GENETIC PROFILING OF *SCHISTOSOMA HAEMATOBIUM* INFECTION, SNAIL HOSTS AND CIRCULATORY ANTIBODIES OF INFECTED PUPILS IN NORTHERN CROSS RIVER STATE, NIGERIA.

BY

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DECLARATION

I, Djieyep Noundo Armand Claude, with Registration Number MIC/Ph.D/2013/005 hereby declare that this thesis on "GENETIC PROFILING OF *SCHISTOSOMA HAEMATOBIUM* INFECTION, SNAIL HOSTS AND CIRCULATORY ANTIBODIES OF INFECTED PUPILS IN NORTHERN CROSS RIVER STATE, NIGERIA" is original and has been written by me. It is a record on my research work and has not been presented before in any publication.

Djieyep Noundo Armand Claude — Signature.....

Student — Date.....26/02/2020

CERTIFICATION

We certify that this work titled "GENETIC PROFILING OF *SCHISTOSOMA HAEMATOBIUM INFECTION*, SNAIL HOSTS AND CIRCULATORY ANTIBODIES OF INFECTED PUPILS IN NORTHERN CROSS RIVER STATE, NIGERIA" carried out by Djieyep Noundo Armand Claude (MIC/Ph.D/2013/005) in the Department of Medical Laboratory Science, under our supervision has been found to have met the regulations of the University of Calabar, Calabar. We therefore recommend the work for the award of the Doctorate Degree in Medical Parasitology.

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ABSTRACT

The interactions between age, gender, socio-demographics, genetic variations of *S. haematobium* / snail hosts, and antibody profiles of infected pupils in Northern Cross River State, Nigeria was investigated from March to September 2018. Urine and blood samples from 416 pupils aged 4 -15 years comprising of 224 males and 192 females were collected in randomly selected schools across the senatorial zone. One hundred snails (20 per Local Government Area) with the phenotypic features of *Bulinus* species were also picked from the human-water contact sites. Questionnaires were administered to capture the relevant socio-demographic data. Aliquots of urine samples were filtered after being stained with 1% Carbol Fuchsin for quantitative estimation of eggs per 10 mls of urine while the genomic DNA was extracted from the other urine aliquot and the snipped foot part of the snail hosts. Microsatellite marker Dra1Sh, ITSSh of the *S. haematobium* and the ETTS were amplified using Polymerase Chain Reaction (PCR). The evolutionary distance between *S. haematobium* and the snail host were computed using Neighbour Joining. The sera from the blood samples were screened by ELISA method for Immunoglobulin (Ig) G and IgM respectively. Microscopy revealed that 39 (9.4%) subjects were infected with *S. haematobium* with a mean egg count of 16.97 ± 8.05. The prevalence (P = 0.334) and intensity (P = 0.849) of the infection were not statistically significantly associated with age. Gender was statistically significantly associated with the prevalence (P = 0.031) but not the intensity (P = 0.115) while the Local Government Area (LGA) of residence significantly affected both prevalence (P = 0.00) and intensity ((P = 0.001) of the infection. Age (P = 0.189) and gender (P = 0.112) had no statistically significant influence on the immune profile of the infected study participants. In addition to the three genetic variants observed in humans, a fourth was observed in the infected snails. There was a significant difference in the infection rates of *Bulinus* snails with *S. haematobium* across the LGAs and the pathology associated with each genotype varied significantly with respect to haematuria and proteinuria. Molecular identification revealed *B. forskalii, B cameruniensis, B. truncatus* and *Bulinus globusus* as the snails that serve as intermediate host for *S. haematobium* in the study area with *B. globosus* being the most prevalent (82%) and occurring in all LGAs, and *B. camerunensis* the most infected (100%) with *S. haematobium*. Sensitivities of 57.3%, 80.9% and 41.8% and specificities of 100%, 100% and 95.9% each were recorded for microscopy, serology and haematuria respectively as against PCR. It is concluded that urogenital schistosomiasis remains endemic in Northern Cross River State. The existing control programme should be sustained and further genomics of the parasite be done on a larger scale to enrich the database. Finally, malacological studies directed at generating data be encouraged to reduce the transmission of the disease by the *Bulinus* snails.

Word Count: 473

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
APCs	Antigen Presenting Cells
BLAST:	Basic Local Alignment Search Tool
CDC	Center for Disease Control
CNS	Central Nervous System
cox1:	Cytochrome Oxidase Subunit-1
DAMPs	Danger Associated Molecular Patterns
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESP	Effective Study Participants
gDNA:	Genomic DNA
Ig	Immunoglobulin
IHT	Indicect Haemagglutination Test
ITS:	Intergenic Spacer
IL	Interleukin
IL - 1R.	IL-1 Receptor
LAMP	Loop-Mediated Isothermal Amplification
LRRs	Leucine-Rich Repeats
mtDNA	Mitochondrial DNA
NHM	National History Museum
NOD	Nucleotide Oligomerization Domain

NLRs	Nod-Like Receptors
NTDs	Neglected Tropical Diseases
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PRRs:	Pattern Recognition Receptors
PZQ	Praziquantel
RAPD	Random Amplified Polymorphic DNA
rDNA:	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
TLRs	Toll-Like Receptors
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Schistosomiasis, also known as Swimmer's Itch, Katayama fever, Snail fever or Bilharzia is a parasitic disease caused by the digenetic blood trematodes of the genus *Schistosoma*. The name derives from the Greek word *schistos*, meaning "split" or "divided," and *soma*, meaning "body". The commonest species infecting humans are *Schistosoma haematobium*, *S. japonicum*, and *S. mansoni* although there are two other species, *S. mekongi* and *S. intercalatum* that are more localized geographically. Other species of Schistosomes parasitize birds and mammals and can cause cercarial dermatitis in humans (Arora & Arora, 2009).

Schistosomiasis is endemic in 74 tropical countries in Africa, the Caribbean, South America, East Asia, and the Middle East, most especially in places with poor sanitation, with 62 percent of the burden occurring in countries in Africa. Worldwide, more than 700 million people are at risk of infection with more than 232 million requiring annual treatment (Montgomery, 2019). In the 1990's, a national survey among school children aged 5 to 14 years reported a presence of schistosomiasis in all 36 states of Nigeria including the Federal Capital Territory, Abuja, with about 20 million people infected (NSCP, 1996). The geographic distribution of the various species of schistosomes is dependent on the distribution of the species of their intermediate freshwater snail hosts (Gryseels *et al.*, 2006).

S. haematobium is the most common of the Schistosomes, infecting about 110 million people globally, mostly in sub-Saharan Africa (Chitsulo *et al.*, 2000). School-age children who live in these areas are more exposed because they play in infected water bodies. Residents or visitors to endemic areas exposed to contaminated freshwater are also at risk of infection. The prevalence, transmission and intensity of the disease is related to many parameters such as socio-economic status, awareness, human behaviour, and biological factors which influence the interactions between human and the surrounding ecosystem (Mazigo *et al.*, 2015).

S. haematobium may be sparsely distributed in the world but probably has an African origin. The parasite tends to be less focal, more prevalent, and more widely distributed throughout the continent than other human schistosomes (Van der Werf *et al.*, 2003). The use of molecular tools in identification and the exploration of host-parasite compatibilities has answered many evolutionary questions over the years and its applications has engineered the establishment of database platforms to promote knowledge on the intermediate host diversity and population structure (Raghavan and Knight, 2006; Goordy *et al.*, 2016). Advances in the production of effective genetic markers such as random amplified polymorphic DNA (RAPDs) ribosomal gene (rRNA), and the mitochondrial cytochrome oxidase I (COI) have created a robust and reliable taxonomy, particularly in the complex *Bulinus* group and widened knowledge on the epidemiology of schistosomiasis (Rollinson *et al.*, 2001; Kane *et al.*, 2008). In the Southern part of Africa, the *truncatus* group of Bulinid snails are responsible for transmission while in the Northern part it is the *africanus* group that is more involved (Joubert *et al.*, 1991). Akinwale *et al.*, (2011) observed that the predominant strain in

South West Nigeria is *Bulinus truncatus* and while *Bulinus globusus* was also documented. The northern strains of *S. haematobium* are not infective to the southern intermediate snail hosts and vice versa (Fransden, 1979).

It has been observed that the increase in the incidence of urinary schistosomiasis in sub-Saharan Africa is likely to be related to the construction of dams and irrigation canals (Adamu, 2010). The dynamics of the disease are associated with communities where agricultural practices, fishing and recreational activities bring the population into contact with snails infested waters. Most of the population of Cross River State is involved in agriculture and the geography of the state sustains the thriving of the snail intermediate host. The prevalence of *S. haematobium* may not be unconnected to these facts (Ejezie *et al.*, 1991).

The relationship between the genetic diversity and the morbidity caused by *S. haematobium* infection has been investigated with conflicting results. Variations in intensity of the disease outcome caused by the parasite have been observed within communities and even between different regions of Africa (Bouwer *et al.*, 2003). Depending on its severity, different outcomes of the disease have been observed. Signs and symptoms such as haematuria, anaemia, dysuria, stunting, uraemia, bladder cancer, and urosepsis, have been documented in the course of urinary schistosomiasis (Nmorsi *et al.*, 2005). The reasons behind these discrepancies in clinical outcome remain ambiguous. Although the species of *S. haematobium* is considered to be morphologically identical, some phenotypic variations between parasite strains or populations have been observed in a number of biological characteristics such as

infectivity, virulence dynamics of transmission and drug susceptibility (Imbert-Establet and Combes, 1986; Morand *et al.*, 1996; Sire *et al.*, 1999; Gyseels *et al.*, 2006). Curtis *et al.* (2001) postulated that these differences may be attributed to the genetic variations of the parasites. Brouwer *et al* (2001) observed that the genetic diversity in the parasite populations is more predominant among children and that this variability may direct the immune response and the clinical outcome of the disease.

In as much as some diagnostic techniques have their limitations, adequate diagnosis of *Schistosoma* infections remains very important to generate results that will influence decisions on evaluation of chemotherapy, estimations of prognosis and assessment of morbidity, and other control measures (Feldmeier *et al.*, 1993). Most epidemiological assessments of the burden of schistosomiasis have relied on microscopy which is tedious and time-consuming, probably because it is a relatively easy and cheap tool for detecting and counting eggs in urine samples in many endemic countries (Legender *et al.*, 2002). However, inadequate sensitivity as a result of great fluctuations of egg output is well known, and the difficulties in meeting the multiple sampling requirements for classical parasitological diagnosis often lead to suboptimal results (Hove *et al.*, 2008). While it is important to screen endemic communities to assess the prevalence of the disease, screening the snail intermediate hosts can help to assay the evidence and extend of environmental contamination and the environmental risks of infection. At selected transmission foci, monitoring the prevalence and intensity of infections in the snail have been reported to be particularly useful for assessing any environmental impact that an intervention may have (Abath *et al.*,

2006). Even in cases of chemotherapy-based control, it is not out of place to study the snails in order to monitor their impact on the disease transmission (King, 2009).

The location of the adult worm in the definitive host complicates studies of the genetic diversity of natural *Schistosoma* populations. Some molecular epidemiological studies have provided opportunities to investigate many important topics such as the contribution of parasite genetics to variation in disease burden and pathology, the genetic consequences of various control activities for parasite populations, their dynamics in endemic areas, as well as the spread of drug resistance (Norton *et al.*, 2010; Gower *et al.*, 2011). However, it is important to study the genetics among the various strains that are responsible for infections in an endemic area since this genomics will go a long way to throw more light on the epidemiology and clinical manifestations of the disease, and even promote new diagnostic, preventive and control measures (Norton *et al.*, 2010; Gower *et al.*, 2011). The whole Genome Analysis (WGA) of *Schistosoma* DNA has been demonstrated to provide reliable templates for genotyping microsatellite loci (Valentim *et al.*, 2009). It is believed that molecular tools will allow researchers to make use of genetic variations to monitor the effectiveness of treatment, understand the impacts of treatment on the gene pool and population structure of *Schistosoma* parasites, and establish whether movement of humans from endemic foci or non-treated areas introduces new genetic variants into local populations (Rollinson, 2009). Recent studies using an internal transcribed spacer (ITS)-based real-time polymerase chain reaction (PCR) for the detection of *Schistosoma* DNA in urine samples has shown high sensitivity and specificity when performed on controls and known microscopy-positive samples (Aryeetey, 2013).

The prevalence of schistosomiasis tends to have a strong correlation with age in areas where the disease is endemic with the intensity of infection being higher in pre-puberty children and lower in older age brackets. Post-treatment evaluations in endemic communities suggest that children usually become heavily re-infected while older subjects, if re-infected, bear a lesser burden than before (Butterworth *et al.*, 1994). An experiment on the response to schistosome-like synthetic epitopes revealed that children produced more antibody than adults irrespective of the *Schistosoma* species.

Humans and other higher animals are periodically exposed to microorganisms and their survival is related to their ability to effectively recognise pathogens and mount an adequate immune response. These responses may be humoral, with the production of antibodies or cell mediated. Antibodies, a product of the humoral response, belong to the immunoglobulins (Ig) superfamily, secreted by plasma cells in response to the antigen that stimulated their production (Burton and Woof, 1992). Extensive research has highlighted the role of natural IgM in health, infection, inflammation, and autoimmune diseases. Recent studies revealed the early involvement of natural IgG in infection, and its contribution to pathogen clearance (Panda *et al.*, 2013).

1.2 Rationale of the Study

Human schistosomiasis is endemic in some communities in Cross River State (Useh and Ejezie, 1999; Okpara *et al.*, 2003; Inyang-Etoh *et al.*, 2009). Control programmes of schistosomiasis in endemic communities have focused mainly on drug

distribution. Despite an established control programme with Praziquantel distribution every two years, the disease tends to persist in this geographic area. Adie *et al* (2014) reported prevalence as high as 58% in some areas of Northern Cross River State. The emergence of resistance may not be unconnected to the selective susceptibility of the parasite variants to the control measures, their infectivity to intermediate snail hosts and immune response of the residents which are believed to direct the course of infections in an endemic area. The role these parameters play in the epidemiology of urinary schistosomiasis in Cross River State in whole and Northern Cross River State in particular has never been explored. Although molecular approaches have been used on local scales across Africa to differentiate between snail hosts population and have gone way further to ascertain their phylogeny they still aren't fully explored. The dynamics of the disease in this area might well be associated to the genetic variations of the parasite, the snail intermediate hosts and the immunological profiles of the infected individuals; hence the need to study the interaction between the parasite genetics from the human and snail hosts, snail genotypes, and the host response.

1.3 Justification of the Study

Agnew *et al.* (1993) observed that no evidence of in-vivo immune mediated killing of the adult worm has been documented. Schistosomes like many other helminths evade host responses allowing them to survive in the blood stream (Mencl *et al.*, 2000). There is a slow development of appropriate immune responses to worm antigens probably because schistosomes are very long-lived parasites and the host becomes exposed to these antigens only after parasites die either as a result of ageing

or drug intervention (Woolhouse and Hagan, 1999). Over the years, control programmes against schistosomiasis have continually focussed on the application of antihelminthic on the definitive hosts. It is believed that control measures directed at the intermediate hosts as well is desirable and will strengthen schistosomiasis control. Hence understanding the extent of involvement of the snail hosts in the transmission in areas where antihelminthic have been distributed cannot be overemphasized. This study will provide an insight on the parasite/mollusc ecology in this part of the country, the epidemiology of the disease, the antibody profiles of the resident pupils and recommend further approaches in the control of the disease.

1.4 Hypotheses

1.4.1 Null Hypothesis

The transmission of *S. haematobium* among pupils of Northern Cross River State is not influenced by the host immune response.

1- The dynamics of urinary schistosomiasis is not associated with the genetic variants of the parasite

2- The genotypes and distribution of the snail host variants do not influence the transmission of *S. haematobium*

1.4.2 Alternative Hypothesis

1- The host immune response influences the dynamics of urinary schistosomiasis.

2- The transmission of the disease is associated with the genetic variants of the parasite.

3- The transmission of urinary schistosomiasis is associated with the genetic variants of the snail host.

1.5 Aim and Objectives

1.5.1 Aim

The aim of this work was to study the dynamics of *S. haematobium* infection such as hosts response, *S. haematobium* genotypes and snail host genetic profile in Northern Cross River State, Nigeria.

1.5.2 Objectives

The specific objectives of this study were to:

1- Determine the prevalence and intensity of urinary schistosomiasis in pupils in Northern Cross River State and its association with socio-economic status and past medical history

2- Study the schistosome IgG/IgM antibody response in selected pupils.

3- Determine the genetic variations of *S. haematobium* responsible for infection among pupils attending primary schools in Northern Cross River State.

4- Determine the biodiversity of the *Bulinus* snails responsible for infection and their involvement ratio in the study area.

5- Determine the clonal relatedness between the human and snail host variants of *S. haematobium* in the study areas

6- Assess the performance of microscopy, serology, haematuria, proteinuria, haematuria/proteinuria against that of PCR in the diagnosis of urinary schistosomiasis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pathologic Species of Schistosomes

Schistosomiasis is a freshwater snail transmitted parasitic disease caused by a dimorphic trematode of genus *Schistosoma* that lives in the bloodstream of humans. Humans are usually infected by five species of Schistosomes, namely *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma mekongi,* and *Schistosoma intercalatum*, but the main burden of disease in sub-Saharan Africa is usually attributed to two species, namely, *S. mansoni* and *S. haematobium* and are referred to as the major human schistosomes (Utzinger *et al.*, 2009). *Bulinus* snails transmit *S. haematobium* which is the chief cause of urinary schistosomiasis in Africa and in the Arab world (Gryseels *et al.*, 2006). The disease has some time ago been misconceived as the male equivalent of menstruation and has subsequently been celebrated as the coming of age for young males in rural endemic communities (Amazigo *et al.*, 1997).

2.2 Morphology

The trematodes include two major groups, the *Aspidogastrea* and the *Digenea*. Most *Digenea* which are the flukes of medical importance are hermaphroditic (i.e they possess male and female reproductive systems). Attachment to the host is brought about by one or two suckers (though sometimes missing). Some groups (the Schistosomes) are diecious (sexes are separate) (Rhode, 2001) in nature (Figure 1). Adult Schistosomes share all the fundamental features of the digenetic trematodes;

they are bilaterally symmetrical with oral and ventral suckers and a body covering of a syncytial tegument. The digestive system consists of the oral sucker, the pharynx, oesophagus and blind-ending caecum. The area between the tegument and alimentary canal is filled with a loose network of mesoderm cells, and an excretory or osmoregulatory system based on flame cells that do not openly communicate with the surrounding tissue (Rohde, 2001).

Adult worms reside in venous plexuses in the definitive host, with each species having its predilection site. Males are colourless, shorter and stouter than the females, measure about 1 cm in length and possess a gynaecophoric canal used for holding the female during copulation. They have a rather small oral sucker at the anterior part of the body and a larger pedunculated sucker a bid behind the oral sucker. The gynaecophoric canal extends from the ventral sucker to the posterior end. Small tubercles cover the cuticle dorsally and tiny spines are found on the suckers and gynaecophoric canal (Morris and Threadgold, 1967; Silk *et al.*, 1969). The genitalia consist of four clustered testes, each attached to a vas efferent that combine to form a very short vas deferens, which in turn dilates immediately into a larger and globular seminal vesicle that runs to the genital pore located slightly behind the ventral sucker.

Females, filled with deposits of haematin, are usually reddish-black, cylindrical, have pointed tuberculated extremities and measure two cm in length. The small and elongated ovary lies at the posterior half of the body from where the oviduct arises, turns and runs anteriorly. The vitelline gland is located at the posterior quarter of the body. Its ducts run parallel with the oviduct and unite near the ovary to form the common duct which is surrounded by the shell gland.

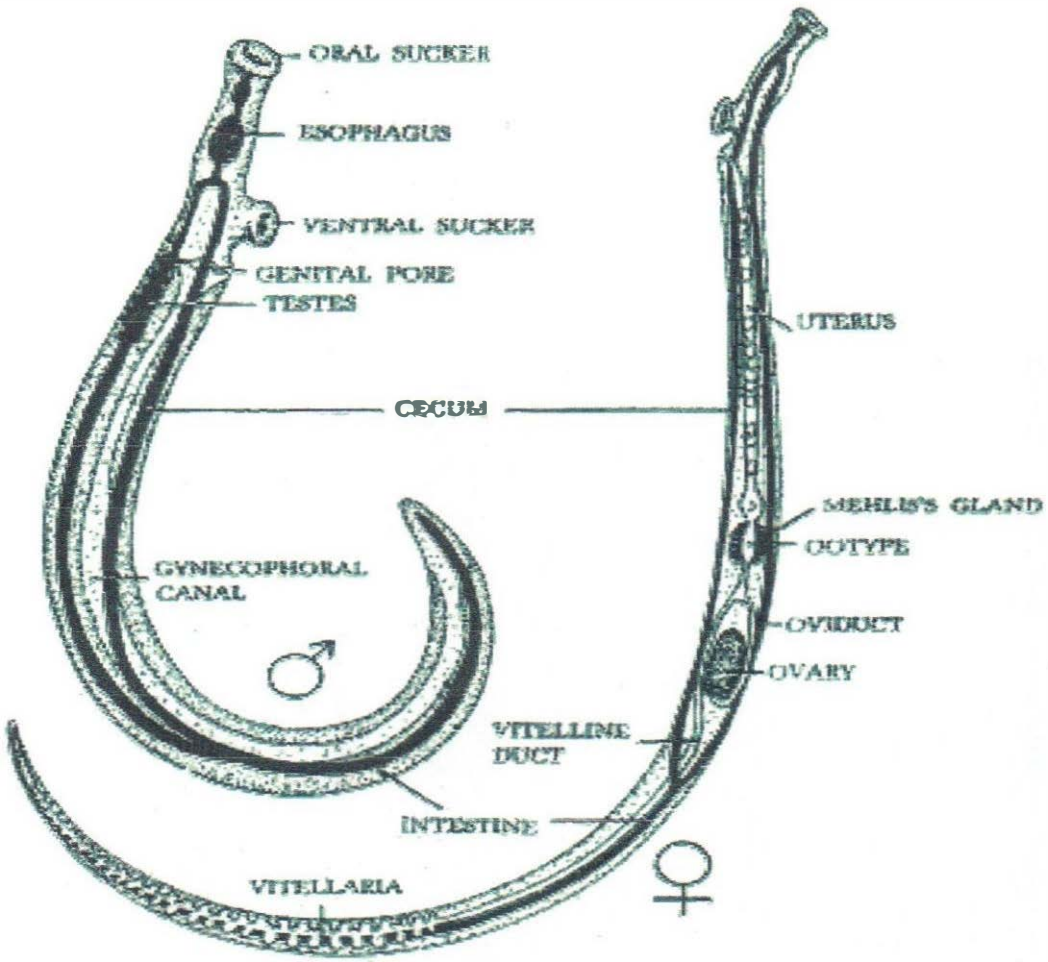

Figure 1: Morphology of Adult *Schistosoma*.

