanus in Africa are found in Kenya, and they include *Bulinus africanus, Bu. Globusus, Bu. nasutus,* and B*u. ugandae. Bulinus ugandae* has not been observed to transmit *Schistosoma* (Brown, 1994). The main intermediate snail hosts for *S. haematobium* in Kenya are *Bulinus nasutus* and *Bulinus africanus. Bulinus africanus* reside in altitudes of 1800m and above where the water temperature is low (18℃) and can survive eight months of drought. They are mainly found in large reservoirs and permanent streams. *Bulinus nasutus* species are distributed in Western and Nyanza provinces in Kenya, and their main habitat is seasonal ponds with muddy water (WHO, n.d.). *Bulinus ugandae* and *B. globusus* are uncommon within the country (Kenya). However, they are found in large numbers on L. Victoria shores and neighboring marshes. *Bulinus trancatus* snail species are found in water reservoirs that find their way to irrigation canals. *Bulinus forskalii* and *B. tropicus* are found in seasonal water sources, mainly

small pools of water located near farms and residual ponds (WHO, n.d.). *Bulinus* and *Biomphalaria* are all hermaphrodites. They have reproductive organs of the female and male sexes and can self-fertilize or cross-fertilize. A single snail is capable of populating a whole non-snail habiting environment when introduced alone. The snails lay eggs in batches of 5 to 40, normally enclosed in a yellow or colorless jelly-like material for *Biomphalaria* and *Bulinus* snail species, respectively. The egg hatches in six to eight days, and the young snails mature within 1 to 2 months, depending on environmental conditions and species type. The main factors that affect snails' egg laying include food availability and temperature. A mature may live for a year, or more where up to a thousand eggs are laid (Jordan, P.; Webbe, G. & Sturrock, 1993). Water velocity less than 0.3m/s, optimum water temperature (18°C to 28°C), little turbidity, water level, a slope below 20m/km, mud substrate that is firmly attached to the ground, a small amount of organic pollution, and partial shade conditions favor the survival of snails belonging to genus *Bulinus* and *Biomphalaria* (Chimbari et al., 2020; Hailegebriel et al., 2022; Rabone et al., 2019). In Africa, most irrigation systems lack proper drainage, thereby increasing the chances *of Schistosoma* transmission. Improper drainage systems provide a suitable habitat for snail survival, such as enough aquatic vegetation. There are different snails in the different habitats neighboring L. Victoria and inland fresh water bodies. The study seeks to identify snail types present in River Asao that are intermediate hosts for schistosomiasis since it extends from inland areas to shore line areas of Lake Victoria that is, the river.

Schistosoma mansoni is most prevalent as it is found in 55 countries, namely, the Arab states, North African countries such as Egypt and Sudan, and a West African country that is Libya, Sub-Saharan African countries, South America such as Brazil, some Caribbean islands, and Venezuela, as shown in Figure 2.2 (Olveda et al., 2013). The Middle East and a bigger part of Africa has most cases of schistosomiasis comprising of 110 million or more individuals getting infected annually. African countries that are endemic for schistosomiasis are about 53 countries (Barakat, 2013; Olveda et al., 2013).

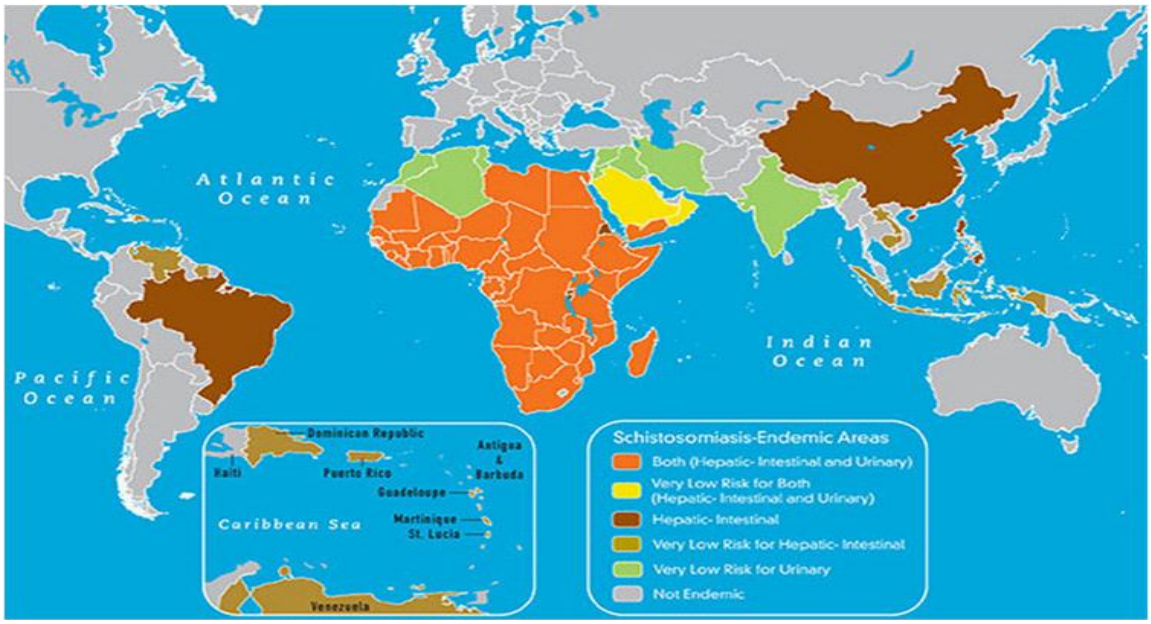

Figure 2.2: A map showing global distribution of schistosomiasis. Source: US Centers of Disease Control and Prevention (Olveda et al., 2013)

2.5 Snail Identification and Cercariae Detection

Polymerase chain reaction technology is the best alternative for microscopy methods for diagnosis, in particular, use of real-time PCR for identifying and differentiating of *S. haematobium* and *S. mansoni* (Kjetland et al., 2009; Ten Hove et al., 2008). Also, a multiplex real-time PCR method can detect and quantify *S. haematobium* and *S. mansoni* infections in endemic areas and proved to be more efficient in detection (Ten Hove et al., 2008). High resolution melting (HRM) analysis is a post-PCR analysis directly quantifies already amplified DNA. The real-time PCR work well without need for multiplex method. The cost of rt-PCR is low compared to standard. The HRM has been applied previously in studies relating to patients with clinical conditions but has less applications in the diagnosis of parasitic infections, particularly in molecular studies of some helminth and protozoa infections (Gudnason et al., 2007). The PCR-HRM assay has not been used in detection and quantification of parasite DNA in snail vectors. Techniques for qualitative detection of cercariae of the avian schistosome and *S. japonicum* have been developed to test for presence and absence of the former in intermediate snail hosts(Driscoll et al., 2005) and also *S. mansoni* in water (Abbasi et al., 2010). Previously PCR has been used for *S. mansoni* eggs quantification in animal and human stool and quantification of *S. mansoni* DNA (Ten Hove et al., 2008). Limited studies have examined the

impact of dose-dependent *Schistosoma* exposure on infection establishment in the snails (Mutuku et al., 2014b). The studies have been limited as the outcome measure depended measurement of cercariae shedding. The extent to which infection rates in snails depend on initial exposure to miracidia has not been studied in western Kenya. Previous studies show that a highly infected snail sheds a higher number of cercariae, and a lowly infected one sheds a lower number of cercariae (Mutuku et al., 2014b). The cercariae are transformed sporocysts within the snail that are shed by infected snail during conducive conditions. The relationship between sporocysts and cercariae is not exploited by use real-time quantification and thus the study focused on correlation of *Schistosoma mansoni* cercariae shed by snails and the quantity of its DNA (sporocysts DNA) in snails by the use of a real-time technique. The sporocyst gradually increase within the snail over one month period. The real-time PCR quantifies DNA contents but don't reflect the times at which the snail was penetrated by a miracidium. Therefore, the study used the real-time PCR to detect the quantities of sporocyst with the snail on varied number of weeks post infection with the same number of miracidia under laboratory conditions. This will help us predict the time unto which a snail is infected by miracidia from the point of miracidia entry in a snail by obtaining the gradient of perfect relationship. Investigation of intermediate host snails provides important knowledge on areas with high risks of *Schistosoma* transmission (Abe et al., 2018). An important component in understanding the epidemiology of schistosomiasis involves detection of infection within snail intermediate hosts. The most common approach in to is the induction of cercarial shedding by snails followed by microscopic examination (Haggerty et al., 2020). Studies indicate that the infection rates of snails differ according to snail species in question (Kariuki et al., 2017). The gold standard microscopy technique may therefore not reflect the actual *Schistosoma* parasite prevalence in transmission site. A quick and sensitive real-time PCR technique wasdeveloped as an alternative but has been applied in limited settings in Coastal Kenya (Kariuki et al., 2005). There is need to measure the prevalence of *Schistosoma* infection within snail species collected in river Asao in western Kenya by microscopy and PCR.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area

The observational and analytical study was done in Western Kenya that is one of the areas in Kenya which is endemic for bilharzia and the disease parasite distribution in intermediate snail host is not available in the specific area of the study. Some studies have shown very high prevalence rates of the disease among school going children living close to inland waters or along the lake region (Odiere et al., 2012; Sang et al., 2014; Sassa et al., 2020). The focus put on a river that flow towards Lake Victoria to ascertain the focal point or the source of infection among the sampling sites within the river. R. Asao was the target study area running from site Ps1 (S 00 19.052', E 035 00.399' to site Psn (S 00 16.823', E 034 55.632') within Kisumu County (figure 3.1*)*. Total area sampled starting from Ps 1 to Psn was 16 km. Part a; a map of Kenya. Part b; Area targeted for the field survey with yellow pins dropped to mark study site, that is, along R. Asao where 16km was sampled from Ps1 towards Psn where the river enters the lake as shown in the figure 3.1 below.

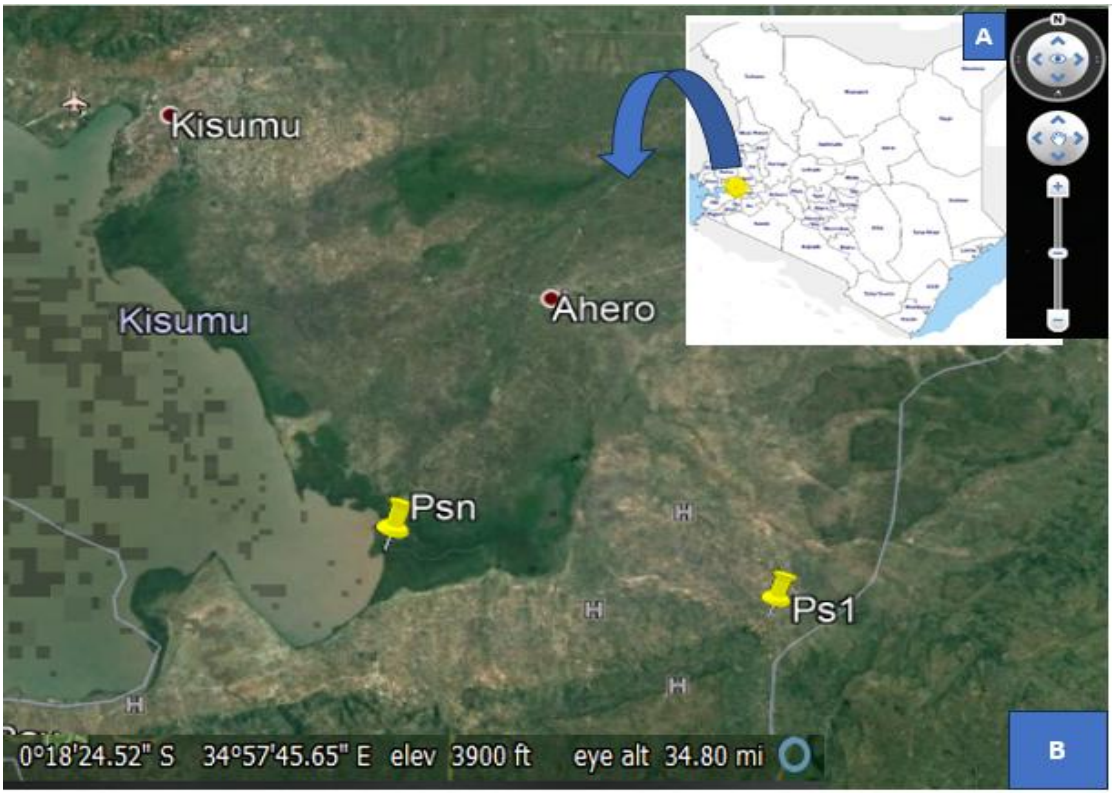

Figure 1.1: A map of Kenya and the sampling site area

3.2 Site Characterization

The sampling procedure targeted dry season (October to December year 2019) of the year in the region where the water flow of the slow to sustain snail's survival (Chimbari et al., 2020) Sampling was done at the riverbanks characterized by submerged, emerging or floating vegetation. A 0.05km m section was sampled and considered as the sampling site. The distance between two sampling points was 0.45km. The distance was chosen to reduce chances of snails being carried by water they snails are disturbed sampling activities, other human activities, animals crossing or rainfall that may interfere with water speed where the average speed is supposed to be 3m/s (Chimbari et al., 2020). Biotic and non-biotic factor are same and that there are no much deviations affecting the snail's availability of one part of the other and thus infection prevalence as well. The sampling area was located from a Google map showing the distance of the location from the lake and the coordinates (*figure 3.2*). This was also complemented with a Garmin GPS 12xl navigator on the field sites. Only the selected site was sampled. Water depth was measured using a meter rule and water velocity of the sampling site was obtained by use of a stopwatch and a 10-liter bucket (Othman et al., 2017). Water velocity was calculated as the time taken to fill a bucket (10 liters) (Othman et al., 2017)). The dominant land cover that surrounds the sampling site was noted down as natural, farmland, semi-urban or urban and for each site, photos were taken using a camera.

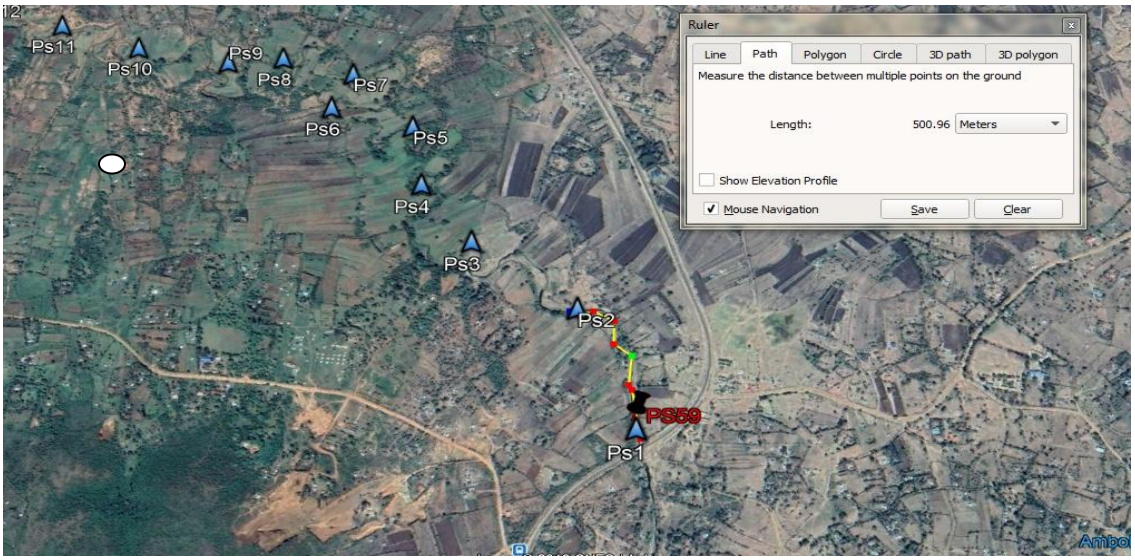

Figure 3.2: A detailed map of study site with blue arrows indicating the sampling point. The ruler on the top right measured the distance between sampling points in a Google Earth map.

3.3 Study Design

The study design was a cross-sectional. Snails were collected from River Asao in one dry season, snail types identified and their distribution found among sampling sites. All snails collected from the field were counted as total samples and *Schistosoma* infection prevalence was done per site and per snail type using both microscopy and real-time PCR techniques.