Source: Arora and Arora (2009).

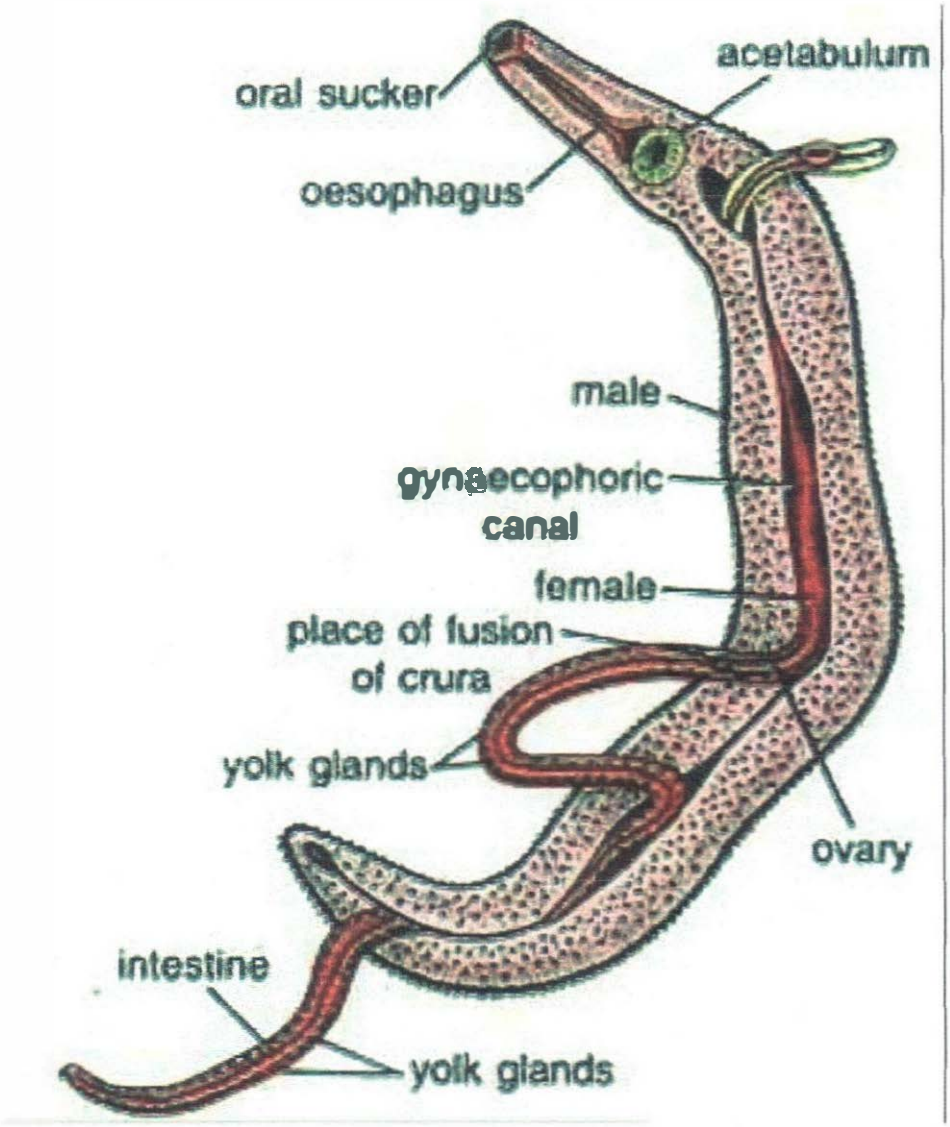

Figure 2: Female *Schistosoma* Lying in the Gynaecophoric Canal of the Male

Source: Bongdap (2018).

This common duct elongates as uterus to the genital pore located below the ventral sucker (John and Petri, 2006;) (Figure 2).

The eggs are compact, elongated spindles with a short terminal spine, non operculated and fully embryonated when laid, and measure about 140 x 50cm in size (Figure 3). The miracidium is highly organized. The posterior half is occupied by a mass of germ cells. It has two large unicellular penetration gland, a well-developed excretory system and a central nervous system. The anterior boring papilla is attached to two small penetration glands between which lies a primitive digestive sac. The sporocyst is a thin-walled elongated sac-like structure (John and Petri, 2006).

The cercariae are highly organized structures with an oval bulbous upper half and a lower half tail-stem that bifurcates distally, together measuring 400 x 80 picometer. The tail fork of the human schistosomes is less than half of the tail-stem and is covered with very tiny spines (John and Petri, 2006). The large oral sucker is anterior to the body while the prominent ventral sucker is located in front of the junction of the body (Figure 4). The emerging cercariae from the snail possess five pairs of unicellular penetration glands some of which secrete lytic ferments that facilitate the penetration of the final host's skin. The anterior two pairs are coarsely granular readily visible and possess a duct which runs forward to the margins of the oral sucker. The digestive tract starts with a minute mouth in the middle of the oral sucker, followed by a small narrowed tube ending in a small bi-lobed dilatation near the middle of the body. The rudiments of the genitalia are located behind the ventral sucker as a mass of cells. The excretory system consists of three pairs of flame cells in the body and one in the tail (Arora *et al.*, 2009; John and Petri, 2006).

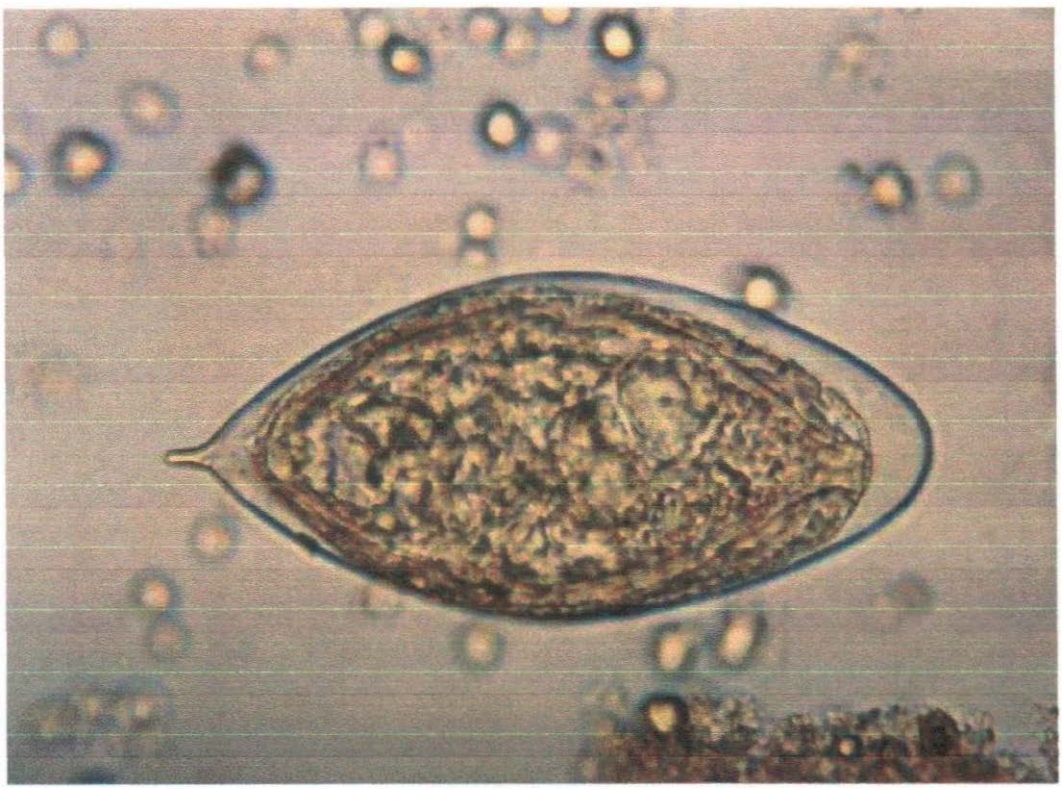

Figure 3: Egg of *Schistosoma haematobium* with the Characteristic Terminal Spine

Source: Bongdap (2018).

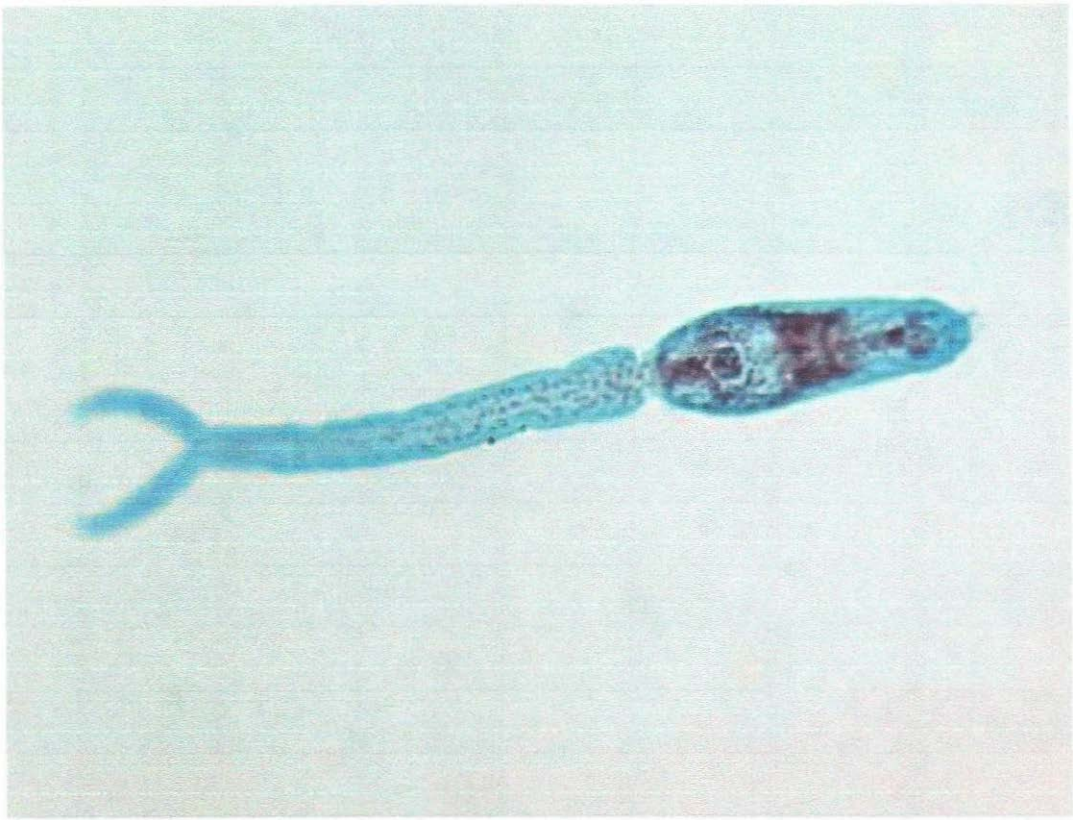

Figure 4: Morphology of *Schistosoma* Cercaria

Source: Arora and Arora (2009)

2.3 Life Cycle of Schistosomes

The life cycle of Schistosomes is complex (Figure 5). The eggs hatch in fresh water in the presence of light and sufficient warm temperature to release a free swimming miracidium which remain viable for up to 48 hours and use external stimuli such as light and snail-derived chemicals to seek out and penetrate the intermediate snail hosts of the genus *Bulinus* where they encyst (Hira, 1970; Noble *et al.*, 1989). Inside the snail, the miracidia shed their coat and develop into mother sporocysts. The sporocysts enlarge and the germ cells within begin to form daughter sporocysts after two weeks (Noble *et al.*, 1989). Free swimming forked-tailed cercariae are released in thousands per day four weeks after the initial penetration of the miracidium and this process can last for months. They survive for up to 72 hours and use water turbulence and skin-derived chemicals to seek for human hosts (Wilkins, 1987). When contact is made, the cercariae attach and penetrate the unbroken epithelium within half an hour, shedding their tail in the process (Schmidt *et al.*, 1996). When man enters the water, the cercariae penetrate healthy human skin often between the hair follicles by means of anterior spines and the cytolytic secretions of the cephalic glands (Muller, 1975).

In the skin, the cercariae heads transform into larvae (Schistosomules) which pass several days in the skin before entering the venous circulation and eventually migrate to the lungs. They then migrate through the circulatory system to the portal circulation where they mature into adult worms. In the case of *S. haematobium*, the young flukes migrate after a period of about three weeks to the veins of the urinary bladder to copulate.

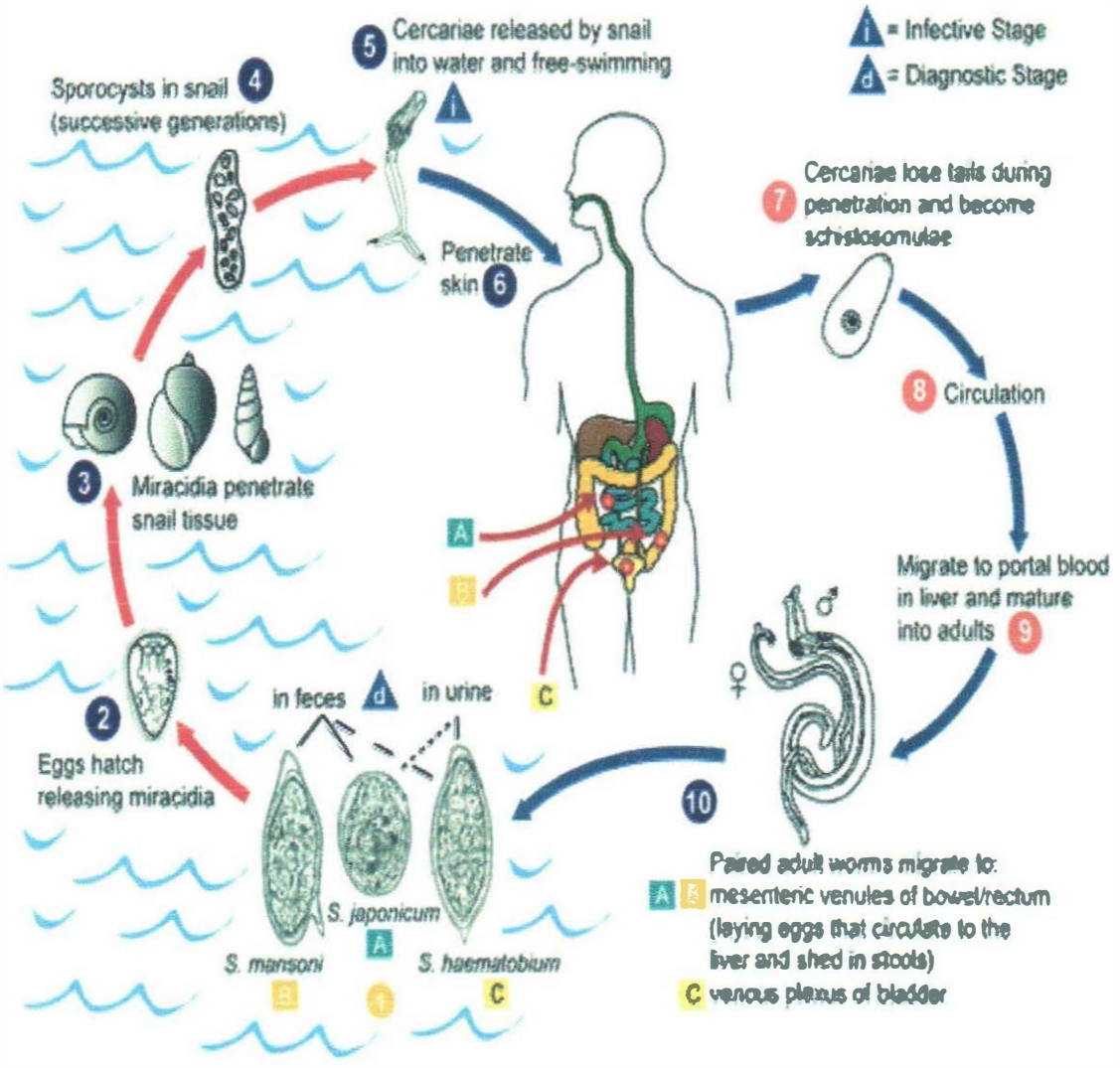

Figure 5: Life Cycle of Schistosomes.

Source: Arora and Arora (2009)

infection (Byram and von Lichtenberg, 1977). The pathology of UGS may be acute or chronic.

2.4.1 Acute Schistosomiasis

In the acute phase, a mild maculopapular skin lesion may develop within hours after cercarial penetration. This cercarial dermatitis also known as ''swimmer's itch'' which are uncommon with the major human Schistosomes may resolve over several weeks but can be fatal. In the case of *S. haematobium*, the invading cercariae probably cause a minimal immunogenicity but this reaction is more significant when it is caused by species that rely on other primary hosts. This self-limited process may re-occur more intensely with subsequent exposures. Despite the antigenic diversity other symptoms of acute schistosomiasis are mediated by the innate immune system and starts with the deposition of eggs into the host tissue which antigenic stimuli are by far more immunogenic than the earlier phase of disease (Gaafar *et al.*, 1992; Gaafar *et al.*, 1993; El Ridi *et al.*, 1997). Eventually, the clinical disease of *S. haematobium* infection is barely noticed until oviposition starts (Gryseels *et al.*, 2006). Individuals that have the ability to mount a strong immune response to the adult worms (usually IgE) may be at advantage over those with tepid responses in that they might overcome the infection (Hagan *et al.*, 1991). Other symptoms may include fever, myalgia, fatigue, malaise, non-productive cough, haematuria, dysuria and abdominal pain and occurs in people who are infected for the first time (Ross *et al.*, 2007)

2.4.2 Chronic Schistosomiasis

Chronic schistosomiasis is far more common than the acute form probably because its pathology results from the eggs-induced immune responses such as granuloma and fibrosis. The eggs require an intense immune response to aid their migration through the body. The adult worms may not be attacked by the immune system and may live for years in the blood stream since it can absorb host proteins and become coated with host antigens (Brouwer *et al.*, 2003). The disease causes a range of morbidities, the development of which seems to be influenced to a large extent by the nature of the induced immune response and its effects on granuloma formation and associated pathologies in target organs (Figure 6) (Dunne *et al.*, 1999; Cheever *et al.*, 2000).

Urinary schistosomiasis is a chronic infection of the circulatory system which affects the urinary tract of man. The pathology of the disease is due to the deposition of the eggs in the bladder and the ureters which elicits a chronic granulomatous injury. Though many eggs eventually pass into the bladder lumen and are excreted, a good number of them is trapped within the host tissue, triggering an immune response that progressively results in adaptive tissue changes with subsequent pathologies (Odegaard, 2014). The predilection sites for *S. haematobium* are the vesical and venous plexuses although the parasite in some instances could be retained in the haemorrhoidal plexus of the veins or terminal tributaries of an inferior mesenteric vein.