3.4 Experimental Design

Snails collected from sampling sites were checked for infection using microscopy and rt-PCR to determine snail prevalence in R. Asao. Cercariae shed from *Schistosoma-positive* snails were counted, and the count was correlated with sporocyst DNA inside the snails as measured by real-time PCR. To test for the rt-PCR in quantifying the parasites' DNA, hatched miracidia quantified by real-time PCR. After that, snails were infected in the laboratory using miracidia, and later, the sporocysts in the infected snails were quantified weekly for five weeks as seen below in figure 3.3.

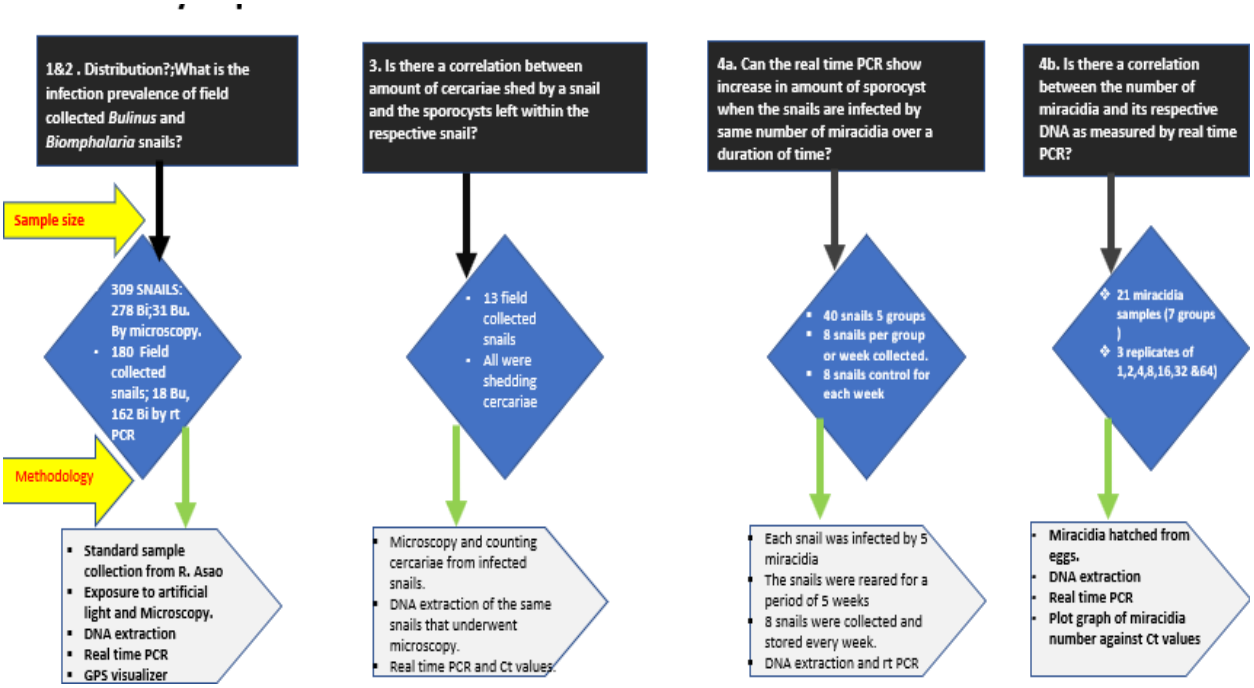

Figure 3.3: Experimental design flow diagram

3.5 Snail Sampling and Collection

At each sampling site, each side of the river was sampled for snails. A sweep net or a snail catcher with a 2 m metal handle was used to collect snails. The sampling procedure involved dragging a sweep net or a snail catcher 1m towards the riverbank at an angle of 45° and shaking the vegetation gently to enable the snails attached to vegetation to fall into the net (Ouma et al., 1989). The snail catcher was used in areas where the sweep net could not pass through the vegetation. Alternatively, snails resting vegetation and under shallow depths of the river were collected using long forceps or directly with gloved hands. Collection was repeated several times to ensure a thorough coverage of the sampling site. The time during the day in which the snails were collected was noted.

Plate 1: Images for *Biomphalaria* and *Bulinus* shells.

Shells of *Biomphalaria* (a) They have a discoid shell and *Bulinus* snails (b) Shell's shape is sinistral, the height measure is bigger than width, ovate, or higher to nearly cylindrical, inversely conical, and turreted according to the spire height (Mandahl-Barth, 1962) .

After collection, the snails were put into separate tubes according to their genus and counted per site using some of the most common morphological differences described in below as per Mandahl-Barth (Table 2.1).

Table 3.1: Morphological differences between *Biomphalaria* and *Bulinus* snails

***Biomphalaria* snail**	***Bulinus* snail**
➢ They have a discoid shell. ➢ Shell height is not more than 2.5mm high ➢ Shell is ultra-dextral, orbicular, and biconcave ➢ Shell diameter is about 7mm to about 22mm. ➢ The whorls count varies between 3.5 and almost 7. ➢ The shell is a lighter or darker brownish horn-colour, sometimes almost white, in other cases more reddish and very often concealed by a grey or black coating.	➢ Shell is sinistral, invariably having greater height than width, inversely conical, ovate, or higher to nearly cylindrical, and turreted according to spire height. ➢ Shell height begin from 4mm to 23mm ➢ The number of whorls is usually from 4 to 5 or some forms may be greater than 7 ➢ Aperture is high wide with low spire, and relatively narrower in forms with a high spire. ➢ In colour the shell appears from nearly clear white to dark brown

3.6 Transport and Sample Storage in Snail Screen House

All snails collected per sampling day were put in separate labeled 3L troughs. The separation was based on the different sampling locations and the different genera. They were carefully placed inside a 7 liter cooler box to avoid desiccation. They were transported to the laboratory at ITOC (International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus) for identification and check for *Schistosoma* cercariae. The snails from the field were transferred into separate troughs containing dechlorinated tap water and oxygen supply from an air pump. Sizable (3cm diameter kale could feed three snails for three days) pieces of kale were boiled, left to cool, and then fed to the snails in the rearing troughs.

3.7 Determining Infected Snails and Parasite (Cercariae) Load

3.7.1 Microscopy Detection

The snails from the rearing troughs were rinsed with distilled water to remove any cercariae that could be in the rearing troughs and then put into 24-well plates and distilled water added to half a well (not exceeding three-quarter). The 24-well plates were then placed indoor and artificial light source was used to ensure the snails are exposed to standard amount of light (shades unvaried amount of light unlike to sunlight)(Sharif et al., 2010). Natural sunlight was not used since it varies in intensity in different days and times of the day as well. After 1- 2 hours of exposure, the snails were observed under dissecting microscope for detection of cercariae.

To determine infection rates by microscopy, the 24-well plate (Figure 6b) was placed on the stage of the dissecting microscope (Mg: X5 is the maximum). All wells were examined for presence of cercariae. Water that contained cercariae was collected and put into a separate labelled vial. The infected snails were then separated from the uninfected ones.

The cercariae shed by the infected snails was stained by adding 15 µl of trypan blue (McMahon & Rohr, 2014) for every 500 µl of the water containing cercariae in a micro-invertebrate counter plate. After staining by 20 µl of Lugol's iodine put on to stain further and kill the cercariae for easy counting. The cercariae were left for five minutes to die after addition of Lugol's iodine (figure 5a). The cercariae number was recorded. When the amount of the sample was less, 1-2 drops of the iodine was added to 200µl of the sample containing trypan blue was added into a petri dish and let stand for about 1 minute. The parasite load was determined as the number of cercariae per volume of water. Cercariae counting was repeated thrice per sample by pipetting 250µl of the water with the cercariae. The water was then stained with Lugol's iodine and then an average number of the counted cercariae was calculated. The same was done for each single cercariae shedding snail for three days and then overall average number of the cercariae shed by the snail was calculated.

A microscopic image of cercariae stained with iodine. *Schistosoma mansoni* cercariae characterized with a fork tail (indicated by an arrow) and the branched tail, normally folded. b. A 24-well plate. Snails were place in the 24- well plate (b) with three-quarter fill of water and a positive snail shed Cercariae (a) as shown in figure 3.4 below.