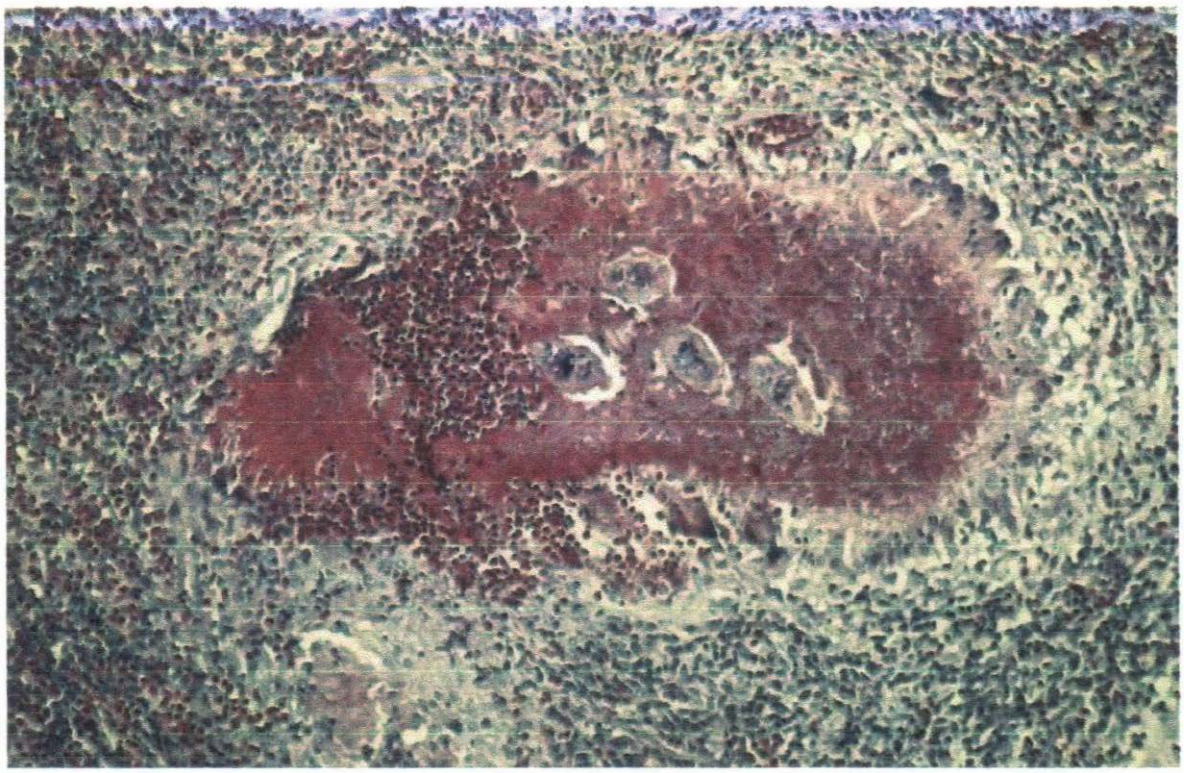

Figure 6: Schistosome Eggs (four ovals in middle) in the Bladder Surrounded by a Granuloma (dark pink area).

Source: YG (2016).

The earliest noticeable symptom is a painless haematuria with the passage of eggs in the urine and there is an elevated tendency to urinary tract co-infections and the progressive urothelial neoplasia develops into an especially aggressive type of bladder squamous cell cancer referred to as urothelial carcinoma (Gelfand *et al.*, 1967; Brand, 1979). On the mucosa, there is an initial hyperaemia and petecha followed by polyps or raised areas with granular mucosa. Other progressive changes include glandular hyperplasia (cystic), squamous metaplasia (leukoplakia) and ulceration. The epithelium covering the calcified eggs at the bladder base assumes a "sandy patch" appearance. Marked fibrosis around the ureters cause a bladder-neck obstruction and eventually results in hydronephrosis and hydroureter. An investigation of the relevance of haematuria and proteinuria in the diagnosis of urinary schistosomiasis on children aged 9 to 14 years revealed that there is a strong correlation between the intensity of dipstick proteinuria/haematuria, frequency of visible haematuria, and egg count of the parasite suggesting that haematuria and proteinuria are sensitive indicators of urinary schistosomiasis with haematuria being a more sensitive parameter (Murare and Taylor, 1987). A good number of the eggs produced do no reach the vesical lumen and may be trapped in tissues or carried away with the blood stream to ectopic sites such as skin, lungs, brain, muscles, adrenal glands, genitalia, and eyes where they initiate additional clinical conditions such as granulomatous inflammatory responses which are the main causes of pathology in human hosts (Odegaard, 2014).

2.4.2.1 Urogenital Schistosomiasis

The involvement of the prostatic gland, seminal vesicles, epididymis, testis, penis and urethra in both genders is possible (Pearce and Andrews, 2002). *S. haematobium* infection has been described in the male genital organs, with both leukocytospermia, gross haematospermia and possible infertility as disease outcome (Corachan *et al.*, 1994; Leutscher *et al.*, 2005). Nmorsi *et al.*, (2007) carried out some ultrasonographic investigations on some volunteers infected with *S. haematobium* and reported ten pathological conditions including abnormal wall thickness, abnormal shapes, irregular bladder wall, masses, echogenic particles, residual volume, pseudopolyps, calcification, hydroureter and hydronephrosis. The incidence of these findings was more significant in children than adults. In females, eggs penetrating the urinary system may proceed to the genitalia and form granulomas in the uterus, salpinx and ovaries. In addition to the bladder pathology, 30% to 75% (depending on the population studied and examination techniques) of *S. haematobium*-infected females develop genital pathology detectable on examination as 'sandy patches' (physical stigmata of tissue-entrapped eggs) on the cervical mucosa with the vagina, upper tract and external genitalia less commonly affected (Kjeland *et al.*, 2008; Jourdan *et al.*, 2011; Kjeland *et al.*, 2012). Although the onset and progression of cervical lesions in girls have not been properly elucidated, sandy patches of the cervix are pathognomonic for *S. haematobium* infection for the female genital system (FGS) with their pathology not very different from those in the bladder including predisposition to sexually transmitted diseases (especially HIV), dyspareunia and infertility (Helling-Giese *et al.*, 1996; Kjeland *et al.*, 2005). It has been observed however that urogenital

schistosomiasis (UGS) is usually established before puberty, thereby leading to lifelong pathological consequences (Hegertun, 2013).

2.4.2.2 Neuroschistosomiasis

The central nervous system (CNS) involvement is caused by the embolised eggs from the portal circulation to the cerebro-spinal cord via the paravertebral venous plexus (Houston *et al.*, 2004; Mohammed *et al.*, 2007). When the CNS is involved, it can result in transverse myelitis. This is definitely the worse clinical outcome of schistosomiasis and has signs and symptoms such as increased intracranial pressure, myelopathy and radiculopathy. It is believed that the oviposition during an aberrant migration of the adult worm to the brain and spinal cord early in the course of the disease might be responsible for this. The massive granuloma induced by the presence of numerous eggs at these ectopic sites result to complications such as encephalopathies with delirium, blurred vision, seizures, motor deficit and ataxia while the spinal complications include lumbargia, lower limbs radicular pain, muscle weakness, sensory loss and bladder dysfunction and myelopathy of the lumbosacral region as the commonness complain (Ferrari, 2004; Carod-Artal, 2008).

2.4.2.3 Schistosomiasis and Salmonellosis

Schistosomiasis and typhoid fever have a parallel geographical distribution with the highest burden of both NTDs falling on children under 15 years of age (Alcaron *et al.*, 2007; Stothard and Gabrielli, 2007; Baker *et al.*, 2016). Few studies have been carried out in Africa in an attempt to elucidate the immunological responses to co-infections with the two pathogens (Igwe, 2014; Mohager *et al.*, 2014; Modebe *et al.*, 2014; Salem *et al.*, 2015). The entrapment of Schistosome eggs in tissues

ultimately leads to granuloma formation and subsequent fibrosis that makes the tissue more prone to infections by many other organisms including *Salmonella* species (Gryseels *et al.*, 2006). It has been observed that infection with *Salmonella* species is more difficult to treat when associated with Schistosomiasis and that even when successful, there is a tendency for the disease to relapse except if the worms are eliminated after the patient is treated with a schistosomicidal drug. It was therefore postulated that *Salmonella* is able to persist in the body by attaching to the schistosomes, hiding and multiplying in them (Ross *et al.*, 2001; Muniz-Junqueira *et al* 2009; Barnhill *et al.*, 2011). The isolation of *Salmonella* species from the tegument culture of Schistosome adult worm has demonstrated a symbiotic association between the two organisms (Young *et al.*, 1973). Other literature revealed that *Salmonella* colonized only the worm's digestive tract opening, where they use their pili for adhesion (LoVerde *et al.*, 1980; Melhem and LoVerde 1984). It has also been believed that the Schistosome-induced altered immune responses allows for bacterial multiplication inside the reticuloendothelial system cells (el-Hawy *et al.*, 1985). Since the parasite is not sensitive to antibacterial drugs, it is not impossible that *Salmonella* that are inside the worm reached by the drug and may account for therapeutic resistance or failure of typhoid fever under these conditions (Gendrel *et al.*, 1984).

2.4.2.4 Schistosomiasis and Malaria

The morbidity and mortality of schistosomiasis either alone or in association with other parasitic infections cannot be overemphasized in developing countries. It has been demonstrated in animal models that there are some immunological, biological and histological changes in the host response to a parasite in the presence

of another one (Guyatt and Snow, 2001; Diallo *et al.*, 2010; Mboera *et al.*, 2011; Broker *et al.*, 2012) suggesting that there might be some interactions between immune responses induced by the simultaneous presence of both parasites (Friedman *et al.*, 2005; Adegnika and Kremsner, 2012). Concomitant parasitic infections in humans are common, as for example the infections by schistosomiasis and malaria, two of the parasitic diseases with the heaviest economic and social burdens. It has been observed that *S. haematobium*-infected individuals are more susceptible to malaria mainly because of the influence of IL-10 on cytokine and antibody responses (Diallo *et al.*, 2004; Courtin *et al.*, 2011). Jemaneh *et al.*, (1994) noted that there is a synergic effect of the coinfection as concurrent chronic schistosomiasis and malaria increase childhood morbidity. Other literatures on malaria and schistosomiasis co-infection in children suggest that *S. haematobium* has a protective effect of *Plasmodium falciparum* as the malaria parasites densities are negatively associated with *S. haematobium* infection (McDevitt *et al.*, 2004) and that there is an age-dependent protection in children infected with urinary schistosomiasis against acute *P. falciparum* malaria (Kloos *et al.*, 1978; Sousa-Fegueiredo *et al.*, 2012).

2.4.2.5 Schistosomiasis and HIV and/or Hepatitis

An accelerated hepatic dysfunction and an elevated risk of hepatoma are observed in co-infections with Hepatitis B or C than with hepatitis alone (Medscape, 2015). Infection with Schistosome represents a risk factor for HIV-1 transmission and the disease progression in endemic countries. It is believed that the disease impairs the immune system and results to a higher viral load (Stoever *et al.*, 2009; Mbabazi *et al.*, 2011; Kjetland *et al.*, 2012). Although in both sexes, these conditions result in

activation of the immune system which may facilitate HIV entry and binding to HIV-susceptible cells, the incidence seems to be higher in females (Secor 2006; Kjetland *et al.*, 2012). Literature suggest that Hepatitis B surface antigen (HBsAg) and Hepatitis C virus (HCV) infections are potentiated by Schistosome infections and may be attributed to contaminated needles and blood transfusion during parenteral therapy of the disease. Schistosomiasis increases the risk of chronic infection since it prolongs the hepatic inflammation after an Acute Viral Hepatitis (Ghaffar *et al.*, 1991).

2.4.2.6 Other Pathologies

Massive infections ultimately result to hepatic disease. A severe pipestem pattern (Symmer pestem) fibrosis may occur. While the liver functions may still be normal, periportal fibrosis might progress to portal hypertension with potential complications such as splenomegally, ascitis, oesophageal variceal bleeding and portosystemic collaterals through which eggs can reach the lungs and cause pulmonary granulomatosis and fibrosis. A pulmonary hypertension and a frank cor pulmonale eventually results and has a high mortality rate (Lapa *et al.*, 2009). Schistosomiasis due to *S. haematobium* is a disease of the urogenital tract but due to its manifestations in severely infected children, some systemic implications may be observed during and beyond the period of active infection such as anaemia (mainly as a result of iron deficiency due to haematuria), chronic pain, diarrhea, exercise intolerance, malnutrition, growth retardation and recurrent urinary tract infections (UTI) (Kassim and Stek, 1983; PCD, 1998; King *et al.*, 2005; Talala *et al.*, 2008). Schistosomiasis is responsible for a huge proportion of kidney failure (Van der Werf and Vlas, 2001). Given that there is a higher tendency of type 2 immune expression in response to

schistosomiasis, it is not impossible that a beneficial effect of this disease be observed in medical conditions that are known to be associated with type 1-driven immune pathologies such as Crohn's disease, type 1 diabetes and multiple sclerosis (Cardoso *et al.*, 2012).

2.5 Epidemiology of Urinary Schistosomiasis

Schistosomes are small flukes which live in the bloodstream of infected people, mainly children. In many irrigation canals, and rural communities, where there is lack of adequate sanitation facilities and clean water supplies, people have daily contact with infected water bodies (Xu *et al.*, 2006). *S. haematobium* is distributed in Africa, Middle East and southern Europe and this distribution is parallel to that of the snail hosts *Bulinus* and *Physopsis* species (Gryseels *et al.*, 2006). Peoples at risks are those living in or travelling to areas where schistosomiasis occur, and those who come in contact with fresh water where the snail intermediate host dwells. Different settlements usually make use of adjacent streams and this interconnectivity might cause different degrees of transmission of the disease. Where the interactions are high, a greater incidence of the parasite may be observed and subsequently a greater infestation of the water sources and a higher infection rate. The prevalence of the disease in the upstream communities' influences that of those downstream (Xu *et al.*, 2007). Although freshwater lakes and streams are usually identified as the source of the disease, man-made reservoirs and irrigation systems are increasing as well as population growth and rural exodus are becoming implicated in in the spread of the disease in some countries. The slowed flow caused by dams and irrigation canals is a conducive habitat for snails (Chitsulo *et al.*, 2000; Mc Manus and Loukas, 2008).

Indeed, the geographic spread of the disease may not be unconnected to water resource engineering issues in developing countries as well as the migration of infected populations. Evidence suggests that snail's infection with *S. haematobium* miracidiae increase from July to November at normal water levels (N'goran *et al.*, 1997).

The mortality of schistosomiasis is considered next to that of malaria amongst parasitic diseases, as it kills an estimated 280,000 people every year in Africa alone It is disease is regarded as a neglected tropical disease, although it still has an estimated 732 million persons that are vulnerable to infection worldwide in renowned transmission areas (WHO, 2016). It is believed that that 90% of those living in hyper endemic areas will be infected at some point in their lives, usually before the age of ten while in less endemic areas, 25% to 40% of the residents would have acquired infection at the age of 35 years (Alcaron *et al.*, 2007; Stothard and Gabrielli, 2007). In sub-Sahara Africa, 192 million are estimated to be infected with the two forms of schistosomiasis (intestinal and urinary), with Nigeria recording the largest number of infection with about 29 million cases. Previous literature has observed that schistosomiasis has existed in the Northern Nigeria since antiquity and its origin traced to the upper Nile valley through the Fulani invaders (Blair, 1960). The disease is more prevalent in school-aged children, adolescents, and young adults who also suffer from the highest morbidity and mortality (Hortez and Kamath, 2009). Nearly two-thirds of the cases of schistosomiasis however are of the urinary type caused by *S. haematobium* infection (Verle *et al.*, 1994).

In the Niger Delta Community of Odau, Nigeria an overall estimated prevalence of 83.3% has been recorded (Agi and Okafor, 2005). A study in Ogun State

recorded an overall prevalence of 32.2% for urinary schistosomiasis (Morenikej and Idowu, 2007) with the children in the age group (8-10 years) being the most affected. Thirty-seven percent (37%) of the infected individuals had haematuria, but no significant association was noted between the prevalence of the disease and gender. Uwem *et al.*, (2010) reported a prevalence of 58.1% with a mean count of 1.17 eggs/10 mls of urine among pre-aged (1-6 years) school children in a rural community near Abeokuta in Ogun State. This prevalence and intensity were not associated with gender. A similar survey in Nassarawa State documented a prevalence of 30.5% of urinary schistosomiasis among children of four primary schools in Keffi town (Ishakelu *et al.*, 2012). This prevalence had no significant association with gender as well. Amuta and Houmsou (2014) reported an overall prevalence of 55.0% (ranging from 36% to 64.0% in various communities) in Guma Local government area (LGA) of Benue State. However, these prevalences revealed that there was no significant difference in the distribution of schistosomiasis by genders of participants. Okworri *et al.*, (2014) reported a prevalence of 44.3% in Gadabuke and Garagwa communities of Toto LGA of Nassarawa State with no significant association with gender. A recent literature highlighted a prevalence of 45.6% of urinary schistosomiasis among school aged children in three communities of Kwarra State with an overall mean count of 127.9 eggs/ 10 mls of urine significantly associated with age and gender (Babamale *et al.*, 2018)

In Cross River State, infections are found mostly in the Northern and Central parts. These are the areas associated with intensive agricultural practices especially rice paddies found in the swampy parts. In 2003, a survey in Biase and Obudu Local

Government Areas of Cross River State revealed that *S. haematobium* infection had a prevalence rate of 61.5% and 58.6% respectively while Adim, another endemic community of the Cross River Basin had a prevalence of 19.8% among pre-primary school children (Okpara *et al.*, 2003; Okpara *et al.*, 2007). Okon *et al.*, (2007) reported an overall prevalence of 35% with a mean count of 58.8 eggs/ 10 mls of urine not associated with gender in primary school children in Abini Community. Inyang-Etoh *et al.* (2009) randomly screened children aged 5 – 17 years of age in two communities and did not found a case of urinary *schistosomiasis* in Ukwelo-Obudu community, but reported a prevalence of 4.5% in Abini. He also observed a correlation between haematuria ($r = 0.81$), proteinuria ($r = 0.71$) and the infection. Akpan *et al.*, (2017) recorded a prevalence of 4.5% and 1.6% in Nkarasi and Edor communities of Ikom LGA respectively. The intensity of the infection varied from 6 – 25 eggs/ 10 mls of urine and had no significant association with gender. Although the data above suggest a decrease in the prevalence of the disease probably due to an increased awareness and possibly adoption of some control measures, there might still be some foci of endemicity since all communities cannot be surveyed and a control programme sustained until the disease eradication is achieved. The prevalence of the disease tends to be determined by the drug distribution exercise within communities as data suggest a progressive resurgence of the infection as time goes on. Emini and Edema, (2019) recently reported a prevalence of 9.3% with a mean count of 2.35 ± 8.65 eggs/ 10 mls of urine in school-aged children in Abini, Biase LGA. Many parasitic diseases are influenced by environmental factors. Recently, when mapping urinary schistosomiasis in Cross River State, Adie *et al.*, (2014) observed that land use,

increase in temperature and soil type (deep latrine) were significantly associated with increase in infection while a dense vegetation and an increase in altitude rather reduced the infection rate, becoming null above 500 metres of altitude.

2.6 Immunobiology of Urinary Schistosomiasis

Schistosomes are good examples of multicellular pathogen that survive immune response that human develop against intruding organisms. Urogenital schistosomiasis is caused by *Schistosoma haematobium*. To establish the infection in man, the parasite undergoes a complex life cycle involving the skin penetration, the migration, the maturation and subsequent mating and finally the oviposition. Highly immunogenic eggs are deposited within the bladder and other pelvic organs; this stimulates a sequential immune response and immunopathology that will direct the course of the disease (Odegaard, 2014). The immune response to urinary shistosomiasis can be classified into two.

2.6.1 Innate Immune Response to Urinary Schistosomiasis

At the beginning, there is a release of proteases by the cercariae to enable them digest the unbroken skin where they will penetrate. The elastase activity of this serine protein on keratin, fibronectin, laminin or collagen eventually favours the cercarial penetration (Lightowlers *et al.*, 1988; Salter *et al.*, 2002). It has been demonstrated on animal models that the excretory/secretory (ES) activity of the cercariae induces an oedema and neutrophil infiltration in the skin but does not initiate a protective immunity. Rather, the first interaction between the Pathogen Recognition Receptors (PRRs) of the innate immune system and the ES directs the immune response (Viera *et al.*, 1986; Teixeira *et al.*, 1996). As the disease establishes, the worms are believed

to produce some immunological factors (like IL-10 and eicosanoids) that are involved in the regulation of the host immune system as well as a possible modulation of the immune mechanisms mast-cell activation, T-cell apoptosis and/or the skewed activation of antigen presenting cells (APCs) (Machado *et al.*, 1996; Moore *et al.*, 2001; James and Green, 2004). Although many studies have examined innate immune responses (including TLR expression and function) in response to intracellular pathogens, fewer have studied the interaction of the multicellular helminth parasites and the innate immune system. TLR2 appears to regulate the activation of dendritic cells (DCs), stimulating the production of IL10. This interleukin induces the suppression of Th-1 cytokines and modulates the conversion of Th-1 to Th-2 that typically abrogates the antigenic challenge (Van der Kleij *et al.*, 2002). In order to escape the detection by the host immune system, the adult worms are capable of coating themselves with the host antigens (Black, 2005).

A study on the immune responses between those individuals who are susceptible and those who are resistant to re-infection has revealed that there is a correlation between Immunoglobulin-E responses to worm (and not the egg) antigens and immunity, which implicates IgE in the protective immunity (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993). While there is a relatively ineffective reaction to both cercariae and adult worms, *S. haematobium* eggs induce a strong immune response; for a long time, there has been a stagnation on studying the early mechanism due to the inability to recognise newly infected individuals. This phenomenon was explained with the mouse model of direct oviposition (Fu *et al.*, 2012). The eggs induce an immediate and mixed response from resident and recruited

innate immune cells. This initial response encompasses the activation of some immune modulators (such as TNF-α) as well as cytokines involved in type 2 responses (such as CCL11). This leads to the recruitment of innate immune cells and parallel recruitment and activation of adaptive system. Schistosome eggs immune responses are known for their degree of persistence as this event results in the development of highly specialized reaction referred to as granuloma, a feat in the bladder given the scarcity of leucocytes in non-infected tissues. This has been demonstrated at as early as 4 days in the mouse model (Fu *et al.*, 2012).

Macrophages and eosinophils aggregate and segregate the eggs from the surrounding tissues. Eggs become progressively engulfed by giant cells which in turn are surrounded by a mesh of epitheloid macrophages, eosinophils and some neutrophils on the granuloma periphery. Although the polyps are not observed here (Smith *et al.*, 1977), this egg- centered granuloma is histologically similar to that observed in humans (Sadun *et al.*, 1970; Von-Lichtenberg *et al.*, 1971; Cheever *et al.*, 1977; Cheever *et al.*, 1978; Ghoneim *et al.*, 2011).