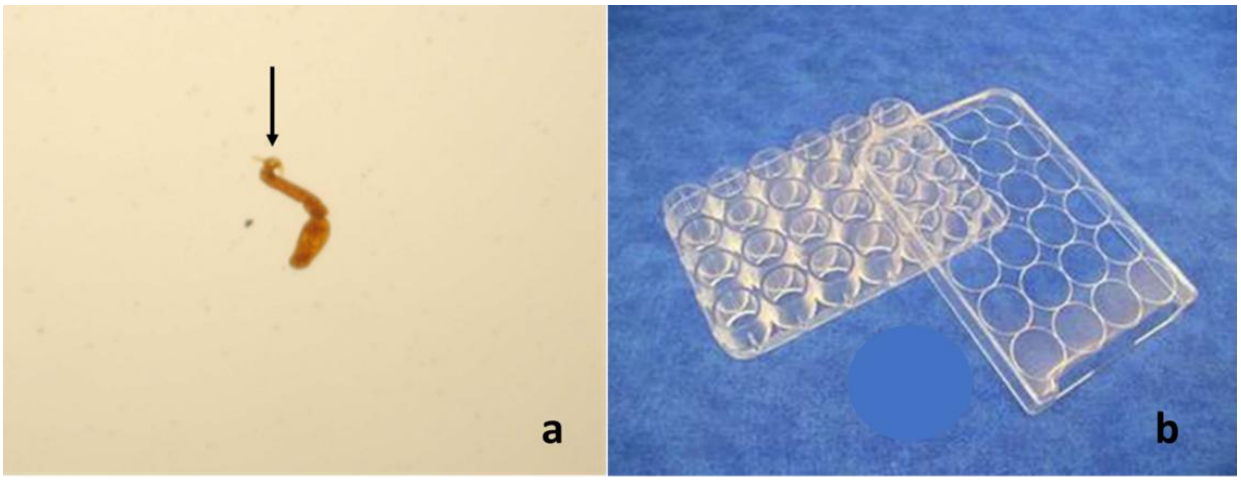

Figure 3.4: *Schistosoma* cercariae image (a) and a 24-well plate and its top cover (b).

3.8 Determination of Parasitic Load By Real-Time PCR HRM Analysis

3.8.1 DNA Extraction

The snail soft tissue was removed from the snail's shell by use of scalpel and sharp forceps and then homogenized in 300µL of cell lysis buffer (100mM Tris-HCL, pH 8 0, 1.4M NaCl, 20mM EDTA, 2% Hexadecyltrimethylammonium bromide (CTAB), 0.2% 2-mercaptoethanol) while incubating in a heating block for an hour at 65°C, removed and cooled to room temperature. After cooling, 100ml of Protein precipitate solution was added, vortexed, and placed in ice for 5 minutes then centrifuged at maximum speed (14,500*g at 4℃) for 5 minutes and all the floatable was removed and placed in a new eppendorf tube. Isopropanol (300ml) was then added to the supernatant, mixed by inverting 100 times and then centrifuged for 1 hour. All the supernatant was pipetted off after centrifugation and 300µL 70% of ice-cold ethanol was put on to the eppendorf tubes which were then centrifuged for 30 minutes at maximum speed (14,500*g at 4℃) and then the ethanol was pipetted off and discarded. The eppendorf tubes with the DNA were then air dried on a tissue paper overnight and in the morning the pellet was dissolved in 50µL of double distilled water /nuclease-free water and then placed in a heat bath for an hour at 65°C. The sample DNA was left in the fridge and at -20°C for PCR.

3.8.2 Detection of *Schistosoma* Parasite by PCR

A conventional PCR was used to test published primers done by Sady and others where the PCR conditions could be adjusted to ensure the primers were working using positive controls, although the primers were previously used to examine target *Schistosoma* parasite in stool and urine samples (Sady et al., 2015). An annealing temperature range was between 55°C and 60°C,

2 µl (HotStar Taq DNA Polymerase, QIAGEN), published primers 0.5 µl forward (ShmF-5′-GGATTGATTTGTGCTATGGC-3′) and reverse (ShmR5'- CACCGCCWAYCGTAAATAA-3'), 1-2µl of the snail's DNA, and an amount of molecular water to add the reaction mixture to a volume of 10µl. A segment of 267 bp in cox1 mitochondrial DNA (mtDNA) of both *S. haematobium* and *S. mansoni* was amplified by ShmF and ShmR primer pairs adopted from study done by Littlewood and others (Littlewood et al., 2006). This variable region has relatively good amplification with many conserved regions and structures and most abundant within the cell (Littlewood et al., 2006). PCR machine was set at 95°C for 10 minutes for initial activation, 35 cycles for 30 s for denaturization at 95°C, 58°C for 1 minute 30 seconds for annealing, 72°C for 1 min and 72°C for 7 minutes for extension and final extension respectively.

3.8.3 Gel Electrophoresis

A 2% agarose gel was made ready by weighing 2 mg of the agarose in a conical flask where 100ml of 1X TAE buffer was added, mixed by gently swirling and then heated for two minutes and allowed to cool to 65°C where 5 µl of Ethidium Bromide DNA gel stain (Invitrogen, Waltham, MA, USA) was added and the mixture drawn into a gel plate with well fitted combs and then allowed to solidify. When the gel hardened, it was taken to a gel tank and the PCR product was transferred to the wells and then electrophoresis run at 125 V for 30 minutes. Finally, the results were observed on a UV transilluminator. Gel electrophoresis procedure was used to help get the annealing temperatures for primers used to test for *Schistosoma* parasite by use of positive control samples.

3.8.4 Real-Time PCR-HRM Assay

Same conditions used for primer optimization (55°C-60°C) in conventional PCR were used as a trial to run samples in real-time PCR.

The final volume for real-time PCR was of 10 µl with 1X of MeltDoctor HRM Master Mix,0.25 µl of each primer (ShmF and ShmR), 1 µl of DNA extract and the rest of the was deionized water (4.5 µl). PCR cycling for HRM curve conditions consisted of one cycle of activating enzymes in 15 minutes at 95°C, amplification for 35 cycles that had denaturation step at 95°C for 30 s and an annealing step at 58°C for 90 seconds and a subsequent melting step to begin directly in the same real-time PCR machine by increasing the temperature to; 95°C for 10 s, 58°C for 1 min for denaturization and annealing respectively, HRM reaction at 95°C for 30 seconds and

annealing in 15 seconds at 60°C and hold after cycling at 72°C in 420 seconds. The real-time PCR assay was used in detection of infected snails and quantification of *Schistosoma* infection in the snail's DNA. DNA free water was used as a negative control for *Schistosoma* in all the runs made in real-time PCR.

3.8.5 Quantification of *Schistosoma* Parasite (sporocyst) Developing within Snails

Field collected snails were first tested by microscopy for being free of *Schistosoma* parasites *Schistosoma* infection. This was done by first checking whether the snails shed for cercariae, then left for one month period then screened again for infection. Those that did not shed for cercariae were left for experiment. The snails that were free of infection were infected in the laboratory by *Schistosoma* miracidia for experimentation by Akbar Ganatra (Ganatra et al., 2024). The miracidia were obtained from *Schistosoma mansoni* positive children's stool sample. Through the area Chief, consent form was signed by the parents whose kids volunteered to offer their stool for examination. Those with high egg/mg (20eggs/mg) of stool count were selected to offer enough stool for experimentation. All children that tested positive were treated with Praziquantel. The eggs were harvested and hatching was induced by artificial light (Jurberg et al., 2008). The snails were then infected by miracidia with each snail infected with four miracidia that is four miracidia were picked using 100 microliter pipettes, drawn into a petri dish with the snail, and then observed on a microscope as they penetrate the snail. The infected snails were reared in a screen house at ICIPE and collected after every week for five weeks. The snails were then grouped per week and quantified using the real-time PCR. Eight (8) snails for each week were quantified. The primers; forward (ShmF-5′-GGATTGATTTGTGCTATGGC-3′) and reverse (ShmR5'- CACCGCCWAYCGTAAATAA-3'), were used.