2.6.1.1 Macrophages

Macrophages play a major role in the entire innate immune response against *S. haematobium* infection. They are the primary structural constituent of granuloma. Previous experiments have demonstrated their ability to mount strong type 2 immune responses to the eggs including expression of high levels of ARG1, MRC and CHI3L3 (Herbert *et al.*, 2004; Wilson *et al.*, 2007). They have also been implicated in the initial fibrosis of the granuloma and its surrounding tissues (Burke *et al.*, 2010; Chu *et al.*,

2011; Hams *et al.*, 2013) with a mixed population (phenotypic appearance) of type 1 and type 2 macrophages and of both type 1- and type 2-associated cytokine and chemokine signaling pathways (Ray *et al.*, 2012).

2.6.1.2 Eosinophils

The functional role that eosinophils play in parasitic infections still remains elusive despite their tissue infiltration and presence in blood and urine as an indication of such diseases (Eltoum *et al.*, 1992; Reimert *et al.*, 2000). Before the parasite gets to maturity and starts oviposition, eosinophils are capable of killing cercariae and adult worms of *S. haematobium* in a degranulation- and IgE-dependent manner (Verwaerde *et al.*, 1987). Observations of rats that could clear Schistosome infections without developing chronic disease led to believe that it was due to the ability of their eosinophils to degranulate easily in response to IgE-decorated worms unlike those of mice and mammals that was responsible for this character (Rousseaux *et al.*, 1978). Secondary to urogenital oviposition, a high expression of CCL11 and other chemokines modulate the migration of eosinophils to the bladder to unite with macrophages (which due to their size occupy a larger volume and form a granuloma (Fu *et al.*, 2012). These eosinophils, demonstrable histologically in calcified eggs even long after the granuloma has resolved (Sadun *et al.*, 1970; Von-Lichtenberg *et al.*, 1971) possibly serve as an important source of IL-4 ahead of the emergence of the Th-2 response (Molofsky *et al.*, 2013; Heredia *et al.*, 2013) and remain one of the primary sources of IL-13 throughout the evolution of hepatic granulomatous responses (Reiman *et al.*, 2006).

2.6.1.3 Dendritic Cells

Dendritic cells are the principal antigen presenting cells (APCs). As the principal antigen-presenting cells as they constitute a critical bridge between early innate and later adaptive immune responses to *S. haematobium*; they are a necessity for proper Th-2 responses to the parasite eggs (Phythian-Adams, 2010). However, the maturation of these cells or the activation of the corresponding cytokines or other precursor molecules has not been achieved in-vitro even with highly immunogenic soluble Schistosome egg antigens (Kane *et al.*, 2004; Perona-Wright *et al.*, 2006; Van Liempt et al., 2007). Interestingly, these cells display a high ability to induce an activated Th-2 phenotype in naïve T cells in a CD40-, OX40L- and NFκB activation-dependent manner (MacDonald *et al.*, 2002; Jenkins and Mountford, 2005; Blazquez and Berin, 2008).

2.6.1.4 Natural Killer (NK) Cells

The primary and secondary outcomes of schistosomiasis correlate strongly with the relative balance between type 1 and type 2 immune responses. It will be interesting to investigate how these activation programme are initiated, regulated and how they could be manipulated for its possible benefits. NK cells are known to be a major source of IFN-γ and other type 1-associated cytokines and are recruited to Schistosome egg-associated hepatic granulomata (Remick *et al.*, 1988) with their presence corresponding to a local raise in IL-12 levels. Depletion of NK cells results in increased Schistosome egg-associated granuloma volume and fibrosis, whereas their activation (along with many that of several other lineages) by TLR3 ligands has the opposite effect (Hashimoto *et al.*, 1990; Asseman *et al.*, 1996; Hou *et al.*, 2012).

evidences suggesting that macrophages constitute the cells most present in the granuloma. First, T cells quickly infiltrate egg-exposed tissues and can be seen in large numbers in the elementary granuloma four days after the mouse model of bladder oviposition (Fu *et al.*, 2012). Secondly, ahead of the systemic response, T cells present locally and in the draining lymph node strongly express Th-2 associated cytokines (e.g. IL-4 and IL-13) suggesting that they might have been the activators of the immune response in the granuloma. In humans, these cells are later found spread all over the granuloma and in lymphoid follicles at its surroundings (Odegaard, 2014). Finally, interfering with factors that modulate the production of Th-2 such as IL-4 has resulted in reduced granuloma formation and a more complicated disease (Herbert *et al.*, 2004; Kouriba *et al.*, 2005; He *et al.*, 2008; Isnard *et al.*, 2011). Supported by the above data therefore, it could be postulated that the initial innate immune response generates Th-2 cells, which then initiate the granuloma formation through IL-4 production, later recruit eosinophils via IL-5, and promote fibrosis via IL-13. Literature on serological studies in humans suggest that elevations in Th-2 cytokines are also associated with suppression of their Th-1/Th-17 counterparts via a raised level of IL-10 although at a lower level than that of Th-2 mediators (Burke *et al.*, 2009). Several experiments have demonstrated that highly immunogenic (soluble) egg antigens stimulate IL-10 production which in turn suppresses T cells immune responses (Wahl *et al.*, 1997; Lundy and Boros, 2002; Teixeira-Cavalho *et al.*, 2008). This cascade reaction probably attenuate the host immune pathology associated with schistosomiasis. The induction of Th-1/Th-17 immunomodulators is not directly associated with infection but rather strongly correlate with aggravated urogenital

morbidity and end-stage pathology while the activation of Th-2 precursors leads to disease sequelae (Burke *et al.*, 2009). Correspondingly, polymorphisms that either impair type 2 or enhance type 1 responses are both associated with increased prevalence and gravity of urogenital schistosomiasis infection intensity in endemic areas (Isnard and Chevillard, 2008; Maizels, 2009). The balance of type 1 responses in relation to type 2 could be exploited as an important determinant of the control of *S. haematobium* infection since it can contribute to infection resistance (Odegaard, 2014)

2.6.2.2 B Cells

Despite the important role played by T lymphocytes in the fight against the egg-related pathologies, B lymphocytes are more directly involved in infection clearance and resistance as observed in endemic areas where B cells induced immune response (mainly IgE) has been associated with resistance to *S. haematobium* infection (Ji *et al.*, 2008). Although *S haematobium* specific IgM is very common in such populations (Nash, 1978), complicated pathology is restricted to a very small group of individuals without evidence of infection and can only be detected by some B cells related characteristics such as *S. haematobium*-specific IgE, anti- adult worm antigen (AWA) IgG1 and anti-Sh13 IgG3 as well as high IgE. (Grogan *et al.*, 1997; Naus *et al.*, 1998; Burke *et al.*, 2009). However, the immunity provided by these antibodies which tends to increase with age are directed not against the highly immunologic eggs but against the adult worm, the cercariae and possibly many other targets since the anti-*S. haematobium* antibody repertoire diversity other than these specific targets remains a powerful predictor of resistance (Naus *et al.*, 1998).

2.7 Genetics of *S. haematobium*

2.7.1 Phylogeny and Hybridization of *S. haematobium*

For many years, various approaches to the phylogeny of the *Schistosomatidae* were based on morphology, intermediate snail host and its geographical distribution (Rollinson and Southgate, 1987). Progressively, some advanced molecular tools eased a better understanding of the relationships between many of the 24 known species of *Schistosoma* (Lockyer *et al.*, 2003; Loker and Brant, 2006; Webster *et al.*, 2006). It has been shown that the African species so far examined, including *S. mansoni* and *S. haematobium*, share a common Mitochodrial DNA gene different from that of *S. japonicum* and related species. As assumed, the closely related species to the *S. haematobium* group were not the African Species *S. mansoni* and *S. rodhaini* in the *S. mansoni* group but, in fact, a clade including the Asian Species *S. indicum, S. spindale and S. nasale* (Webster *et al.*, 2006). The *S. haematobium* Species group are currently known to be made up of 8 species, some of which are of medical (*S. haematobium, S. intercalatum, S. guineensis*) and significant veterinary (*S. bovis, S. mattheii and S. curassoni*) importance. It has been reported that many of the species of the *S. haematobium* group such as *S. mattheei* and *S. haematobium* in southern Africa (Wright and Ross, 1980) and *S. guinieensis* and *S. haematobium* in Cameroon (Webster *et al.*, 2005) cross-breed experimentally as well as in nature. Zoonotic hybrid Schistosomes may develop when humans and livestock are in contact with the same water bodies, and such cross-breeding may severely alter the parasite's biological characteristics, its transmission patterns, the clinical course of infection and the drug sensitivity (Webster *et al.*, 2013; Poole *et al.*, 2014).

2.7.2 Genetic Markers in the Study of the Genetic Diversity of *S. haematobium*

Random amplified polymorphic DNA (RAPD) primers represent sensitive markers for exploring genetic variability and degree of gene flow among parasitic populations. RAPD-PCR was developed in an attempt to discriminate between species, strains, and individuals (Williams *et al.*, 1991). With such technology then, it became feasible to investigate population genetics of Schistosomes with few available sequence data (Welsh and McClelland, 1990). RAPD primers performed a whole genome scan, identifying a large number of loci and emerged as a powerful tool in distinguishing both inter- as well as intraspecific variations (Barral *et al.*, 1993; Neto *et al.*, 1993). Many researches have used these markers to explore the genetic variability within Schistosome populations in different hosts (Minchella *et al.*, 1994; Sire *et al.*, 1999). Because the procedure makes use of a very small amount of DNA, it can easily be obtained from the cercaria or miracidium thereby eliminating the task of isolating the parasite from the host (Shift *et al.*, 2000). Many factors influence patterns of genetic variability of different parasites, like the mating system, the intermediate hosts and vehicle of parasite transmission (Van den Broeck *et al.*, 2014). In the life cycle of *S. haematobium* there is an alternation of host and generations and the bionomics of the two hosts can influence the genetic variability (Gower *et al.*, 2011)

Unlike with *S. mansoni* where the use of microsatellites has proven very resourceful in the study of genetic variations within and between parasite populations, little progress has yet been made with the analysis of *S. haematobium.* In order to strengthen traditional methods and provide new information to improve success

towards schistosomiasis control, the scientific community joined efforts to assess the *Schistosoma* genomic information, starting in 1994, as an initiative of WHO/TDR (UNICEF-UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases) (LoVerde *et al.*, 2004; Oliviera *et al.*, 2008). At that time, only a few hundred expressed sequence tags (ESTs) from *S. mansoni* were available (Franco *et al.*, 1995). The WHO/TDR support initiated the possibility to generate data that could be translated into new tools for schistosomiasis diagnostics and treatment; That became the starting point of *Schistosoma* nuclear genome studies. Nuclear and mitochondrial genomes of *S. haematobium* have been sequenced from an evolutionary perspective and the DNA barcoding of the mitochondrial sequences is used to assess the biodiversity of the parasite (Nahum *et al.*, 2012). Progress on *S. haematobium* research has rather stagnated as there have been no breakthroughs in the areas of omics, high throughput drug screening, reverse vaccinology, molecular imaging, bioengineering, structural biology, and molecular immunology. Inasmuch as this dearth may be ascribed to the fact that it is a neglected tropical disease (NTD), the same cannot be said of S. *japonicum* and *S. mansoni* where results have been observed. Other limitations to these researches could as well be attributed to the following situation (Rollinson *et al.*, 2009).

2.7.2.1 The Absence of Mouse Animal Models

Groundbreaking studies on mouse models for *S. mansoni* and S. *japonicum* infections have enabled molecular and cellular immunologists to unravel the complexities of schistosome-mediated immunopathogenesis (Wynn *et al.*, 2004; Fairfay *et al.*, 2011). Subsequently, from 2011 to 2012, advanced researches have been

carried out on genetically modified knock-out mice to also identify co-factors, and lead to an improved understanding of *S. haematobium* carcinogenesis (Nair *et al.*, 2011) while microinjection of *S. haematobium* eggs in the bladder of mice reproduced urinary tract fibrosis and other bladder pathologies similar to human urogenital schistosomiasis (Fu *et al.*, 2012). It is predicted that an expansion of such studies will lead to new insights into parasite-induced immunopathogenesis, carcinogenesis, Female Genital Schistosomiasis (FGS), and HIV/AIDS co-infections.

2.7.2.2 In Vitro Systems and Completed Genome Projects

The first report of the genetic manipulation of *S. haematobium* by short interfering ribonucleic acids (siRNAs) into parasite eggs, and subsequent culturing of eggs, miracidia, schistosomula, and adult schistosomes (Rinaldi *et al.*, 2011), portend enormous promise for analyzing novel *S. haematobium* genes. It is believed that such in-vitro studies would complement ongoing in-vivo projects in mice. On the other hand, work in mammalian cell lines and transgenic Schistosomes will provide additional insights into parasite-mediated carcinogenesis (Bothelo *et al.*, 2009; Rinaldi *et al.*, 2012).

2.7.2.3 Related Omics Projects

In 2012, the 385-Mb genome of *S. haematobium* was completed at 74-fold coverage (Young*et al.*, 2012; Mitreva, 2012). Although there was a consistent synteny between the roughly 13,000 genes of *S. haematobium* and the other two schistosomes, many specific *S. haematobium* genes were singled out, among which those encoding novel drug targets, estrogen-synthesizing enzymes that might be involved in neocarcinogenesis of eggs in situ, and molecules with immunomodulatory functions.

However, many of these barriers have recently been surmounted to an extend and a new era of *S. haematobium* research is now opened.

Whole Genome Analysis (WGA) is useful because it increases the small amount of DNA obtained from each developmental stage of the parasite to quantities that allow for multiple Polymerase Chain Reactions (PCRs), replicate PCRs and subsequently estimation of genotyping error rates (Valentim *et al.*, 2009). Given the completed whole genome sequence of *S. haematobium*, and the rapidly advancing genetic manipulation technologies, new insights that could lead to the development of a new generation of tools are emerging and may help to eliminate the disease (Brindley and Hotez, 2013).

Sequence variation has been demonstrated in the complete mitochondrial genome of *S. haematobium*, showing population level differences (WHO, 2012). The mitochondrial genome of *S. haematobium* has also been sequenced. It comprises of 36 genes with two ribosomal genes (large and small subunit rRNA genes), and 22 transfer RNA (tRNA) genes, as well as 12 protein-encoding genes (atp6, cob, cox1–3, nad1–6, and nad4L). The atp8 gene, which is present in other phyla, is absent from Platyhelminthes (Le *et al.*, 2002). Moreover, each *Schistosoma* mitochondrial genome contains a long noncoding region that is divided into two parts by one or more tRNA genes, which is found in other Platyhelminthes and vary in length according to each taxon. The gene order in the mitochondrial genome of *S. haematobium* is obviously different from other taxa (Littlewood *et al.*, 2006; Lawton *et al.*, 2011). The unique gene order rearrangements identified in this specie constitutes valuable information to its phylogeny. Partial *cox*1 molecular data of *S. haematobium* has shown extremely

low levels of genetic diversity within and between *S. haematobium* populations and is divided into two distinct groups; Group 1 clustered around a highly common, persistent and widespread mainland African haplotype (H1) and Group 2 more diverse and unique to the Indian Ocean Islands (Webster *et al.*, 2012; Webster *et al.*, 2013).

Several studies have used *S. haematobium* and related species tandem-repeat sequences in a conventional and real-time PCR for the detection of *Schistosoma*-specific DNA in stool, urine, and serum samples as well as the SSU rDNA, 28S, and ITS sequences and mitochondrial genes (Table 1) (Verweij and Stensvold, 2014).

Real-time PCR targeting the *Schistosoma* ITS2 sequence used on vaginal lavage samples and has proved to be a reliable tool in the complicated diagnosis of female genital schistosomiasis (Kjeland *et al.*, 2009; Downs *et al.*, 2013). The second internal transcribed spacer (ITS2) of *S. haematobium* sequences from Kenya has been reported to be nearly identical (99%) to conspecific sequences from Egypt, Mali, and Niger (Barber *et al.*, 2000).

Specific species of *S. haematobium* have been identified and characterized using the *S. haematobium* repeated sequences Sh73 and Sh77 (73bp and 77bp long, respectively) which were present in all Schistosomes of the *S. haematobium* group members tested Simple PCR using these primers was able to differentiate between *S. haematobium*, *S. bovis*, and the other animal schistosome, after agarose chromatography using a 100 bp ladder, sufficient for identifying a single parasite without having to do a nested PCR (Abassi *et al.*, 2012).

TABLE I

Examples of Studies that have used Tandem Repeats of *S. haematobium* in Conventional and Real-Time PCR Detection in Specimens

Organism	Thermocycling	Identification	Marker	Specimen
S. haematobium	PCR	Gel electrophoresis	*S. haematobium* tandem repeat	Adult worm
Schistosoma genus	PCR	Gel electrophoresis	28S	Urine
S. haematobium, *S. bovis*, *S. intercalatum*	PCR	Gel electrophoresis	ITS	Urine
S. haematobium	Multiplex real-time PCR	Hydrolysis probe	*cox1*	Stool
Schistosoma genus	Real-time PCR	Hydrolysis probe	ITS2	Urine
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Cerebrospinal fluid
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Urine
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Urine
S. haematobium	PCR	Gel electrophoresis	*nad1* and *cox1*	Urine
Schistosoma genus	Real-time PCR	Hydrolysis probe	28S	Stool, urine, serum
Schistosoma genus	Real-time PCR[c]	Hydrolysis probe	ITS2	Urine
Schistosoma genus	Real-time PCR[c]	Hydrolysis probe	ITS2	Urine, vaginal lavage

Source: Adapted from Verweij and Stensvold (2014).

2.8 The *Bulinus* Species of Snails (The Intermediate Hosts)

About 350 snail species are estimated to be of possible medical or veterinary importance. Many species of freshwater snail belonging to the family *Planorbidae* serve as intermediate hosts for the highly infective *Schistosoma* larvae although infection with the parasite does not necessarily require any direct contact between snails and human (WHO, 1997). Those of the genus *Bulinus* harbour the asexual stages of *S. haematobium* and serve as intermediate host (Brown, 1994).

2.8.1 Habitat

Bulinid snails are aquatic snails that live under water and cannot usually survive elsewhere. They flourish and breed almost in all types of freshwater bodies ranging from small temporary ponds and streams to large lakes and rivers bodies with suitable temperature and pH where the aquatic vegetation at the fringes provide food, microhabitats and protection from strong water currents and mostly safety for their eggs. They are not found in salty or acidic water (Ofori *et al.*, 1999; Yirenya-Tawiah *et al.*, 2011).

2.8.2 Morphology

Early species descriptions were grouped into genera based on shell characteristics, especially while in the field before using other methods of identification. Although their use in phylogenetic studies has posed some dilemma, shell and internal anatomy of snails have been exploited for in the identification and differentiation of *Bulinus* species, (Inaba, 1969; Brown, 1994; Stothard and Rollinson, 1996). It is believed that shell characteristics are of limited value for the identification

of *Bulinid* species (Stothard *et al.*, 1997). However, some characteristics such as distal genitalia, prostate, shell and radula teeth have been useful in the identification of snail species (Walter, 1968; 1969, Mimpfoundi and Ndassa, 2005). Some observable differences and/or similarities such as the number and the structure of radula have been of higher importance in molluscan taxonomic relationships at the species level and have been exploited in the classification of gastropods (Arularasan *et al.*, 2011). Although many factors such as food, seasonal changes and sexual differences affect the structure of radula (Carlos and Helena, 2003; Matthews-Cascon *et al.*, 2005), some radula characters are believed to be usually the same within the same species (Fretter and Graham, 1994). Other anatomical characters such as kidney, nervous system, pneumostome, tentacles and digestive system have also been in snail identification with varying degree of reliability in speciation (Jackiewicz, 1990; Paraense, 1994; 1995; Ponder and Waterhouse, 1997; Samadi *et al.*, 2000).

2.8.3 Life Cycle

All species are hermaphrodite, and therefore capable of self- or cross-fertilization; consequently, a single snail at a new habitat can populate it. The optimal temperatures for reproduction are usually between 22 °C and 26 °C although the snails can easily survive between 10 °C and 35 °C. The seminal fluid is produced by the male organ while the eggs are produced by the female organ. Cross-fertilisation is achieved by pressing the front parts of their feet together and shooting sperm-filled darts into each other. Sexual maturity can be reached at 1 year (Krina, 2017). The eggs are laid at intervals in batches of 5-40, each batch being enclosed in a mass of jelly-like material often attached to plants or similar surfaces in the water. Some species

lay their eggs out of the water, and their young return after hatcing. The young snails hatch after 6-8 days and reach maturity in 4-7 weeks, depending on the species and environmental conditions. A snail lays up to 1000 eggs during its life, which may last more than a year (Betterton *et al.*, 1983; Goll and Wilkins, 1984). A few weeks after mating and laying eggs, the hatchlings emerge from their egg, small and defenseless against many predators that sneak around, such as beetles, birds, turtles and even other snails.

2.8.4 Ecology

The snail densities vary significantly with the season. Within each habitat, snail distribution may be patchy with its detection requiring an examination of different sites. Bulinid snails are usually found in shallow water near the shores of lakes, ponds, streams and irrigation canals where the water plants and mud are rich in decaying organic matter. They also occur on rocks, stones or concrete covered with algae or on various types of debris. Plants serve as substrates for feeding and oviposition as well as providing protection from fast flowing water as well as predators such as fish and birds. Most species can survive outside water for short periods but die when exposed on to dry land in the dry season; but a proportion of some snail species are able to survive the drought buried in the mud bottom by sealing their shell opening with a layer of mucus. They rapidly recolonize the ecosystem after the rains return (Diaw *et al.*, 1989, Greer *et al.*, 1990).

2.8.5 Genetics of the Snail Intermediate Host

Sapp and Loker (2000) observed that the mollusc host infection with the schistosome parasites is species specific and often localized with previous literature showing that the adaptation of *S. haematobium* to parasitism within the snail host is influenced by both the host and parasite genes (Has and Haberl, 1997; Rollinson *et al.*, 1998). The current geographical changes have led to global changes in the distribution of many species resulting in some hybridization as in the case of Schistosomes where offspring have shown a higher virulence and invasive ability (Adema and Loker, 1997). Extensive studies on snail's genome differentiation directed towards identifying genes related to the snail's immune responses so as to prevent the Schistosome parasites survival in them was subsequently recommended (Adema and Loker, 1997). The introduction of snail hosts with parasite resistant genes into the resident population to replace or cross-breed with the susceptible snail species in endemic areas will alter the parasite transmission and also will be more friendly to the natural ecosystem than molluscicides (Abe *et al.*, 2018).

Molecular biomarkers have played important role in species identification (Hebert *et al.*, 2003; Akinwale *et al.*, 2015). The application of PCR *Dral* and sm17 in the early detection of *S. haematobium* in infected *Bulinus* species has helped to strengthen snail surveillance and boost schistosomiasis control efforts (Hamburger *et al.*, 1998; Hamburger *et al.*, 2004). In Morocco, the simultaneous use of PCR, *Dral* PCR and Sh110 SmSI PCR has unraveled the Schistosome parasites that infect the *Bulinus* group of snails (Armanir *et al.*, 2014). *B. truncatus* and *B. forskalii* have been identified using molecular methods in Chad (Moser *et al.*, 2014).

TABLE 2

Some Studies that have been done across Africa with Characterisation of the *Bulinus* species of Snails in Various Endemic Foci

S/N	Country	Snail species
1.	Nigeria	*Bulinus globosus, Bulinus forskalii, Bulinus* species.
2.	Chad	*Bulinus truncatus, Bulinus forskalii*
3.	Angola	*B. canescens B. angolensis, B. crystallinus, B. globosus,*
4	Egypt	*Bulinus truncatus, B. canescens Bulinus truncatus, B. globosus, B. senegalensis, B. tropicus,*
5	Cameroon	*B. camerunensis, B. globosus, B. forskalii,*
6.	Senegal	*Bulinus truncatus, B. senegalensis, B. umbilicatus*
7.	Tanzania	*B. globosus*
8.	Madagascar	*Biomphalaria pfeifferi*
9.	Malawi	*B. globosus, B. nyassanus*
10.	Cote D' ivoire	*B. forskalii, B. globosus*
11.	Equitorial Guinea	*B. forskalii*
12.	Niger	*B. umbilicatus*

Source: Adapted from (Abe *et al.*, 2018).

In Nasarawa State in Nigeria, the mitochondrial gene cytochrome oxidase I (*cox1*) has been used to establish that *B. globosus* and *B. forskalii* were the intermediate hosts of *S. haematobium* and that using nucleotide blast homology on genbank to study the sequence data of *B. globosus* from Nasarawa State, it was revealed that they had a phylogenetic relationship with those from other West African countries such as Burkina Faso, Senegal and Niger when BLAST, with a monophyletic lineage but formed a paraphyletic relationship with *B. globosus* species from East Africa. *B. forskalii* had a similar pattern, clustering to form a monophyletic relationship with species from Burkina Faso (Mogtedo barrage), Niger (Tondia) and Senegal (Thiekeene Hulle) (Abe, 2015).

The infection status of *Bulinus truncatus* (identified by PCR-RFLP technique) has been assayed with *Dra I* gene repeat in Southwest Nigeria (Hassan *et al.*, 2016) while Akinwale *et al.*, (2015) established the population structure of *B. globosus*, *B. forskalii*, *B. camerunensis* and *B. senegalensis* in schistosomiasis endemic communities of Ogun State, Southwestern Nigeria through the application of PCR-RFLP on the snails ribosomal ITS region.

2.9 Diagnosis of Schistosomiasis

Different laboratory techniques are documented for the diagnosis of schistosomiasis. They include the parasitological, the immunological and the biomolecular methods (Carneiro *et al.*, 2013; Hoy *et al.*, 2014; Hinz *et al.*, 2017). The diagnostic approach to schistosomiasis for people that have just returned from endemic areas is different from that of residents. While serology is the best screening tool for the first group, microscopy to assess egg burden and polymerase chain reaction

to check for parasite genotypes respectively remain the most indicated procedures for the second category of people. However, these later approaches may not be sensitive for cases of less than three months (Meltzer *et al.*, 2006; Meltzer *et al.*, 2013; Soentjens *et al.*, 2014).

2.9.1 Rapid Assessment Procedure (RAP)

The primary target of every epidemiological surveys is usually to evaluate the impact of the disease on the livelihood of the populations in order to be equipped with the required data for necessary control measures. Unfortunately, the application of conventional diagnostic techniques in these studies is not always possible because of some factors such as limited resources, time, social compliance and cultural barriers among others. In the case of urogenital schistosomiasis, RAP provides a fast and cheaper screening method in communities of high prevalence as it makes use of simple questionnaires to evaluate the distribution of the disease in selected study of studies (Chitsulo *et al.*, 1995).

Literature suggest that RAP can be used in developing as well as developed countries to evaluate morbidity through community members' perception and in the utilization of health services patterns and can contribute significantly to the health planning (Ross and Vaughan, 1986; Tanner, 1989). In structured organisations like schools, this method has been applied with success since teachers with little training have proved competent enough to detect the presence of haematuria (Lengeler *et al.*, 1991; Useh and Ejezie, 2004).

2.9.2 Urine Macroscopy

In order to find their way to the lumen of the urinary bladder for further excretion in view of completing the life cycle, the eggs of *S. haematobium* puncture the capillaries of the vesical plexuses (Woodruff and Bell, 1978). The resulting haemorrhage coupled with the tissue immune response to the egg are reflected in the urine as haematuria and proteinuria and can be detected using simple laboratory techniques like strip tests. WHO guidelines for preventive chemotherapy, recognizes the prevalence of haematuria, in addition to egg count-based criteria as effective measures for locating communities with high, moderate, or low risk for schistosomiasis (WHO, 2006). However, in areas of low endemicity the situation can be incorrectly evaluated depending on the infection prevalence (Gray *et al.*, 2011; King and Bertsch, 2013). This approach has been exploited in the diagnosis and mapping of the disease in Cross River State of Nigeria (Adie *et al.*, 2014). However, it is not very sensitive or specific since it is not all haematuria that are caused by Schistosomes and this symptom will occur only after oviposition to and subsequent migration of eggs across the tissues to the bladder. Mild infections may not produce haematuria as it is associated with egg output (Ejezie and Ade Serrano, 1981; Mott *et al.*, 1985). Vester *et al.*, 1991 reported that false positive results could be as high as 15% in girls who have been circumcised because of the constant or intermittent bleeding associated with this condition. Likewise, it may not be uncommon that haematuria due to normal menstruation is attributed to the disease.

The assessment of micro-haematuria in urine using dipsticks has also been considered to be a good diagnostic and relatively inexpensive indicator and a

potentially accurate proxy for diagnosis of heavy *S. haematobium* infections in an endemic community and can be used for the identification of high-transmission areas (Briggs *et al.*, 1971; Savioli *et al.*, 1990). Recent literature has highlighted their usefulness as indicator of *S. haematobium* in a setting with a history of two decades of preventive chemotherapy (Knopp *et al.*, 2013). Because there is reduction in haematuria levels after treatment against schistosomiasis, the reagent strips can be used to assess drug efficacy, with the most appropriate assessment time of six weeks after treatment (Stete *et al.*, 2012). Ochodo *et al.*, (2015) observed that the detection of micro-haematuria on test strips had a sensitivity of (75%, 95% CI) and specificity (87%, 95% CI) in a study that involved 102,447 participants.

2.9.3 Urine Microscopy

Microscopic identification of eggs in the urine is the most practical routine of diagnosis of *S. haematobium* infection (Gray *et al.*, 2001). The recommended time for sample collection is between noon and 3:00 P.M. Characteristic eggs with terminal spines are present in greatest numbers in the last part of the urine passed after exercise and can be present in the stool. The concentration of the eggs by centrifugation and subsequent examination of the deposit can enhance the diagnosis. Egg count can be achieved through filtration technique using a Nucleopore membrane across which a standard volume of urine is filtered and the total number of eggs subsequently counted (Utzinger *et al.*, 2015). A modified filtration technique developed by Ejezie and Ade-Serrano (1980) has been successfully replicated by other researchers in Cross River State and has shown not only to be very reliable, but also to have the advantage of preserving the eggs for a later count (Useh and Ejezie, 1999; Inyang-Etoh *et al.*, 2009).

Although this method is easy to perform, infections with very light intensities fail to be diagnosed particularly if they are affected by the daily variation of ova output (Melchers *et al.*, 2014; Utzinger *et al.*, 2015). To address this deficiency, Gray *et al*, (2011) recommended the use of other methods such as centrifugation, sedimentation, miracidium hatching and flotation technique.

2.9.4 Antibody Detection.

Various immunodiagnostic tests have been developed to overcome the challenges posed by false negative and false positive results from microscopy (Wilkins and Keystone, 2013; Berry *et al.*, 2014). They include intradermal test and ELISA. The sensitivity and specificity vary widely among various serological approaches based on the type of antigen preparation (crude, purified, adult worm, egg, cercarial) and the test procedure. FAST-ELISA test is 95% sensitive and 99% specific for *S. haematobium*. The presence of antibodies is indicative of schistosomiasis at some time and cannot be correlated with clinical status, worm burden, egg count and prognosis (Tsang & Wilkins, 1997). The detection of antibodies is better in some cases but has some limitations in its application. A positive reaction may occur with a patient that is not excreting eggs; it has been found very useful in the detection of an initial active infection in young children who are not yet egg patent but might require special care (Stothard *et al.*, 2011; Poole *et al.*, 2014).

The test is of great importance in field studies to determine the prevalence of the disease particularly where patient have a low egg count and can also help is checking for resurgence of the infection after implementation of a control program. It

can also be used for screening of travelers. When the infection is established with eggs passed in urine, the commercially available immunodiagnostic kits are less sensitive and less specific than repeated urine sampling for microscopy probably due to the cross-reactivity with antigens from other helminths (Bergquist *et al.*, 2009). Most procedures detect IgG. IgM, or IgE against soluble worm or egg antigen through enzyme-linked immunosorbent assay (ELISA), Indirect haemagglutination test (IHT), or immuofluorescence test (IFT). For some time however, a cercarial antigen ELISA has since been in use for serological diagnosis of the disease (Ross *et al.*, 2007; Chand *et al.*, 2010). Urine strips detecting circulating cathodic antigens have been on sales and have been useful for field studies of *S. mansoni* and *S. haematobium* but seemed not to be sensitive and/or specific enough for definitive studies (Stothard *et al.*, 2009).

Detecting circulatory worms or eggs antigens using monoclonal or polyclonal antibodies in serum, urine or sputum in infected individuals has progressively been considered an upgrade of the routinely used diagnostic techniques. A monoclonal antibody-based antigen detection technique has recently been developed and shows a very high diagnostic accuracy (Van Dam *et al.*, 2013). It uses an up-converting phosphor-lateral flow to detects parasite-excreted antigens in urine or serum at very low concentrations (Corsjens *et al.*, 2014). Its application in a close to elimination setting in Tanzania revealed that it was several folds more sensitive than a single urine filtration (Knopp *et al.*, 2015).

Although adult *S. haematobium* worms reside in the blood vessels or urinary bladder and are therefore strictly blood parasites, their eggs pass through the bladder

wall and are excreted in into the urine. The number of eggs, especially for travelers, is often very small, and therefore, large quantities of urine are concentrated to improve the sensitivity of microscopic examination. Because infection is usually very light in settings where the disease is not endemic, serology is much more sensitive than microscopy for the diagnosis of schistosomiasis in travelers (Gryseel *et al.*, 2006). Recent studies have shown that the detection of *Schistosoma*-specific DNA can achieve a higher sensitivity for the diagnosis of acute schistosomiasis than serology (Clerinx *et al.*, 2011; Wichmann *et al.*, 2013).

2.9.5 Molecular Techniques

Methods for the diagnosis of infectious diseases stagnated for two to three decades. Since the introduction of PCR however, few major advances in clinical diagnostic testing have been made although new technologies are still being investigated. Many tests that form the backbone of the "modern" microbiology laboratory are based on very old and labour-intensive technologies such as microscopy (both wet and stained preparations). This traditional approach is readily performed when there is a trained microscopist and is cheaper to achieve since the reagents and equipment are less costly (Kompali-Cristo *et al.*, 2005). However, the ability to accurately identify the parasite might interfere with the sensitivity of the procedure. The antibody detection on the other hand may not be very specific since many parasites may share the same antigen or may be false positive since the circulatory antibody may persist even after the pathogen is eliminated.

In order to enhance the identification and characterization of the parasites, molecular biology was considered and became progressively established and a recognised a diagnostic tool in parasitology (Jardim *et al.*, 2006). In recent years, research has been focused on alternative methods to improve the diagnosis of parasitic diseases. These include immunoassays, molecular-based approaches, and proteomics using mass spectrometry platforms technology. Molecular parasitology represents an emerging field in Microbiology Diagnostics. This technique demonstrates the antigenic components or DNA segments of the parasites and are not influenced by environmental factors thereby ensuring reliability of results (Scott, 1995; Portela-Lindoso and Shikanai-Yasuda, 2003).

Since the start of molecular approaches for the detection of parasites, several techniques have been developed with an increase sensitivity and specificity. Parasites that could not be demonstrated using conventional techniques are now diagnosed and can subsequently be managed before any major harm is caused to the susceptible individuals. Many molecular techniques and primers have been described for the qualitative and quantitative analysis of *Schistosoma*-specific DNA in clinical samples, with a specificity of about 100%, but with varying sensitivities, ranging from equal to higher than that of traditional microscopy techniques depending on sampling technique for the latter (Aryeetey *et al.*, 2013; Schwartz *et at.*, 2014). For the detection of DNA in urine, the sensitivity is further increased by the use of concentration procedures such as sedimentation or filtration, the latter being more indicated for population-based surveillance in more remote endemic regions (Kenguele *et al.*, 2014; Lodh *et al.*, 2014). The approach allows for detection of the *Schistosoma* DNA in other

samples including semen and vaginal lavages thereby facilitating the diagnosis of genital schistosomiasis (Kjetland *et al.*, 2009) and can also be used to type strains and detect hybrids between human and animal Schistosomes.

Current laboratory diagnostic methods for the identification of parasites include: Polymerase Chain Reaction (PCR) (Monis and Andrews, 1998), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) (Blears *et al.*, 2000), Restriction Fragment Length Polymorphism (RFLP), microsatellite marker method (Quan *et al.*, 2008), Luminex xMAP-based technology (areas of multianalyte profiling) (Dunbar, 2006), Loop-Mediated isothermal Amplification (LAMP) (Parida *et al.*, 2008), and the real-time PCR (Guy *et al.*, 2004). The discovery of Polymerase Chain Reaction (PCR) brought enormous benefits and scientific developments such as genome sequencing, gene expressions in recombinant systems, the study of molecular genetic analyses, including the rapid determination of both paternity and the diagnosis of infectious disease (Novais *et al.*, 2004). It was developed in the 1980s by Kary Mullis, for which he received the Nobel Prize in 1994 (Bruce *et al.*, 1999). PCR allows for the in-vitro synthesis of specific DNA fragments using a DNA-polymerase enzyme, which takes part in the replication of the cellular genetic material thereby synthesising a complementary DNA sequence by making use of a specific small fragment (primer) of the DNA strands to start the synthesis. Primers limit the sequence to be replicated and the result is the amplification of a particular DNA sequence with billions of copies (Mullis, 1990; Novais *et al.*, 2004; Spolidorio and Spolidorio, 2005). An innovation of PCR, Real-Time PCR, became even more important in clinical diagnostics and research laboratories due to its capacity to

generate quantitative results. This technique allowed for a presentation of results in a faster and more accurate fashion than conventional PCR, which was only a qualitative technique (Novais *et al.*, 2004; Kubista *et al.*, 2005).

2.9.5.1 Microsatellites

In genetics, microsatellites are regarded as a section of DNA consisting of very short nucleotide sequences repeated many times at a particular locus on a chromosome, the number of repeats varying between members of the species, and so can be used as a genetic signature. They are Single Sequence Repeats (SSRs) extensively used in plant genetics studies, using both low and high throughput genotyping approaches (Viera *et al.*, 2016). They are used as a marker to determine the genetic diversity, identify important genetic traits, and in forensics, population studies, and paternity dispute. They are abundant in eukaryotic genomes, can readily mutate by losing or gaining repeat units and have been used in parasites of both human and animals. They have a wide variety of applications mainly because they show frequent polymorphism, codominant inheritance, high reproducibility and resolution, require simple typing methods and can be detected by PCR. There is however a low popularity of these genetic markers probably due to their high number which cause some technical difficulties in isolating the parasite (Oliviera *et al.*, 2006; Johnson *et al.*, 2006).

2.9.5.2 Microsatellites and Diagnosis of Schistosomiasis

Microsatellite analyses and sequencing of the mitochondrial DNA contribute to a better understanding of the genetic make-up of both Schistosome and snail

populations. The new procedures to capture the DNA of eggs and larval stages of the parasite simplify more detailed and ethically advantageous studies on the heterogeneicity of the parasite (Rollinson *et al.*, 2009). The cytochrome oxidase sub-unit 1 mitochondrial DNA (CO1*m*DNA) is the gene encoding the specific identity of human Schistosomes and is the PCR target that is able to differentiate between *S. mansoni, S. haematobium, S. japonicum and S. mekongi* (Kato-hayashi *et al.*, 2010). Microsatellites as genomic quirks resulting from recombination or replication errors are simple sequence tandem repeats that may tend to occur in non-coding regions therefore making them ideal for the detection of genetic variations.

2.9.6 Indirect Methods for Diagnosis of Schistosomiasis

The diagnosis of schistosomiasis can also be achieved through indirect methods which can be clinical and sub-clinical by estimation biochemical morbidity markers. These methods however may not be very specific as various signs and symptoms are observed in the course of the disease. The clinical assessment of the patient may be associated with ultrasound, liver biopsy/histology, estimation of some biochemical markers. Changes consistent with schistosomiasis might include peripheral blood eosinophilia, anaemia (iron deficiency, macrocytic), hypoalbuminaemia, increased hypergammaglobulinaemia, urea and creatinine levels. A bladder biopsy is useful where a patient with typical presentation shows no eggs in the urine. A liver involvement of the disease is usually reflected by its characteristic appearance on abdominal imaging and a biopsy may be indicated in a case of co-infection with another liver disease such as viral hepatitis (Gryseels *et al.*, 2006).

be effective after all oral and parenteral routes of administration tested and depended on the host species and the posology to achieve at least 95% of the parasite reduction. The total doses ranged from 200–1,000 mg/kg in mice and from 100–500 mg/kg for *Mastomys* and hamsters. Although PZQ has been the drug of choice for schistosomiasis for many years, its precise mechanism of action remains unknown. When schistosomes come in contact with PZQ in vitro, there is a rapid influx of calcium ions followed by an intense muscular paralysis of the worm.

A vacuolation and blebbing of worm tegumental and subtegumental structures in adults but not juveniles has also been observed (Xiao *et al.*, 2009). It is believed that the drug disrupts the tegument and exposes parasite surface antigens leading to recognition and parasite clearance by the host immune system and this may account indirectly for the difference in sensitivity between juvenile and mature stages. Praziquantel is very effective against the invading stages and slightly less against schistosomules up to an age of 7 days. It is less effective against 2- to 4-week-old juveniles, but is effective again against 5-week-old and older schistosomes. It is equally effective against both sexes of *S. mansoni*, less effective against unpaired and therefore juvenile female worms, but fully effective against single male worms. The efficacy of the drug on *S. mansoni* in mice is not influenced by the strain or the gender of the host, the worm burden or the age of the infection. Given the above considerations, praziquantel promised then to be a very potent antischistosomal drug.

PZQ was first released by Bayer in 1979, after the mandatory toxicological tests and clinical trials were completed and was recommended at 40 mg/kg body weight with a maximum dosage of 2400 mg if the patient was above 60 kg of weight

(Adam *et al.*, 2005). Some early trials of PZQ antischistosomal activities carried out under the auspices of the World Health Organization using standard protocols were recorded. In Zambian children affected by *S. haematobium*, there was only one treatment failure in 79 patients; In Brazil, praziquantel cured 25 of 28 patients with *S. mansoni* infection while in the Philippines the recovery was 60 of 75 people affected by *S. japonicum* (Davies *et al.*, 1979; Katz *et al.*, 1979; Santos *et al.*, 1979). Cure however in these studies was referred to as the complete cessation of excretion of viable eggs.