3.8.6 Correlation between Miracidia Number and Ct Value

Another experiment was set up as a complement to the cercariae number versus RT PCR Ct-value to confirm the correlation between real-time PCR and microscopy in quantification of the infection. Miracidia eggs were harvested and hatched from stool sample which was positive for S*chistosoma mansoni.* The stool sample collection was an extra sample stored in the laboratory after experiments by Mr. A. Ganatra (Ganatra et al., 2024).There was ethical clearance for human stool sample collection. Through the area Chief, consent form was signed by the parents whose kids volunteered to offer their stool for examination. Those with high egg/mg

(20eggs/mg) of stool count were selected to offer enough stool for experimentation. All children that tested positive were treated with Praziquantel. After hatching, they were collected and put into separate eppendorf tubes in the order 1,2,4,8,16,32,64. Three replicates of the same were made and stored for DNA extraction. The amount of water in the eppendorf obtained while pipetting was reduced by allowing the miracidia to die first through putting the sample on ice or in the freezer then later the sample was centrifuged to sink the dead miracidia to the bottom and then extra water was pipetted off. DNA extraction was conducted for each miracidia sample in the eppendorf in different amounts were placed, same as that for real-time PCR detection of *Schistosoma* in snails in section (3.8.1) above. The primers; forward (ShmF-5′-GGATTGATTTGTGCTATGGC-3′) and reverse (ShmR5'- CACCGCCWAYCGTAAATAA-3'), were used. The cercariae number shed by snail with the aid of a microscope and the Ct-value was obtained after running each respective sample DNA in a real-time PCR.

3. 9 Data Analysis

The data collected was entered into a spread sheet then imported into SPSS for analysis. A GIS package – GPS (http://www.gpsvizulizer.com) visualizer was used to analyze number of snails (total collected, infected and uninfected) per site data so that distribution of the snails could be visualized along the river where sampling was done and also the sites with infected snails. Also, a GIS online software (GPS visualizer) was used to provide a visual analysis of the prevalence of the infected snail from each collection sites. SPSS version 20.0 was used for the analysis where regression test was used to determine whether the distance of a sampling site from the river predicts variation in the number of snails available. The data obtained was analyzed by Pearson's test to determine whether a snail that shed higher number of cercariae could have higher amount of the parasite DNA in its body or if a low cercariae number could indicate low amount of parasite's DNA within the snail. Pearson's product moment correlation coefficient was used to correlate between the parasite load in the infected snails obtained through microscopy and real-time PCR technique. Data analysis for sporocyst development within snails was performed in R software version 4.0.2 (R Core Team, 2020). The dataset on Ct value failed to meet assumptions of parametric statistics even after log-transformation (Shapiro-Wilks normality test: $W = 0.56$, $P < 0.001$) and therefore was subjected to Kruskalis-Walli's chi-squared test.

3.10 Ethical Consideration

Project was under Ganatra's project (Ganatra et al., 2024) whose approval certificate was provided by KEMRI Scientific and Ethics Review Units (SERU) - (KEMRI/SERU/CBRD/194/3836). A NACOSTI Research and Ethics certificate was obtained before the project's activities began. Best research practice and ethics was used to conduct this study. During the fieldwork, the scientist humbly asked for permission to cross people private homesteads or farms that could extend to the river basin. Also killing of the snails for extraction of DNA was done humanely by carefully removing the outer shell using a hard forceps or scalpel then submerge them into a lysis 70% for storage or directly into a lysis buffer to begin extraction immediately to avoid elongated agony. Disinfectants were applied on points of exposure in case of any contact with water from troughs containing field collected snails. Collection and handling of data was done by well-trained field assistants.

CHAPTER FOUR

RESULTS

4.1 Snail types and Distribution of Biomphalaria and Bulinus Snail Species along River Asao

Thirty (30) points selected along the river were sampled for snails. A total of 278 *Biomphalaria* and 31 *Bulinus* snails were collected from the sites and used for the study. The snail types identified were *Bulinus globosus* and *Biomphalaria pfeifferi* by the use of identification key by Mandahl-Barth. The distribution of *Bulinus* snails and *Biomphalaria* snail is shown. The size of each circle denotes the number of snails collected per site where a bigger circle indicates high snail number. The arrow points the direction in which the river flows (upstream to downstream). The first circle at the arrow side is Ps1. Yellow circles indicate *Biomphalaria* snails present, orange circles (within the larger yellow circles) indicate *Bulinus* snails are present while the blue triangles indicate either of *Biomphalaria* or *Bulinus* is absent. The distribution patterns and number of the intermediate snail hosts collected per site are shown in figure 4.1 below. The map image is a screenshot from GPS visualizer after the data was analyzed by it.

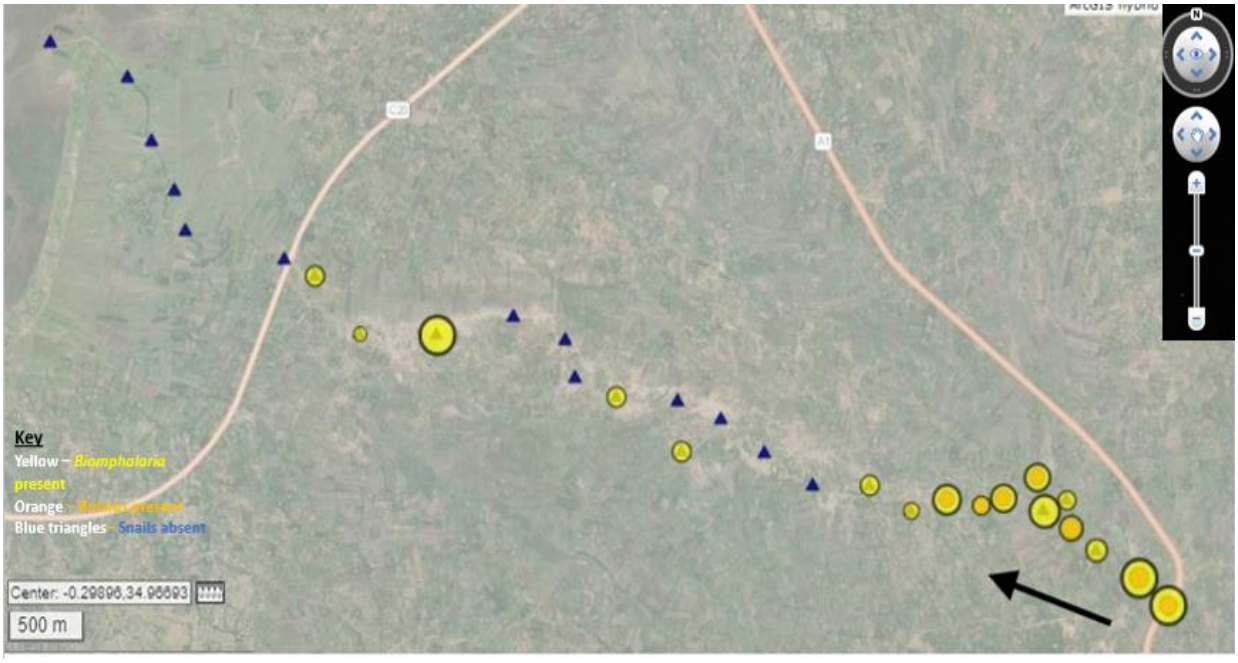

Figure 4.1: Sampling sites along river Asao, Kisumu County, Kenya

An actual representation of the snail number is shown the graph below. Y axis shows snail number and X-axis show the number of sampling sites beginning from site number 1 to 30.

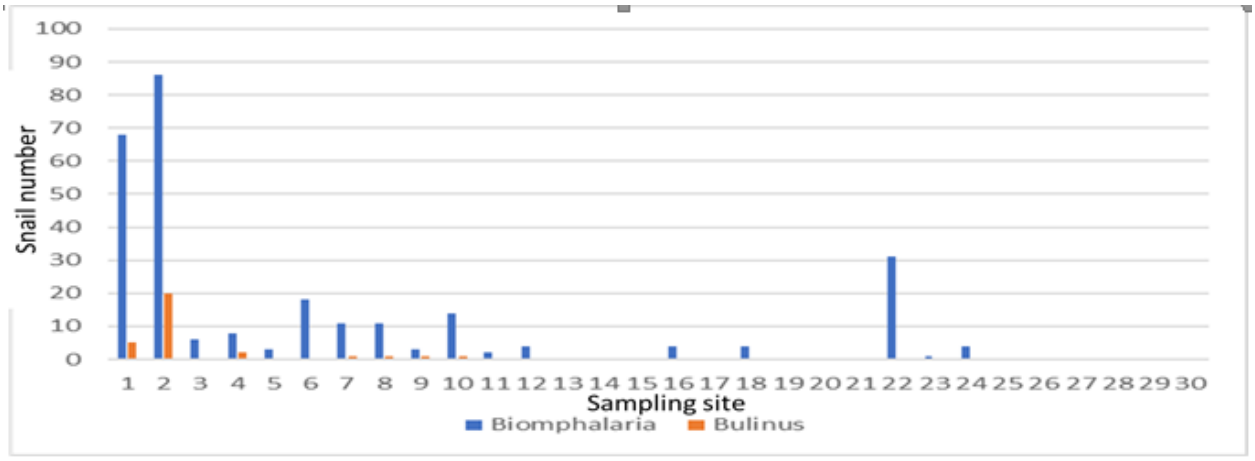

Figure 4.2: A bar graph showing snail numbers in study area.