By using the recommended PZQ dosages the cure rates were: 75-85% for *S. haematobium*; 63-85% for *S. mansoni;* 80-90% for *S. japonicum;* 89% for *S. intercalatum* and 60-80% for double infections with *S. mansoni* and *S. haematobium*. This was the first anthelminthic pharmaceutical product to fulfill the World Health Organization's requirements for population-based chemotherapy of a broad range of parasitic infections (Wegner, 1981; Wegner, 1984). Supported by the above data, PZQ was considered as the drug of choice for treatment of schistosomes and a breakthrough in the treatment of most trematode and cestode infections in most endemic areas (Bouree, 1991; Cobo *et al.*, 1998) and also probably because its ease of administration, its tolerable side-effects, and its cost (Cioli, 1998). Eventually, the treatment and control of schistosomiasis has relied mostly on this drug either alone or in combination with others. Unfortunately, if used alone, this drug may not be well effective for a good control program since it is not effective against the schistosomulae. Currently in Cross River State of Nigeria, PZQ is distributed to all primary schools every two year in view of reducing the high prevalence of the disease. Although absolute clinical

resistance to the drug has been described, the drug resistance trend might still be considered a rising threat and the need for investigating other therapeutic measures becomes an option (Doenhoff *et al.*, 2008; Melman *et al.*, 2009). A separate trial of PZQ in combination with placebos statistically failed to show a better treatment than PZQ alone (Hou *et al.*, 2008).

2.10.1b Artemisinins

Despite it availability as a common herb and its use in Chinese medicine for thousands of years as a remedy to a wide range of ailments, *Artemisia annua* (*qinghao*) became known to the world for its antimalarial properties only in the 1970s (Tu, 2011). The active pharmacophore (Artemisinin) is a potent sesquiterpene lactone that has a characteristic peroxide bridge and the active metabolite is dihydroartemisinin. It has a short half-life, poor bioavailability and the foresight of a global need beyond plant cultivation triggered a search for semi-synthetic and completely synthetic alternatives (Ho *et al.*, 2014). The most widely used artemisinin-derivatives are the first-generation semi-synthetics artesunate and artemether. By the nineties, evidence was accumulating on their prophylactic uses (de Clerq *et al.*, 2000; Shuhua *et al.*, 2000). Literature suggest that all artemisinin-based therapies have common antischistosomal therapeutic characteristics. They are highly effective against juvenile infections but have only a moderate effect against adult worms suggesting that the clinical benefits of the drug are mainly in prophylaxis (Keiser *et al.*, 2007; Utzinger *et al.*, 2007; Keiser *et al.*, 2012; Li *et al.*, 2014). Field trials have highlighted the efficacy of artemisinin based therapy or artemisinin based combined therapy (ACTs) in the control of schistosomiasis (Inyang-Etoh *et al.*, 2004; Sissoko *et*

- Protecting the susceptible snails from being contaminated with infected urine. The provision and use of latrines to reduce the contamination of water bodies which may serve as sources of infection, and modern biocomposting toilets appear to be effective in killing parasite eggs when used properly. Public enlightment campaigns may also influence the social behavior.
- Controlling the snail hosts population This may be achieved by the strategic use of molluscicides (niclosamide or copper sulphate), draining marshes and swamps, and clearing channels of vegetation. The introduction of snails with resistant genes in endemic foci for cross-breeding with the susceptible local ones.
- Protecting people from patronizing cercariae-infested waters. Irrigation practices can be modified to avoid long-standing still waters, and different or improved crops can be used which are less dependent on lengthy immersion in water.
- Developing and sustaining a control program in endemic communities.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the Study Area / Map

The study was carried out in northern Cross River State, Nigeria. The State is situated within the tropics, between latitudes 5° 32' and 4° 27' North and longitude 7°50' and 9° 28' East. Rainfall in the hinterland is between 120 and 200 mm annually with maximum precipitation occurring from July to September.

Cross River North consists of the five Local Government areas (Figure 7) that constitute Cross River North Senatorial District namely: Ogoja, Obudu, Yala, Obanliku and Bekwarra. It has a land mass of 4,466 square kilometres which represents 20.8% of the entire State land mass (21,481 square kilometres). It lies within two major relief demarcations; a lowland region ''Ogoja Syncline" characterized by seasonal flood plains and swamps good for rice cultivation found at the south western section and a high plateau at the north eastern portion. This geo-political zone is found at the North western extremities of Cross River State within longitude 8° 20 and 9° 26' East of the Greenwich Meridian and latitude 6°14' and 7° 10 north of the equator (Ottong *et al.*, 2010). The region is bordered in the north by Benue State; in the South by Obubra and Ikom Local Government Areas, in the East by Cameroon Republic and the West by Ebonyi State. The zone is a part of the greater upper Cross River region often described by orthodox historians as "fragments of the Earlier World", "Splinter Zone" and "Human Cluster" (Erim, 1990).

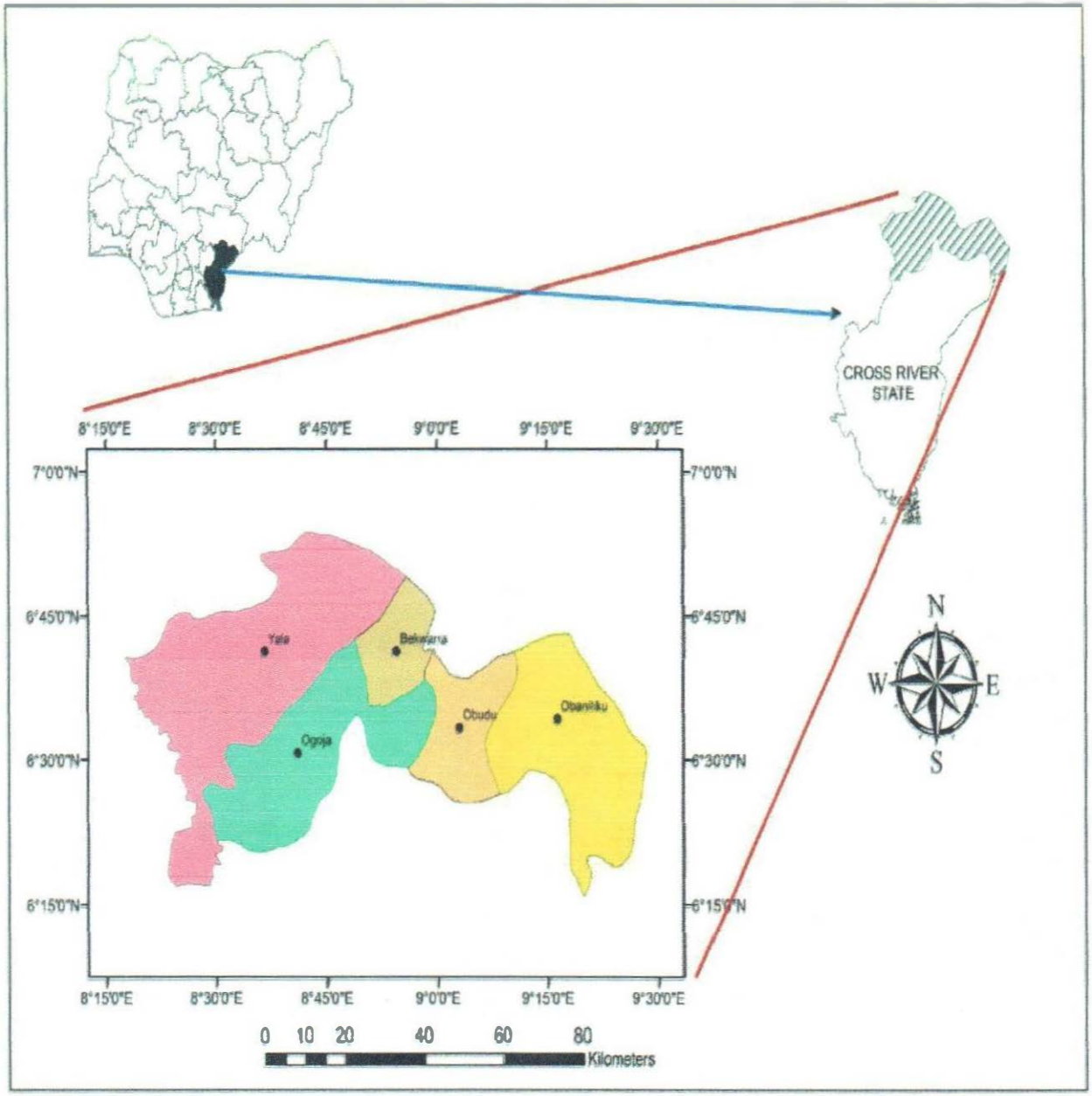

Figure 7: Map of Northern Cross River State, Nigeria (Study area).

Source (GIS ADSU, 2017).

The climate of Cross River North senatorial district has two varieties. These are the tropical grassland climate and the montane climates. The tropical grassland climate is associated with the lowland section of the region and characterized by two well defined seasons-wet and dry seasons. The annual rainfall varies between 2,500 mm to 3,500 mm, distributed unevenly between March and November and is of the orographic type majorly.

The dry season lasts from the latter part of November to the early period of March when dry winds have much influenced on the climate. The mean annual temperature of this climate ranges between 25° C to 28° C daily maxima are also common on the high plateau of Obudu and Obanliku. These LGA have an altitude of 1,575.76 metres above sea level sub-temperate climate that is influenced by height and is characterized by alternating dry and wet seasons (Udo, 1978; Ottong *et al.,* 2010).

The predominant economic activities in Cross River North senatorial district is farming which involves the cultivation of crops such as Rice (*Oryza sativa L.*), Yam (*Dioscorea* species), Cassava (*Manihot esculenta Krantz*), Groundnut (*Arachis hypogaea*), Cocoa (*Theobroma cacao L.*), vegetable of different types, Maize (*Zea mays L.*), etc... Besides, fishing, palm wine tapping, mining of sand/gravel, lumbering (around Obanliku), transportation, salt mining, civil service, hospitality, trading etc... are also associated with the people of the area. Fifty-five markets create a meeting point for buyers and sellers, and also serve as central places for political and social activities. Markets such as Abuochiche, Okuku, Okpoma, Bendi, Sankwala, Ishibori, Mfuma, Wanikade, Obudu and Mbok among others are attended by people across the

country and even beyond. The popularity of most of these markets predates colonization (Chinyere and Lequome, 2012).

3.2. Study Population

The study population comprised of primary school children aged from 4 years and above irrespective of gender attending five selected primary schools, one in each LGA of northern Cross River State that have lived in the communities for at least one year and whose guardians had complied with the study. Snails with the phenotypical appearance of *Bulinus* species collected at swamps and rivers in the environs of the schools where there is regular human contact with the water body were also studied.

3.3 Sample Size

3.3.1 Determination of Sample Size

The sample size was calculated using the formula below (Cochran, 1963) and a prevalence of 41.68% as reported by Adie *et al*., (2014) for the prevalence of urinary schistosomiasis in Cross River State was used.

Using the formula

$$N = \frac{Z^2Pq}{e^2}$$

N = is a sample size

Z^2 is abscissa of normal curve that cuts and tails = $(1.96)^2$

e is the level of precision = 0.05

P = Current prevalence = 41.68% (Adie *et al*., 2014)

Q = 100 - p = 100 – 41.58 = 58.32%

$$N = \frac{Z^2Pq}{e^2} = \frac{(1.96)^2 (p) (q)}{(0.05)^2}$$

$$N = \frac{(1.96)^2 (0.4168) (0.58.32)}{0.0025}$$

$$N = 374$$

P = 0.5 (maximum variability) 95% CI and +5% precision.

The final sample size was 374 +42 = 416

The number of study participants per schools in each LGA was directed by the general macroscopy of the urine samples and the pupil's compliance with the exercise; thus 100 pupils were sampled in Bekwarra LGA, 80 in Obalinku, 80 in Obudu, 100 in Ogoja and 56 in Yala LGA.

3.3.2 Snail Sample Collection

Thirty snails collected at the human - water contact sites of selected water bodies in each LGA were used for the study. In Bekwarra LGA, Itiem River and Rice swamp was selected. In Obalinku, the commonest human contact site with Irisha Ukwo River was explored. In Obudu, Okprini Stream, Ayal in Olugwan community rivers were sampled. In Ogoja LGA, the sample sites were Ataroba rice swamp in Mbenkpen Ibil and Utom rice swamp in Ishindede Ibil. Uguaba rice swamp of Uguaba Ijamah community was the selected site for Yala LGA.

3.4 Ethical Approval and Consent.

Approval was sought from the Ethical Committee of the Cross River State Ministry of Health (See Appendix 1) before samples collection started. The authorities of the selected schools were informed of the study through advocacy visits. The purpose/objectives, methods of the study and its possible benefits were clearly

explained to the school authorities and stakeholders. Consent was obtained from the parents/guardians before sampling. The samples collected for the study as resolved with the effective study participants (ESP) were solely used for the defined purpose.

3.4.1 Inclusion Criteria

Children whose parents/guardians had no objection to the study, who have lived in the community for a minimum of one year and who willingly presented themselves for sample collection were considered effective study participants (ESPs).

3.4.2 Exclusion Criteria

Children below 4 years and female candidates who were menstruating were not considered. Any child who has not lived for a minimum of one year in the community and anyone who refused to be part of the study was not considered.

3.5 Study Design

3.5.1 Allocation of Numbers

Urine and blood samples were collected from each (ESP). After filling the questionnaire and allocating a serial number, the pupils were given pre-labelled sterile universal containers. After providing the urine samples, their blood samples were collected through venepuncture and the test tube given the same number as the urine. These dual samples were so numbered progressively for identification and laboratory numbers were used during sample analysis to ensure individual confidentiality. At the end of the study, Praziquantel was given to the infected pupils at 40 mg/kg body weight.

3.5.2 Administration of Questionnaire

After the pupil consent was sought, a questionnaire (Appendix 2) to capture the socio-demographic data and medical history was filled by the members of the research team for each study participant prior to sample collection.

3.5.3 Collection of Urine

The effective study participants were given sterile universal containers in which they provided urine samples. The exercise was carried out as from 10 am when the pupils came out for break and continued until the early in the afternoon when the last volunteer was sampled to ensure maximum yield of schistosome eggs (Chen and Mott, 1989). Pupils were asked to exercise a bit after the first micturation and force out into the container the extra urine that will come thereafter. The urine samples were screened on the field for *S. haematobium* as described below.

3.5.4 Collection of Blood Samples and Extraction of Serum

A total of 2mls of venous blood was collected from the same ESPs into plain tubes and allowed to stand undisturbed until clotting had occurred. After clotting, the samples which were labelled with their corresponding urine numbers, were gently dislodged from the walls of the tubes and spun at the field at 4000 rpm for five minutes using a bucket centrifuge powered by a generator. The sera were pipetted into pre-labelled micro vials and kept frozen until serological tests were done.

3.5.5 Collection of Snails

Led by a field guide to all the water bodies described in the questionnaire, snails with the phenotypic appearance of *Bulinus* species (Kristensen, 1987) were

searched for at human water contact sites and collected into a clean pre-labelled transparent rubber bowls containing 70% alcohol.

3.6 Processing of Urine Sample

3.6.1 Urine Macroscopy

The macroscopy of the urine was done to describe its appearance, including colour and turbidity. The presence or absence of blood was also reported.

3.6.2 Detection of Proteinuria and Haematuria

Commercial reagent strips were dipped into the urine samples for the detection of haematuria and proteinuria in the field (Hemastic, Boehringer Mannheim, Germany) and the results were recorded immediately by placing the strip against the provided charts on the strip container.

3.6.3 Detection of Eggs of *S. haematobium*

After vigourous agitation, 10 mls of each urine sample was transferred into a universal container holding 5 mls of 1% aqueous solution of carbol fuchsin (Ejezie and Ade-Serrano, 1980; Useh and Ejezie, 1999). The specimens were preserved this way until the time for filtration. A funnel holding a Whatman filter paper No 1 was suspended on a conical flask to act as the filter system through which the well agitated sample was allowed to pass. Using a blunt-ended forceps, the filter paper was removed with care and placed upwards (filtrate on the surface) on a slide. Microscopic examination of the retained eggs on the filter paper was done using x10 objective and the number of eggs counted was recorded as no of eggs per 10 mls of urine.

3.7 Serology

A sandwich Elisa test was carried out on 186 sera (chosen randomly after ensuring that all those that were positive by microscopy have been selected) for detection and determination of IgG and IgM respectively following the manufacturer's protocol as described below. The analysis was carried out at Molecular Biology Laboratory of the Niger Delta University, Amasoma, Bayelsa State. The test kits were manufactured by "Diagnostic Automation, Inc. Immunodiagnostics; 21250 Califa St, Suite 102 & 116, Woodland Hills. CA 91367. Batch No 3676C65 lot No 234H. The kits were procured from USA and mailed down to the Department of Medical Laboratory Science of the Niger Delta University using EMS courier services.

The concentrated wash buffer was reconstituted as recommended by the manufacturer by dissolving the entire 25 mls of the solution into 475 mls of sterile distilled water to get the working wash solution. All the samples were also diluted 1:40 by pipetting 5 µl of each into 195 µl of the dilution buffer pre-dispensed in micro-tubes. For each micro titre plate, 100 µl of negative control was added to well 1; 100 µl of positive control to well 2, and 100 µl of the pre-diluted test samples (1:40) to the other 94 wells. The plate was incubated at room temperature under air conditioner (about 18°C) for 10 minutes. The content was shaken and washed 3 times with diluted wash buffer. Two (2) drops of enzyme conjugate was added to each well. It was incubated at room temperature for ten minutes. The content was shaken out and washed 3 times with diluted wash buffer. Two (2) drops of chromogen was added to each well. It was incubated at room temperature for 5 minutes. Thereafter, 2 drops of

stop solution were added. A yellow colour indicated the end of the reaction (Appendix 15). Results were read visually by comparing the colour intensity of each sample with that of the Negative and Positive controls.

3.8 Molecular Analysis of Samples

3.8.1 Extraction of Genomic DNA from Eggs

Genomic DNA (gDNA) from 100 urine deposit were extracted for molecular analysis. The extraction was done using the *Quick*-DNA Universal Kit supplied by Zymo Research, USA in the Molecular Biology Laboratory of the Niger Delta University, Bayelsa State. Two hundred (200) microlitres of the stored urine deposits was added to a microcentrifuge tube and 200 µl of Biological Fluid & Cell Buffer added to the deposit. 20 µl of the proteinase K was added to the mixture, mixed thoroughly and incubated at 55°C (on a heating block) for 10 minutes. Four hundred and twenty (420) microlitres of Genomic buffer was added to the digested sample. The mixture was transferred to a Zymo-Spin IIC-XL Column in a collection tube, centrifuged at 12,000 g for 1minute and the collection tube was discarded with the flow through. Four hundred (400) microlitres of DNA Pre-Wash Buffer was added to the column in a new collection tube, centrifuged for one minute and the collection tube emptied. Seven hundred (700) microlitres of g-DNA Wash Buffer was added to the column, centrifuged for one minute and the collection tube emptied. Two hundred (200) microlitres of g-DNA Wash Buffer was added to the column, centrifuged for one minute and the flow through discarded with the collection tube. The column was now transferred in a new collection tube and the DNA eluted with 60 µl of DNA

elution buffer, incubated for 5 minutes and centrifuged for one minute. The flow through in the collection tube contained the eluted DNA.

3.8.2 Extraction of Genomic DNA from Snails

One hundred and fifty snails were removed from 70% ethanol, washed in Tris Boric EDTA (TBE) and the shell crushed. Twenty snails from each Local Government Area with the must muscular foot parts were selected and soaked overnight to remove all traces of alcohol. About 25 micrograms of tissue from each snail was snipped off into sterile microcentrifuge tubes containing 95 µl of nuclease free water, 95 µl of solid tissue buffer and 10 µl of Proteinase K. The mixtures were vortexed and incubated at 55° C for about 3 hours with an occasional gentle mixing until tissues were solubilized. Four hundred (400) microlitres of genomic binding buffer was added to each preparation and vortexed. The mixtures were transferred into zymo-spin IIC = XL columns placed onto collection tubes and spun at 14,000 rpm for 1 min. The collection tubes were discarded with the flow-through and the columns placed onto new sterile collection tubes. Four hundred (400) microlitres of pre-washed genomic buffer was added to each column, spun at 14,000 rpm for 1 min and the tube emptied. Seven hundred (700) microlitres µl of g-DNA wash buffer was added to the column, spun at 14,000 rpm for 1 min. Another 200 µl of g-DNA was added and re-spun at 14,000 rpm for l min but this time the tubes were discarded with the flow through. The columns were now transferred onto new clean sterile tubes, 60 µl of elution buffer was added, incubated at room temperature for 5 minutes and then spun at 14,000 rpm for 1min. The columns were now discarded and the eluted g-DNA recovered in the tubes stored frozen until ready for use.

3.8.3 DNA Assessment for Purity and Quantity

After the DNA extractions, the eluted products were quantified using a NanoDrop ND -1000 Spectrophotometer (NanoDrop Technologies USA). The equipment was initialized with DNAse free genomic water and blanked with gDNA elution buffer. The DNA concentration of each genomic extract was read and recorded in ng/µL while the absorbance 260/280 was used to determine the purity (See Appendices). Satisfactory samples with absorbance ranging from 1.70 to 2.10 were amplified by polymerase chain reaction(PCR) using a thermocycler. Those that were not satisfactory were re-extracted.

3.8.4 Amplification of Microsatellites of *S. haematobium* Genomic DNA from Urine Samples and Snails.

The target region was the microsatellites in the nuclear intergenic spacer ribosomal DNA (ITS rDNA) consisting of ShDraI F (Forward: 5'-TAACAAGGTTTCCGTAGGTGAA - 3') and ShDraI R (Reverse: 3'-TGCTTAAGTTCAGCGGGT -5'). They were obtained from Inqaba Inc, South Africa. The Production code was FD34. These primers have previously been designed by Hamburger *et al.*, (2001). PCR was carried out on 100 samples on a ABI 9700 Applied Biosystems thermal cycler including all those that were positive by microscopy, by immunoglobulin M serology and few Negative for both randomly chosen to amplify the Dra 1 repeats of *S. haematobium* variants. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. PCR amplifications was performed using 6 µl of each gDNA extract

in a final PCR reaction of 30 µl (Plate 2). Thermal cycling conditions was set as follows: Initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds for 35 cycles, initial extension at 72°C for 1 minute followed by a final extension at 60°C for 5 minutes.

3.8.5 Amplification of the ITS of the Schistosome Variants

The ITS of the rRNA genes of three replicates (A_1, A_2, A_3) of each of the schistosomes variants with identical microsatellites were amplified using the ITSSh-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITSSh-R: 5'-TCCTCCGCTTATTGATATGC-3' primers on a ABI 9700 Applied Biosystems thermal cycler. They were obtained from Inqaba Inc, from South Africa. The batch No Production code was FD34. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. PCR amplifications was performed using 6 µl of each gDNA extract in a final PCR reaction of 50 µl (Plate3). The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 53°C for 30 seconds for 30 seconds for 35 cycles; initial extension at 72°C and final extension at 72°C for 5 minutes.

3.8.6 Amplification of Snail 18s rRNA Sequence.

Polymerase Chain Reaction (PCR) was carried out on all extracts for specific amplification of the 18s RNA of the snails using genus-specific primers ETTS 1 forwards: (5'- TGCTTAAGTTCAGCGGGT - 3') and Reverse (3'-TAACAAGGTTTCCGTACGGAA - 5'). They were obtained from Inqaba Inc, from

South Africa. The batch No Production code was FD34. One (1) microlitre of the eluted DNA (Concentration 4 - 6 ng/µl was used as template in a final PCR volume of 25 µl. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. PCR amplifications was performed using 1 µl of each gDNA extract in a final PCR reaction of 25 µl (Plate 4). The thermal cycling was done in a touch down PCR as follows: Initial denaturation at 95°C for 4 minutes, followed by 41 cycles of denaturation at 95°C for 15 secs; annealing was performed at 51°C with a decrease of 0.3°C to 48°C for 1 min for 35 cycles followed by an extension at 70°C for 15secs. The final extension was at 70°C for 3 minutes.

3.8.7 Agarose Gel Electrophoresis

All the PCR products were resolved on 1.5% agarose gel using 10 µl of each amplicon to confirm the various bands against a 100 bp molecular ladder. One and half gram (1.5 g) of agarose power was weighed and dispensed into 100 mls of 1 x TBE buffer (Tris Boric Ethylene Diamine Tetra Acetic acid) and heated in a microwave to completely dissolve and become a clear solution. After the molten agar had cooled, 4 µl of Ethidium bromide was added and gently rocked. The solution was then poured into the electrophoretic casting tray onto which a comb was placed and allowed to cool and solidify for about 30 minutes and then placed in the electrophoretic tank. Ten microliters of the amplicon already mixed with the loading dye was loaded into the hole in the agarose punctured by the comb. A 100 base pairs (bp) molecular ladder and a negative control were also loaded in the gel and the preparation was

subjected to an electric field of 120V for 25 minutes. The resulting bands were visualised on a UV trans-illuminator before proceeding for sequencing.

3.9 Sequencing

Sequencing was done using the Bigdye Terminator kit on a 3510 ABI Sequencer by Inqaba Biotechnological, Pretoria, South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

3.10 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

3.11 Data Analysis and Management

Data gotten from the processing of samples were analysed using SPSS version 20.0. Chi-square and Anova test were used to test difference in proportion at $P = 0.005$ level of significance.

CHAPTER FOUR

RESULTS

4.1: Description of Study Participants

4.1.1 Description of Study Participants according to Local Government Areas (LGAs)

Figure 8 describes the study particitipants according to LGAs. A total of 416 pupils from selected primary schools in Northern Cross River State participated in the study. The macroscopy of the urine influenced the sample size per Local Government Areas (LGAs). The distribution of study participants according to LGAs was Bekwarra 100(24.0%), Obalinku 80(19.2%), Obudu 80(19.2%), Ogoja 100(24.0%), Yala 56(13.5%).

4.1.2 Description of Study Participants According to Age Groups

Figure 9 shows the distribution of study participants by age group. It was revealed that 18 (4.3%) children were aged 4-6 years, 129 (31.0%) were in the age range 7-9 years, 218 (52.4%) were aged 10-12 years, and 51(12.3%) were 12 years old and above.

4.1.3 Description of Study Participants According to Gender

Figure 10 shows the distribution of study participants according to age. It was revealed that the number of males 224(53.8%) that participated in the study was higher than that of females 192(46.2%).

4.1.4 Distribution of Study Participants According to Socio-demographic Factors and Medical History

Table 3 shows the distribution of study participants according to their socio-demographic factors. It was revealed that the level of education of the pupils' guardians was primary school 161 (38.7%), secondary school 192 (46.2%), and tertiary institutions 63 (15.1%). Their occupations ranged from fishing/farming 303 (72.8%), civil servants 61 (14.7%), traders 23 (5.5%), drivers 18 (4.3%) to artisans 11(2.6%). The past medical history showed that 23 (5.5%) participants had never taken PZQ, 100 (24.4%) 6 months earlier, 96 (23.1%) 7-12 months before the sample collection, and 197 (47.4%) over a year earlier. Eight (1.9%) lived where there is a clinic, 98(23.6%) where there is a Primary Health Care Centre and 310 where the only health facility available was a chemist shop.

4.2: Prevalence and Intensity (egg count) of *S. haematobium* Infection Among Pupils.

Table 4 shows the prevalence and intensity (egg count) of *S. haematobium* infection among study participants. The urine samples of 416 pupils were investigated qualitatively and quantitatively for *S. haematobium* infection. The microscopy revealed that 39 (9.4%) were infected with a mean egg count of 16.97 ± 8.05. The distribution of the prevalence according to age revealed that 3 (16.7%) out of 18 participants were infected within the age group 4-6 years with a mean egg count of 18.33 ± 11.84, 14(10.8%) out of 129 in the age group 7-9 years with a mean egg count of 16.93 ± 6.28, 15 (6.9%) children out of 218 in age group of 10-12 with a mean egg

count of 15.80 ± 7.06 and 7 (13.7%) cases out of 51 that were aged 13 years and above with a mean egg count of 19.00 ± 12.38.

Table 5 shows the prevalence and intensity of *S. haematobium* infection among pupils by gender. It was revealed that 27 (12.0%) males out of 224 were infected with a mean egg count of 18.17 ± 8.49 while 12 (5.2%) females out of 192 were infected with a mean egg count of 13.50 ± 5.64.

Table 6 shows the prevalence and intensity of *S. haematobium* infection among pupils by LGAs. The microscopy according to LGAs revealed that Bekwarra LGA had 8 (8%) infected out of 100 with a mean egg count of 26.12 ± 9.12, Obalinku LGA 0 (0%) out of 80 participants, Obudu 3 (3.7%) out of 80 with a mean egg count of 15.07 ± 6.17, Ogoja 27 (27.0%) out of 100 with a mean egg count of 13.00 ± 0.00, and Yala 1 (1.8%) out of 56 with a mean egg count of 11.01 ± 1.00.

4.3: Immunoglobulins (IgM and IgG) Profiles of Study Participants

4.3.1 Immunoglobulins (IgM and IgG) Profiles according to age groups

A Sandwich ELISA serological reaction was carried out on 186 samples (Appendix 16). It was observed that 19(10.2%) pupils had IgM alone, 38(20.4%) had IgG alone while 36(19.3%) had both immunoglobulins.

Table 7 shows the immunoglobulins (IgM and IgG) profiles of study participants according to age groups. It was revealed that out of 10 children aged 4-6 years none (0%) was positive for IgM, 2(0.2%) for IgG and 3(0.3%) for both immunoglobulins. Out of 55 aged 7-9 years, 9(16.3%) were IgM positive, 8(14.5%) IgG positive and 10(18.2%) were positive for both. In the age group 10-12, 7(6.8%)

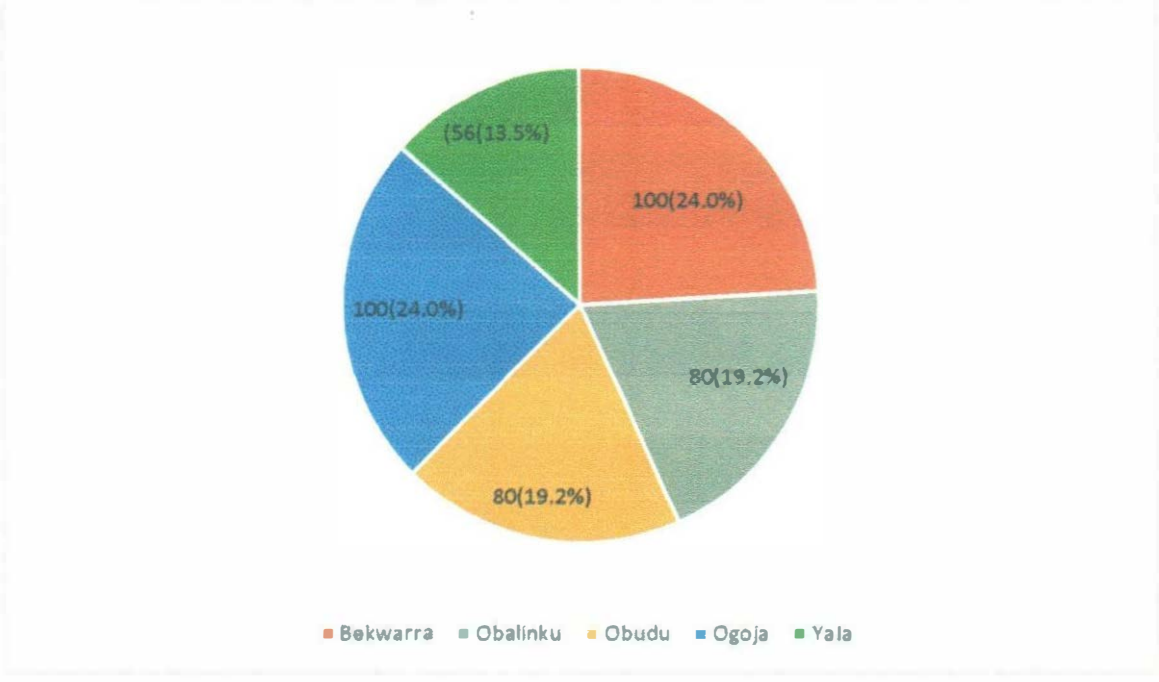

Figure 8: Distribution of the 416 Study Participants According to Local Government Areas (LGAs).

TABLE 3

Distribution of Study Participants According to the parent's Socio-demographic Factors and Medical History

Factor	Category	Number (%)* of Participants
Guardian LoE	Primary	161 (38.7)
	Secondary	192 (46.2)
	Tertiary	63 (15.1)
Occupation	Civil servant	61 (14.7)
	Fishing/Farming	303 (72.8)
	Trader	23 (5.5)
	Driver	18 (4.3)
	Artisan	11 (2.6)
Health Facility	Clinic	8 (1.9)
	P H C	98 (23.6)
	Chemist	310 (74.5)
Last PZQ	Never	23 (5.5)
	≤ 6 months	100 (24.4)
	7 – 12 months	96 (23.1)
	≥ 12months	197 (47.4)

*Percentage based on total number of participants

LoE = Level of education

PZQ = Praziquantel

PHC = Primary Health Care

TABLE 4

Prevalence and Intensity of *S. haematobium* Infection among Pupils by Age

Age Groups	Number (%) Enrolled	Number Positive by Microscopy (%)	Mean Egg Count ±SD
4 -6	18 (4.3)	3 (16.7)	18.33 ± 11.84
7 -9	129 (31.0)	14 (10.8)	16.93 ± 6.28
10 -12	218 (52.4)	15 (6.9)	15.80 ± 7.06
13 - 15	51 (12.3)	7(13.7)	19.00 ± 12.38
Total	416 (100)	39 (9.4)	16.97 ± 8.05
		$\chi^2_3 = 0.407$	$F_{3,35} = 0.266$
		P = 0.334	P = 0.849
		(Not significant)	(Not significant)

TABLE 5

Prevalence and Intensity of *S. haematobium* Infection among Pupils by Gender by Microscopy

Gender	No Enrolled	No Positive by Microscopy (%)	Mean Egg Count ± SD
Male	224	29 (12.9)	18.17 ± 8.49
Female	192	10 (5.2)	13.50 ± 5.64
Total	416	39 (9.4)	16.97 ± 8.05
		$\chi^2_1 = 4.09$ P = 0.031 (Significant)	$F_{1,37} = 2.606$ P = 0.115 (Not significant)

TABLE 6

Prevalence and Intensity of *S. haematobium* Infection among Pupils by LGAs

LGA	No Enrolled	No Positive (%) by Microscopy	Mean egg count ± SD
Bekwarra	100	8 (8.0)	26.13 ± 9.12
Obalinku	80	0 (0.0)	0 (0 ± 0.00)
Obudu	80	3 (3.7)	11.0 1 ± 1.00
Ogoja	100	27 (27.0)	15.07 ± 6.17
Yala	56	1 (1.8)	13.00 ± 0.00
Total	416	39(9.4)	16.97 ± 8.05
		$\chi^2_8 = 58.128$ P = 0.00 Significant	$F_{3,35} = 6.587$ P = 0.001 Significant

TABLE 7

Immunoglobulin M and Immunoglobulin G Profiles of Study Participants by Age

Age groups (years)	No. tested (%)	No Positive for IgM (%) Alone	No Positive for IgG (%) Alone	No Positive for Both (%)
4 – 6	10	0 (0)	2 (0.2)	3 (0.3)
7 – 9	55	9 (16.3)	8 (14.5)	10 (18.2)
10 – 12	102	7 (6.8)	23 (22.5)	16 (15.7)
13 – 15	19	3 (15.8)	5 (26.3)	7 (36.8)
Total	186	19 (10.2)	38 (20.4)	36 (19.3)

$\chi^2_{12} = 16.051$; P value = 0.189; Not significant

TABLE 8

Immunoglobulin M and Immunoglobulin G Profiles of Study Participants by Gender

Gender	No tested (%)	No Positive for IgM (%) alone	No Positive for IgG (%) alone	No Positive for Both (%)
Male	110	11 (10)	28 (25.4)	24 (21.8)
Female	76	12 (15.8)	8 (10.5)	12 (15.8)
Total	186	23 (12.3)	34 (18.3)	36 (19.3)
		$\chi^2_4 = 7.501$;	P value = 0.112;	Not significant

out of 102 had IgM, 23(22.5%) had IgG and 16 both antibodies while for the 19 pupils that were older than 12 years, 3(15.8%) were IgM positive, and 5(26.3%) were IgG positive and 7(36.8%) were positive for both antibodies.

4.3.2 Immunoglobulins (IgM and IgG) Profiles according to gender

Table 8 shows the immunoglobulins (IgM and lgG) profiles of pupils according to gender. Out of males, 11(10.0%), 26(25.4%) and 24(21,8%) were positive for IgM, IgG and both immunoglobulins respectively, while out of 76 female participants, 12(15,8%), 8(10.5%) and 12(15.8%) were positive for IgM, IgG and both Immunoglobulins respectively

4.4 Genetic Variations of *S. haematobium* from Urine of Infected Individuals

4.4.1 Detection of the Genetic Variants

Plate 1 shows the genetic variants of *S. haematobium* from the urine of infected pupils. One hundred urine samples were extracted for molecular analysis and PCR was carried out using microsatellites markers Dra1Sh. Three, two and one band were detected after gel electrophoresis. Twenty-four pupils exhibited one band, 24 two bands, and 20 three bands when visualized against a 100 base pairs (bp) molecular ladder (M). The remaining 32 showed no infection for the targeted region.

4.4.2 Identification of the Different Genetic Variants

Three replicates (A_1, A_2, A_3) of each of the schistosomes variants with identical microsatellites were amplified using the ITSSh genetic marker. Identical bands were observed at 500 bp against the Molecular ladder as shown on Plate 2.

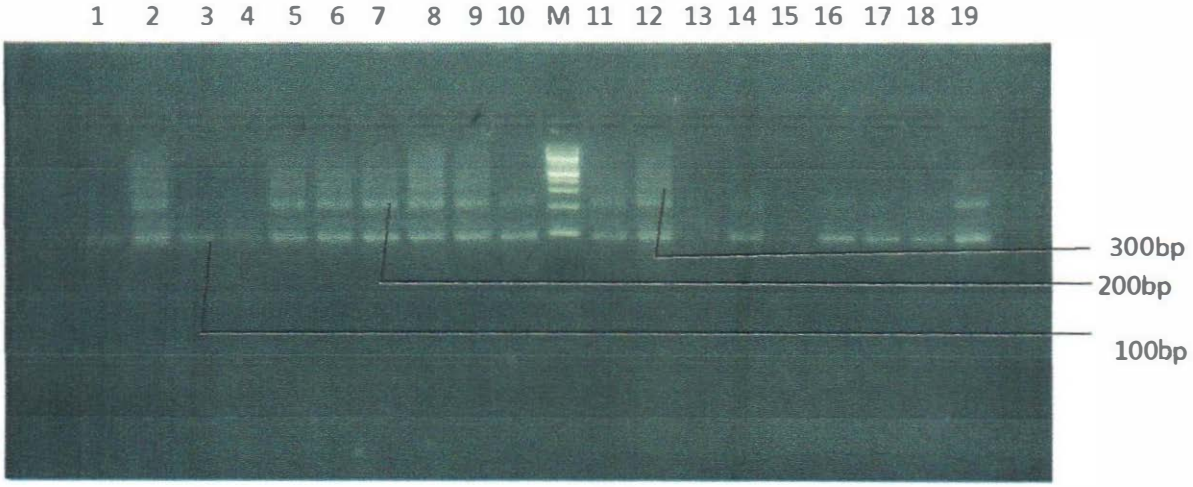

Plate 1: Gel Electrophoresis of Microsatellite isolates. Lane 1,4,13, 15 had 1band of 100bp, Lane 3,10,14,16,17,18,19 had 2bands (200,100bp) Lane 2, 5,6,7,8,9, 11,12 had multiple bands while M represent the molecular ladder.

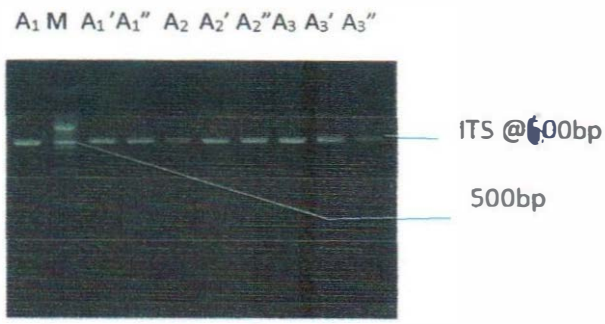

Plate 2: Agarose gel electrophoresis showing the amplified ITS of the *S haematobium* variants, Lanes A_1-A_3 represents the ITS of A_1, A_2, A_3 variants while M represents the 100 bp DNA molecular ladder.

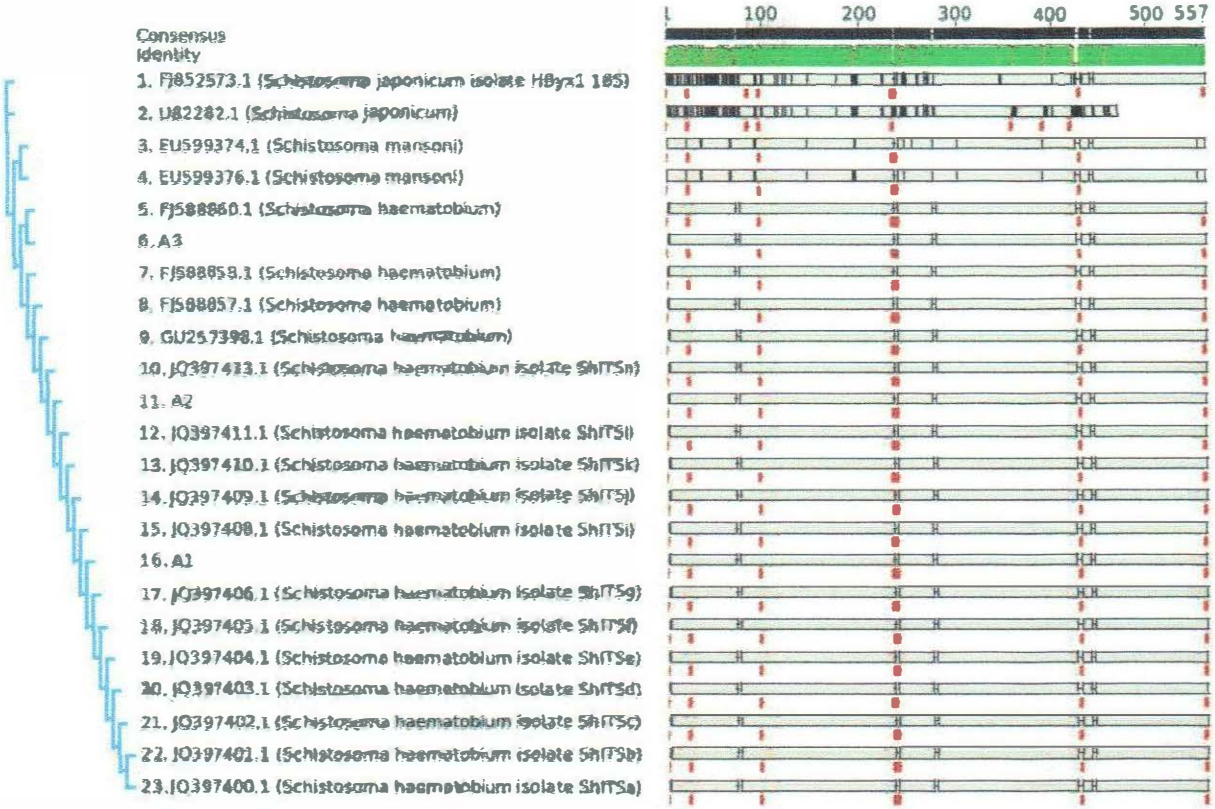

Fig 11: Evolutionary Relationship between the *S. haematobium* Variants and other *Schistosoma* Species

TABLE 9

Geographical Distribution of the Genetic Variants of *S. haematobium* across the Study Area.