4.2 Prevalence of the Infection within *Bulinus* and *Biomphalaria* Snails Collected along the River

Table 4.1: Number of infected snails as detected by microscopy and real-time PCR

Sites with infected snails	*Biomphalaria*		*Bulinus*	
	Microscopy	PCR	MICROSCOPY	PCR
Ps1	4	14	0	0
Ps2	2	10	5	5
Ps3	0	1	0	0
Ps6	0	1	0	0
Ps7	0	1	0	0

4.3 Infection Distribution Map

The green stars in Figure 4.3 below show areas with no infection while the yellow circles show area with *Schistosoma* positive *Biomphalaria* snails and dark circle in the within the second yellow indicates an area with *Schistosoma* positive *Bulinus* snails. The size of the circle indicates the snail number that were positive at that particular site.

Figure 4.3: Distribution map for infected snails in R. Asao.

In the Figure 4.4 below, the orange bars represent uninfected snails both *Bulinus* and *Biomphalaria* while the blue bars represent infected snails of both the *Bulinus* and *Biomphalaria* snails.

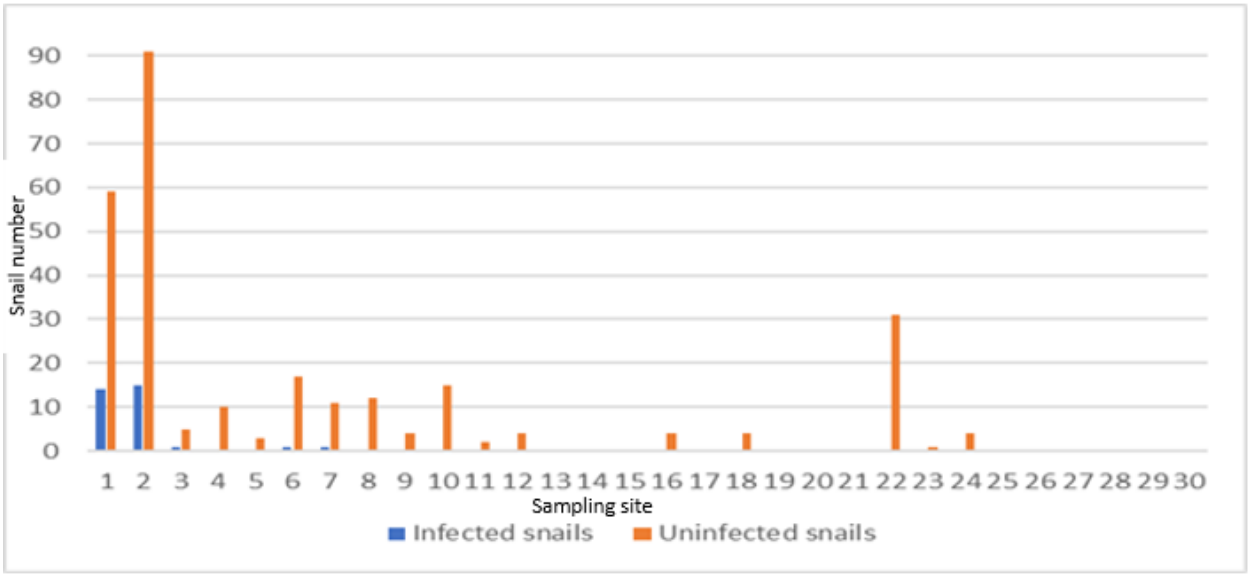

Figure 4.4: A graph showing general number of infected and uninfected snail numbers among sampling sites.

The table 4.2 below shows the number of snail samples collected and the percentage of the found positive by microscopy and real-time PCR. Polymerase chain reaction technique detected higher numbers of infected snails compared to microscopy where the real-time detected presence or absence of the nucleic acids of the parasites.

Bulinus snails tested had 16% positive snails by microscopy and *Biomphalaria* had 2%. In real-time PCR the *Bulinus* snails had 15% positive *Schistosoma* infection while *Biomphalaria* had 17% positive snails. The 5 snails of *Bulinus* and 6 snails of *Biomphalaria* that tested positive by microscopy were confirmed to be indeed positive by real-time PCR.

Table 1.2: Percentage infected snails through microscopy and real-time PCR

Snail specie s	**Microscopy**			**Real-time PCR**		
	Total	**Infected**	**% Infected**	**Total**	**Infected**	**% Infected**
Bulinus	31	5	16	18	5	27
Biomphalaria	278	6	2	162	28	17

4.4 Correlation in parasite load through the microscopic method and the molecular method (RT PCR HRM analysis)

There was a very weak negative correlation between the cercariae number and the Ct value (r= -0.261, P= 0.438). The number of cercariae shed by a snail in below did not significantly correlate with the contents of the *Schistosoma* parasite DNA within the snail. The box plot Figure 4.5 shows the relationship between cercariae number and the DNA amount of the cercariae.

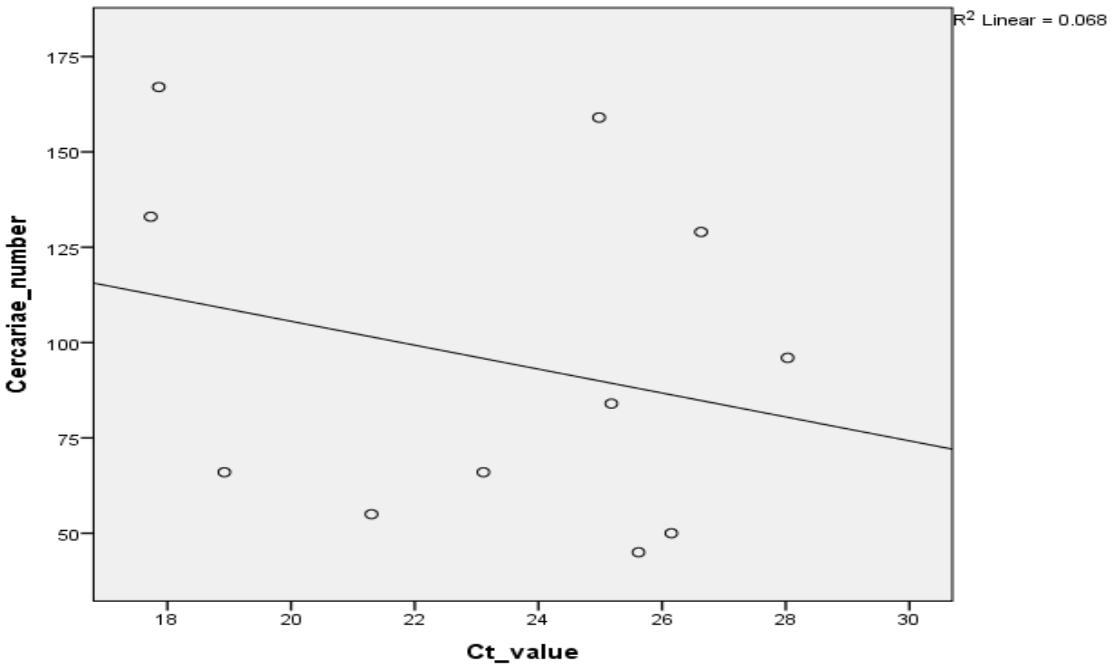

Figure 4.5: A graph showing relationship between cercariae number and correspond Ct values

4.5 Correlation between miracidia number and its corresponding Ct value and determining increase in sporocyst amounts

4.5.1 Miracidia number and its corresponding Ct- values (amount of DNA of the same number of miracidia)

There was a weak negative correlation between miracidia number and the Ct value ($r = -0.312$, $P = 0.192$). This indicates despite the increase in miracidia number, the real-time cannot relate a corresponding amount of DNA of the same miracidia measured as Ct-value by real-time PCR. The Figure 4.6 below shows the relationship between the miracidia number by counting under microscopy and the quantity of DNA for same number of miracidia.

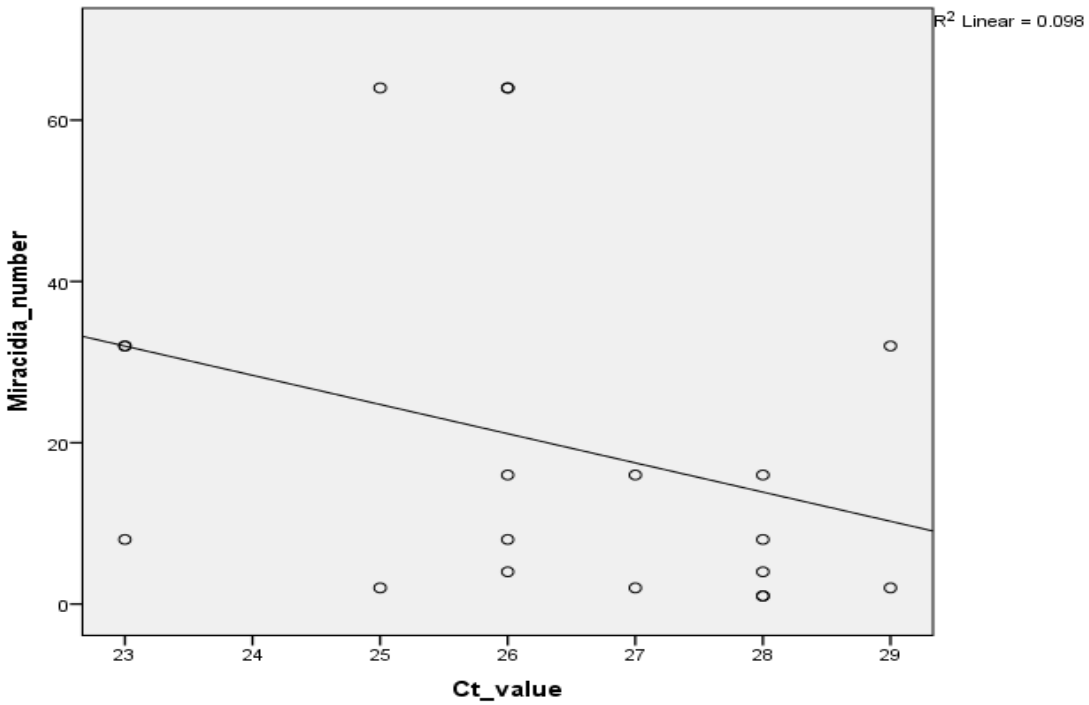

Figure 4.6: A graph showing the relationship between miracidia numbers and their corresponding Ct values.

4.5.2 Quantification of *Schistosoma* Sporocyst within Snails

In Figure 4.7 below, there is one outlier at after 1week post-infection with very low Ct and thus high parasite DNA content relative to samples of the similar. Comparing the P- values between weeks such as week 1 and 2, 1 and &3, 1 and 4, 2 and 4, 3 and 4, 3 and 5 is not significant but week 1 and 5, 2 and 3, 2 and 5, which is significant. In general, the Ct values varied significantly across the weeks after infection of the snails by equal number of miracidia (X^2 14.18, df = 4, P = 0.0067).

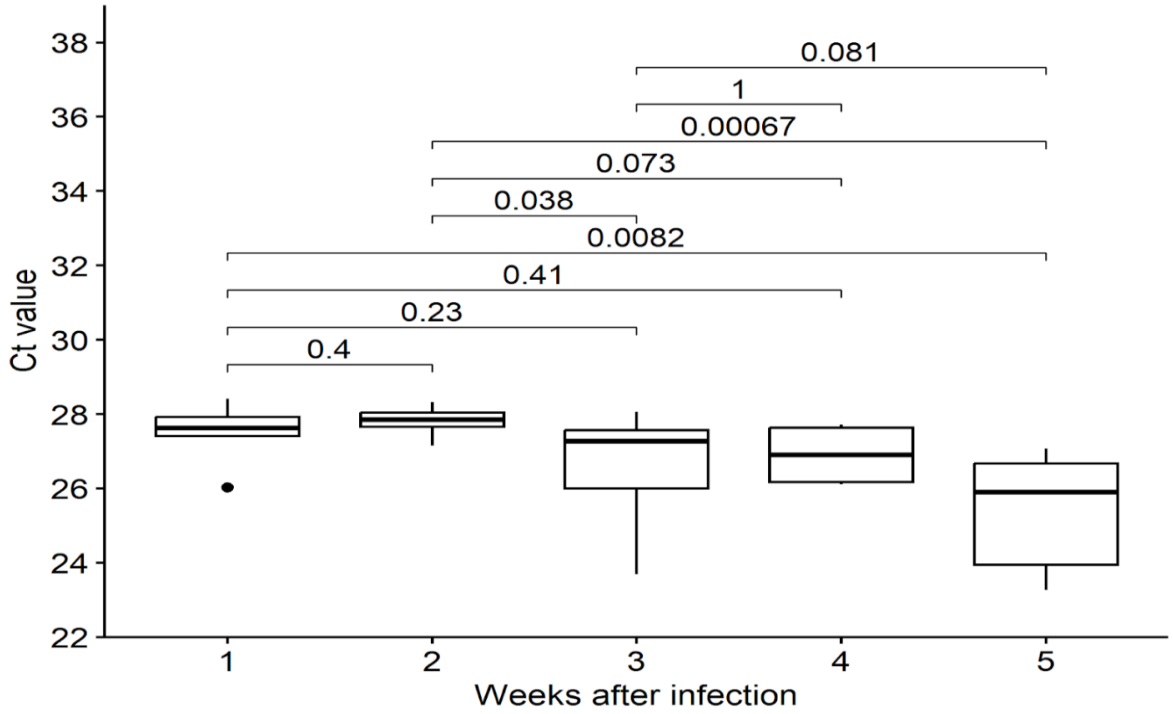

Figure 4.7: A box plot for sporocyst quantity against number of weeks after infection.

CHAPTER FIVE
DISCUSSION

5.1 Introduction

This study identified the first-time distribution of snails from various sampling sites at River Asao, 16 km from L. Victoria. Intermediate snails for schistosomiasis, *Bulinus*, and *Biomphalaria* snails were sampled from the areas to show the distribution and abundance of the intermediate snail species. The study compared the accuracy and precision of two tools used for detecting parasitism by *Schistosoma mansoni* in their respective host snail. Microscopy was used to determine the infection of snails and quantify the level of infection in each snail by counting the cercariae shed per volume of water (microliter), as done in a previous experiment by Hung and Remais (2008). Using real-time PCR to identify and quantify snail infection, then the observation was compared among the two methods.

5.2 Snail types and snail distribution in River Asao

A first-time *Bulinus* snail and *Biomphalaria* snail distribution map were generated as the first exclusive study of this river. The use of the Geographical Information System (GIS) is essential in such a way that it helps in mapping locations where active transmission of schistosomiasis occurs once identified (Belizario et al., 2021). This will enable the application of control measures in the active sites, such as the use of molluscicides (WHO, 2017), environmental methods, biological methods such as snail-predators or introduction of schistosome resistant strains into wild populations (Marques et al., 2014) integrated with MDA for infected individuals and WASH to avoid contact with infested water can be used to stop transmission of the disease and avoidance of open defecation that stops the transmission cycle. The distribution maps of snails collected and the location of infected snails that were identified after identification show overall, the distribution of *Biomphalaria* host snails was higher than that of *Bulinus* such that at each sampling site, there were more *Biomphalaria* snails compared to *Bulinus* (figure 8). The snail types include *Bulinus globosus* and *Biomphalaria pfeifferi*. These findings are similar to those done by Selpha Opisa and others (2011) for the types of snails in inland waters. In some other sampling sites (site 4, 5, 9, 10, 11, 12, 16, 18, 21-24), there were no *Bulinus* snails, while the *Biomphalaria* snails were present. The distribution map will help direct the intervention members concentrate the limited resources towards these active sites.

5.3 Snail Prevalence as Detected by Microscopy And Real-Time PCR

As shown in the current study, areas with a higher number of snails such as site 1 and 2 have a higher prevalence (21% and 14% respectively) of infection than areas with a small number of snails. In contrast, those with a low number of snails have lower prevalence, except site number 22; the number of snails was high, yet no infected snail was found. Only a quarter of the total 16km study area had both *S. mansoni and S. haematobium* infected-snails situated within a small local region. The findings are consistent with those done by Grimes et al. that reported focal nature of schistosomiasis (Grimes et al., 2014) especially in the inland region. This explains that the transmission of schistosomiasis is restricted to a small geographical region where the human host and the intermediate host snails, complete the transmission cycle. The human release infected fecal matter and urine which find their way to water source where the snails are infected, harbor the parasites and later the human gets into contact with *Schistosoma* infected water.

In determining the presence or absence of the infection in snails, the real-time PCR technique detected a 15% infected *Bulinus* snails and 28% infected *Biomphalaria* snails) compared to microscopy, which detected 16% infected *Bulinus* snails and 2% infected *Biomphalaria* snails. At the time of real-time much snails had died during microscopy and hard to recover their DNA. Snail's soft tissues were drawn by bubbles coming from aerators. Our results show that real-time PCR detected *Schistosoma* DNA in more snails than were shedding cercariae, indicating prepatent infection which would otherwise have been unidentified. These findings are congruent with those of (Fuss et al., 2020; Guegan et al., 2019a; Sady et al., 2015), based *on Schistosoma* parasite detection in stool and urine using Kato Katz and real-time PCR. Real-time PCR has a higher sensitivity and can detect prepatent and common infections of the *Schistosoma* parasite before the snail can shed them (Pillay et al., 2014). It has a shorter protocol than microscopy, where the preparation process for checking the shedding of cercariae in snails is long and needs 4-6 weeks or more sometimes before a cercariae is shed (McManus et al., 2018). From the point of entry by miracidia, some snails died as they shift to adapt to the laboratory-rearing conditions or at the end of their life span. The dead snails could have parasites that had not developed into cercariae which are seen through microscopy. Rearing snails to check for cercariae to ensure maximization of microscopy may require qualified personnel. Real-

time PCR could detect the minute amounts of parasites' nucleic acid and thus increase the overall incidence of infection in the field-collected snails. This can accurately estimate infection incidences in a sampling site where the snails are collected. The findings of the study are supported by previous study by Pontes (2003) and others as they observed the real-time PCR is to be more sensitive than microscopy in detecting schistosome parasites, especially in low infection samples. The infection prevalence of schistosomiasis in snails show that *Schistosoma mansoni* 85% is high than *S. haematobium* 15%. This is consistent with study done in Western Kenya, by Sang and others among human populations bordering water bodies with 12.3% prevalence *S. mansoni* and 7.2% for *S. haematobium* (Sang et al., 2014)

5.4 Correlation of *Schistosoma* Parasite Load and Cercariae Shed for Field Collected (R. Asao) Snails

This study showed a weak negative correlation between real-time PCR Ct-value and cercariae number by microscopy (r= -0.261, P= 0.438). These findings are inconsistent to those reported by Mutuku et al where they found that a highly infected snail sheds a higher number of cercariae, and a lowly infected one sheds a lower number of cercariae (Mutuku et al., 2014b). However, since the snails are field collected snails, the number of miracidia infecting the snails were not recorded and that the parasites exhibited low virulence in the snails, slow rate of growth and differentiation. That is the parasites could be low shedders with high with a mixture of low and high wild infections of the miracidia (Le Clec'h et al., 2019).

5.5 Quantifying Increasing Number of Miracidia within Snails and Quantifying Increasing Number of Sporocysts by Real-Time PCR

The study showed that there was a weak negative correlation between miracidia count done with microscopy and its corresponding Ct-value as measured by real-time PCR (r = -0.312, P = 0.192). However, the current finding is inconsistent study done by Hung & Remias (2008) only that they correlated cercariae and its respective Ct value. The insignificant results could be attributed to the close in range of the miracidia counts and the number of replicates in the current study compared that done by hung at 1, 5 and 10 cercariae. After the intermediate snails get infected, the parasite levels within the snail increase within the 4–6-week period. The Ct values varied significantly across the weeks after infection of the snails by equal number of miracidia (ChiSq = 14.18, df = 4, P = 0.0067). The rt-PCR significantly showed the increasing amounts of

sporocyst within snails. There was no significant change within the first (P = 0.4) and second week (P =0.23) since the parasite needed to transform into a sporocyst from miracidia that later develop into a daughter sporocyst, which in turn develop into cercariae (Viana et al., 2018). The study findings showed low quantify of sporocysts was distinguished from high quantity of sporocyst at a later week by the real-time PCR. These findings are consisted with those done by Le Clech and others where they compared high shedders and low shedders population by the real-time PCR (Le Clech et al., 2019). With a proper gradient of the graph between sporocysts quantities between different weeks, the gradient can be used to estimate the time at which a snail last got infection by relative number of miracidia. Also, another factor to consider is that the snails have to be at the time when they have not shedded yet. The real-time PCR is more reliable for snail infection prevalence studies and quantification of sporocysts within the snails.

CHAPTER SIX

SUMMARY, COCLUSION, RECOMMENDATIONS AND SUGGESTIONS FOR

6.1 Summary

The snails sampled from River Asao were not evenly distributed with *Biomphalaria pfeifferi* only species present *Biomphalaria* snail being more than *Bulinus globosus* which is the only *Bulinus* species snail present. Shedding snails were confirmed to be positive by real-time PCR and some non-shedding snails were confirmed to be positive by real-time PCR. Real-time recorded a higher prevalence for the compared to microscopy. Cercariae shed by snail and amount of DNA for the *Schistosoma* sporocyst could not strongly correlate as measured by real-time PCR. The real-time could however show the increase in amount of sporocyst as they asexually increase within the snail but failed to distinguish different amounts of DNA in snail.

6.2 Conclusion

i. The snails sampled from River Asao were not evenly distributed with *Biomphalaria pfeifferi* and *Bulinus globosus* snails present

ii. There was significance difference in the *Schistosoma* infection prevalence between *Biomphalaria* snails 2% by microscopy and 17% by real-time PCR (and *Bulinus* snails 16% by microscopy and 27% by real-time PCR 15% of all positive snails) in River Asao. More snails are found to be positive by real-time PCR (18%) compared to microscopy (4%).

iii. There was a weak correlation between cercariae shed by the snail and the amount of sporocyst present in the corresponding snail. Amount of cercariae shed as observed by microscope may not reflect the quantity of sporocyst present as measured by real-time PCR.

iv. There was a weak negative correlation between miracidia number and its corresponding DNA amount in the snail. There is significance difference of general increase in amount of sporocyst within snails as measured by the real-time PCR.

6.3 Recommendation

6.3.1 Recommendation from the Current Study

i. The study suggest that control measures against *Biomphalaria* and *Bulinus* snails should target the entire length of the river..

ii. Estimation of prevalence of infection in snails should rely on real-time PCR rather than microscopy.

iii. Quantification of sporocyst in the snails cannot be used as an alternative measure of cercariae shed by snails. Both methods should be applied in estimating *Schistosoma* infection rates in snails.

iv. The quantity of sporocyst DNA in field collected snails is not a good predictor of number of miracidium that the snails were originally exposed to nor the time post infection of the snails.

6.3.2 Recommendation for Future Study

i. Sampling for all seasons to monitor the distribution an infection rates of the intermediate snail species.

ii. Molecular identification of snails is very vital especially the intermediate snail hosts. Some snail types have striking resemblance and hence maybe hard to distinguish morphologically.

iii. Community awareness on Schistosomiasis and affordable transmission prevention strategies.

iv. One conducting same experiment should consider addition techniques to reduce impurities in order to improve PCR products and results.

v. In order to have predict history of snail infection by miracidia, snails need to be infected with different number of miracidia and generate their gradient over the weeks post infection.

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APPENDICES

Appendix 1

General information for morphological identification of field collected snails from R. Asao.

The snails of the *Bulinus* group were identified as *Bulinus globosus* had some morphological features as light brown colour of the shell, 4 whorls, and short spire with a blunt apex.

Shell height	Shell width	Aperture width	Aperture height	Number of whorls
9.77mm	6.12mm	6.87mm	3.6mm	4

The snails of the *Biomphalaria* group were found to have the following features; a discoid shell and rounded whorls that increase with width. The table below shows some of the snails' measurements.

SHELL DIAMETER	SHELL HEIGHT	APPERTURE WIDTH	APPERTURE HEIGHT	Number of whorls
8.27mm	3.63mm	3.92mm	3.77mm	4