Genetic Variants	No Examined	LGA				
		Bekwarra	Obalinku	Obudu	Ogoja	Yala
A1	24	3	3	6	11	1
A2	24	6	3	2	13	0
A3	20	5	2	1	12	0
Total	68	14	8	9	36	1

$\chi^2_{16} = 48.99$; P = 0.00; Significant

TABLE 10

Morbidity Indicators Associated with Different Genetic Variants.

Genetic variants	No Examined	Mean Egg Count ± SD	Haematuria	Proteinuria
A1	24	5.67 ± 8.20	10^a	3^a
A2	24	11.33 ± 13.04	11^a	2^a
A3	20	12.70 ± 9.57	8^b	0^b
Total	68	9.7 ± 10.40	29	5
		$F_{2,65} = 3.112$	$\chi^2_4 = 15.31$	$\chi^2_4 = 32.23$
		P = 0.051	P = 0.04	P = 0.00
		Not	Significant	Significant
		significant		

a and b denote the significance between groups

4.4.3 Characterisation of Different Genetic Variants

The three amplicons (A_1, A_2, A_3) were sequenced. The ITSSh nucleotides arrangement revealed a level of similarity of 99 - 100% with other genera when blasted in the NCBI Gene Bank (Figure 11). The evolutionary distance of the three variants was computed using the Jukes-Cantor method and the placement in the ITSSh phylogenic tree revealed that: A_1 is closely related to *S. haematobium* JQ397406 (ShITSi), A_2 is closely related to *S. haematobium* JQ397413 (ShITSn) while A_3 is closely related to FJ588860 (*S. haematobium*).

Table 9 shows the geographical distribution of the various variants across various LGAs in the study area while Table 10 descibes the morbidity markers associated associated with each genotype.

4.5 *S. haematobium* Larval Stages Microsatellites and Genetic Variants of the *Bulinus* snails

4.5.1 Detection of the Genetic Variants

A total of 100 snails (20 per LGA) were extracted and the Dra I Sh of the ITS was amplified to detect the variants of *S. haematobium* larval stages in the snail hosts. Four (4) variants of the parasites were detected as the agarose gel revealed variants with 1,2,3 and 4 bands (Plate 3).

4.5.2 Characterisation of Different Genetic Variants of the *Bulinus* Snails

The 18s ribosomal RNA was amplified using the ETTS primer. When resolved on agarose, the amplicons revealed a single band at 1200 bp when read against a 1000

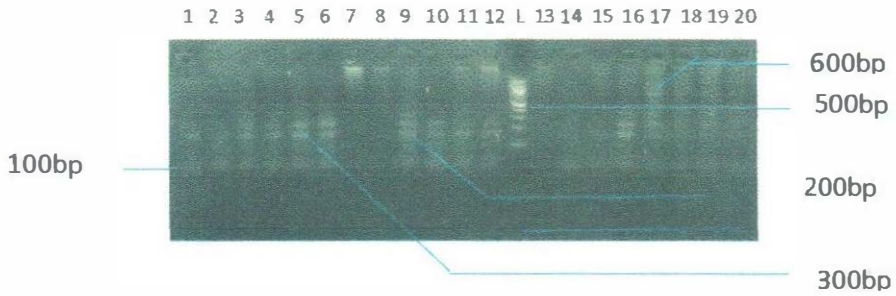

Plate 3: Agarose Gel Electrophoresis Showing the Microsatellites of the *S. haematobium* Cercariae from the Snail Hosts.

TABLE 11

Infection Pattern of the *Bulinus* Snails with *S. haematobium* Across the Study Area.

LGA	No Examined	No infected (%)
Bekwarra	20	11(55.0)
Obalinku	20	2(10.0)
Obudu	20	4(20.0)
Ogoja	20	18(90.0)
Yala	20	1(5.0)
Total	100	36(36.0)

$X^2_4 = 28.72$; P = 0.001 ; Significant.

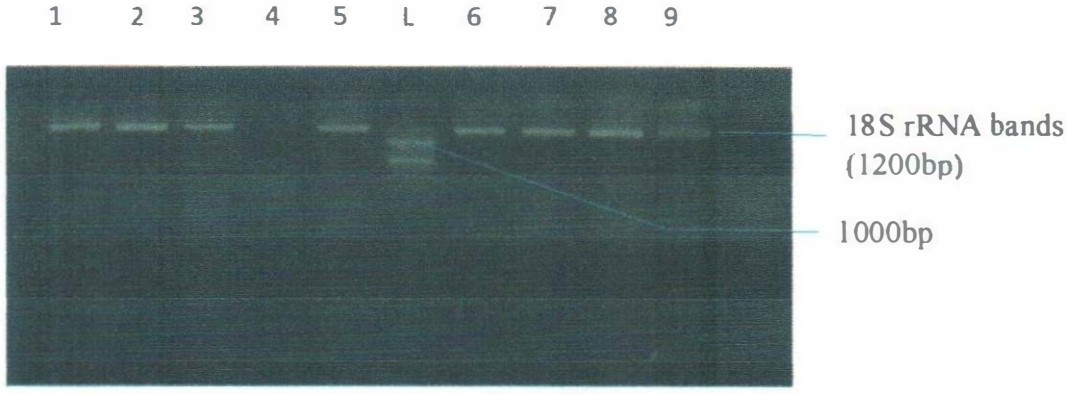

Plate 4: Agarose Gel Electrophoresis showing the Amplified 18S rRNA of the Snail Host. Lanes 1-9 showed the ITS band at 1200bp while lane L represents the 100bp Molecular Ladder.

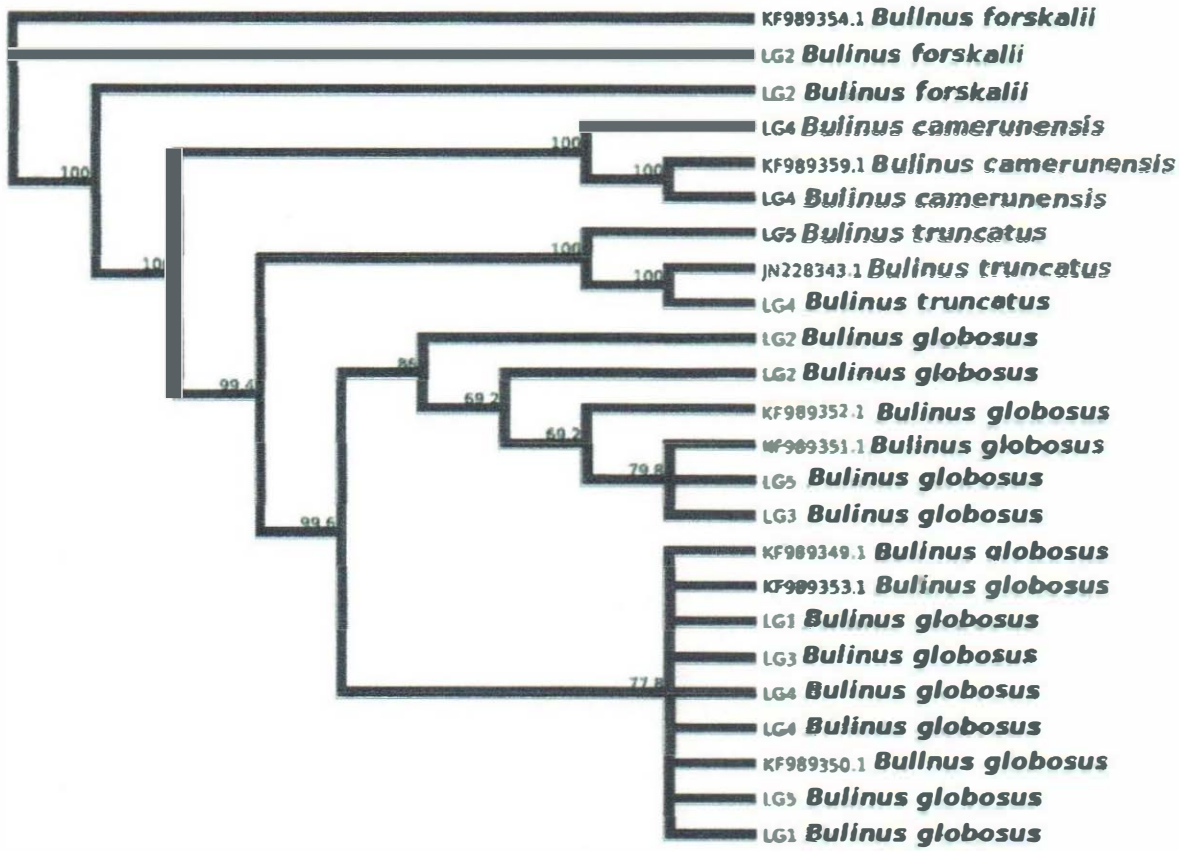

Fig: 12: Evolutionary Relationship Between the Snail's Genotypes and Other *Bulinus* Species.

Key L_1 = Bekwarra

L_2 = Obalinku

L_3 = Obudu

L_4 = Ogoja

L_5 = Yala

bp molecular ladder as seen on Plate 4 . After sequencing, the nucleotides arrangement was blasted in the NCBI Gene Bank. The evolutionary distance of the variants was computed using the Jukes-Cantor method and the placement in the phylogenic tree revealed that: The snails' intermediate hosts of *S. haematobium* in Bekwarra (LG1) are *Bulinus globusus*; The ones in Obalinku (LG2) are *B. globusus* and *B. forskalii;* in Obudu (LG3), only *B. globusus* was found; Ogoja (LG4) was found to harbour *B cameruniensis, B. truncatus* and *B globusus*, while in Yala (LG5) only *B. globusus* was recovered (Figure 12).

4.6: Distribution of *Bulinus* Snails Species per LGA and their Infection Pattern with *S. haematobium*

4.6.1: Distribution of *Bulinus* Snail Species per LGA

A total of 100 *Bulinus* snails (20 per LGA) were characterized. The prevalence of different species per LGA revealed that all twenty in Bekwarra LGA (100%) were *B. globosus.* In Obalinku LGA, 12(60%) were *B. globosus* while 8(40%) were *B. forskalii.* In Obudu LGA, 20(100%) were *B. globosus.* In Ogoja LGA, 10(50%) were *B. globosus*, 6(30%) were *B. cameruensis*, and 4(20%) were *B. truncatus* while in Yala LGA, all the 20(100%) were *B. globosus* (Table 12).

4.6.2: Distribution of *Bulinus* Snail Species by Infection with *S. haematobium*

The distribution of the species of *Bulinus* according to their infection status with *S. haematobium* was done in the study area. Data revealed that 26(31.7%) out of 82 *B. globosus* were infected. For *B. forskalii*, 1(12.5%) out of 8 were infected.

TABLE 12

Distribution of Snail's Genetic Variants per LGA

LGA	No of *Bulinus* Snails Examined	Genotype	Prevalence (%)
Bekwarra	20	*B. globosus*	20(100)
Obalinku	20	*B. globosus*	12(60)
		B. forskalii	8(40)
Obudu	20	*B. globusus*	20(100)
Ogoja	20	*B. globosus*	10(50)
		B. camerunensis	6(30)
		B. truncatus	4(20)
Yala	20	*B. globusus*	20(100)
Total	100		100

TABLE 13

Distribution of *Bulinus* Snail Species by Infection with *S. haematobium.*

Snail Genotypes	No Examined	No Infected (%) with *S. haematobium*
B. globosus	82	26(31.7)
B. forskalii	8	1(12.5)
B. camerunensis	6	6(100)
B. truncatus	4	3(75.0)
Total	100	36(36)
		X^2_3=169.08;
		P= 0.001;
		Significant

For *B. camerunensis*, 6(100%) out of 6 were infected while 3(75%) out of 4 *B. truncatus* were also infected (Table 13).

4.7: Comparison of the Diagnostic Performances of Microscopy, Serology, Haematuria, Proteinuria, Haematuia/Proteinuria against PCR

The diagnostic performance of each tool was calculated using PCR as the gold standard (Wians, 2009). True-positive (TP) were positive for either diagnostic tool, true-negative (TN) were negative for both assays, and false-negative (FN) were positive for PCR alone. A single case of false-positive (FP) was recorded for haematuria while for microscopy and serology, all positive samples were true positive (TP). The sensitivity (TP/TP + FN) was 57.3% for microscopy, 80.9% for serology, 41.8% for haematuria, 6.15 for proteinuria and 11.75 for proteinuria/haematuria. The specificity (TN/TN + FP) was 100% for microscopy and serology respectively, 96.9% for haematuria, 96.5% for proteinuria, and 96.6% for proteinuria/haematuria. The negative predictive value (NPV = TN /TN + FN) was 100% for microscopy and serology respectively, 96.5 for haematuria, 31.4% for proteinuria and 41.9% for proteinuria/haematuria while the positive predictive value (PPV = TP/ TP + FP) was 52.4% for microscopy, 71.1% for serology, 45.1% for haematuria, and 80.0% for proteinuria and proteinuria/haematuria respectively as shown on table 4.8.

TABLE 14
Performances of Diagnostic Methods

Diagnostic Tool	TP	FP	TN	FN	Sens(%)	Spec(%)	NPV(%)	PPV(%)
Microscopy	39	0	32	29	57.3	100	100	52.4
Serology (IgM)	55	0	32	13	80.9	100	100	71.1
Haematuria	28	1	32	39	41.8	96.9	96.5	45.1
Proteinuria	4	1	28	61	6.1	96.5	31.4	80.0
Haematuria/ Proteinuria	4	1	29	30	11.7	96.6	49.1	80.0

Sens: Sensitivity

Spec: Specificity

NPV Negative Predictive value

PPV: Positive Predictive Value

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This study was designed to investigate the genotypes and host variants of *S. haematobium* and the antibody profiles of infected pupils in Northern Cross River State. The biodata, urine and blood samples of 416 pupils aged from 4 to 15 years in some selected primary schools as well as snails from local water bodies, phenotypically presenting with the features of *Bulinus* were collected in Northern Cross River State for the study. A prevalence of urinary schistosomiasis of 9.4% was recorded among the study participants after microscopical examination of the urine samples while haematuria revealed a prevalence of 6.73% and proteinuria 0.96%. Prior to the implementation of this programme, earlier researches in the State reported higher prevalences ranging from 43.5% to 53.8% (Ejezie, 1991; Useh and Ejezie, 1996; Inyang Etoh *et al.*, 2003). The data reported here agrees with those of Emini and Imalele, (2019) who recorded a prevalence of 9.3% in Abini in Cross River State although they are higher than those obtained by Inyang-Etoh *et al.*, (2009) who recorded a prevalence of 0% among Ukwelo-Obudu and 4.5% in Biase school children, and Akpan *et al.*, (2017) who reported a prevalence of 1.6% in Nkarasi and Edor communities of Ikom LGA. The decrease could be ascribed to the Control Programme that has been sustained on primary school's children across the State leading to a post-treatment reduction of disease followed by a progressive rise in the infection rate until a new drug distribution is implemented. A rise and fall pattern in the prevalence is therefore observed over years.

The prevalence of the disease was not significantly (P = 0.334) associated with age. This concur with the findings of Ladan *et al.*, (2011) where no significant association between infection and age was observed in Abarma district of Gusau LGA of Zamfara and those of Babamale *et al.*, (2018) in some selected communities of Kwara State. This might be due to the fact that all study participants were from primary schools and therefore all children are believed to be the highly vulnerable group of individuals (Abed Elhadi *et al.*, 2000). These pupils are of pre to peri-pubertal age, the stage in life that has previously been recorded to have the highest prevalence in some endemic communities (Amuta & Houmsou, 2014; Kabuyaya *et al.*, 2017). Apart from visiting the water bodies routinely for domestic requirements, it is rather common at this age to find children playing in the water bodies as recreational activities thereby increasing their exposure to cercarial penetration.

Males (12.0%) were significantly (P = 0.03) more infected than female (5.2%). Some literatures have highlighted the association between the infection and gender. Abdullahi *et al.*, (2012) described such statistics in 44 LGAs of Kano State. Senghor *et al.*, (2015) observed the same among school children in the district of Niakhar, region of Fatick, Senegal. However, the contrary has been reported among primary school children in Abini (Biase LGA) and school children of Nkarasi and Edor communities of Ikom LGA respectivly (Opara *et al.*, 2003; Akpan *et al.*, 2017). Although the swimming desire and frequency applies to all genders males tend to spend more time in the water bodies as some of them are even involved in fishing.

The intensity of the disease (mean egg count) was 16.97 ± 8.05 and varied between age groups with those aged between 13-15 years being the most affected

(19.00 ± 12.38) and those aged 10-12 having the least intensity (15.80 ± 7.06). However, the difference reported in intensity of infection was not statistically significant (P = 0.849). These findings agree with previous literatures where it was observed that the intensity of the infection is not associated with gender (Obiukwu *et al.*, 2008; Akinboye *et al.*, 2011; Ogbonna *et al.*, 2012). However, it disagrees with Kabiru *et al.*, (2013) and Babamale *et al.*, (2018) who recorded a higher mean egg count significantly influenced by age in Sokoto and Cross River State of Nigeria respectively. The lower severity of the disease in the study area could be associated with the previous PZQ distribution in primary schools, suggesting that many of the positive cases in this study are recent infections in which case the parasite might not have oviposited long enough for the infected individuals to pass out a raised number of eggs in urine. The statistical significance of the intensity of the infection against other variables could have possibly been observed as time goes on.

The intensity of the disease (mean egg count) was also higher in males (18.17 ± 8.49/10 mls of urine) than females (13.50 ± 5.64/10 mls of urine) but was not statistically significant (P = 0.115). A similar observation was made by Akpan *et al.* (2017) and Emini and Edema, (2019) among school aged children of Ikom and Biase LGAs of Cross River State respectively. However, these findings do not agree with Opara *et al.*, (2003) in Obudu and Ladan *et al.*, (2011) in Gusau LGA of Zamfara State where the intensity of the infection was significantly associated with gender. Males tend to have more contact time with the water bodies as their counterparts may be more involved in the household duties and less freedom. It is not inappropriate to link the prolong exposure to multiple infection with different genetic variants of the

parasite. Brouwer *et al* (2001) observed that the genetic diversity in the parasite populations is more predominant among children and that this variability may direct the immune response and the clinical outcome of the disease. Gasmelseed *et al.*, (2014) postulated that the severity of the disease is associated with the genetic variation of the parasite.

The prevalence and intensity of the disease varied each significantly (P = 0.001) with the LGAs with the disease being more prevalent in Ogoja (27%) and not observed by microscopy in Obalinku. The same observation has been documented in previous researches (Adie *et al.*, 2014; Babamale *et al.*, 2018) in some endemic communities of Kwara State. The intensity of the rice farming (and possibly fishing) is higher in Bekwarra and Ogoja LGA due to the altitude that allows for a larger swamp. The same could have been said of Yala LGA but the reduced sample size in that area may not be unconnected to the findings. Another explanation may be that there was a therapeutic failure in Ogoja LGA with a focus of high prevalence probably due to non- compliance or drug resistance or even a high susceptibility of the intermediate/definitive hosts to the variants of the parasite. Ogoja LGA was also found to have more species of the *Bulinus* snails (*B. globosus*, *B. camerunensis* and *B. truncatus*). which could favour a higher transmission rate of *S. haematobium*. Also, *B. camerunensis* that was the most infected specie of snail was found anly in Ogoja LGA. Mazigo *et al.*, (2015) associated other parameters such as awareness, human behaviour, and biological factors which influence the interactions between human and the surrounding ecosystem to the dynamics of the disease.

The molecular analysis of the urine samples revealed three genetic variants of *S. haematobium* (A_1, A_2, and A_3) responsible for infection in the study area, with A_1 occurring in all LGAs. This genotype, as well as A_3 has previously been reported by Webster *et al.*, (2012) as the predominant genotype occurring in thirteen African countries including eight in West Africa among which is Nigeria. Huyse *et al.* (2009) have previously reported existence of the second (A_2) genetic variant as a hybrid of the human and the cattle schistosome occurring in Senegal River Basin (SRB). Van der Werf *et al.*, (2003) observed that *S. haematobium* tends to be less focal, more prevalent, and more widely distributed throughout the continent than other human schistosomes. A significant difference ($P = 0.00$) was observed in the distribution of the three genotypes from infected study participants across the various LGAs in the study area. This discrepancy may not be unconnected to the prevalence of the disease per LGA and could also be due to the sample size per LGA. On the other hand, previous literatures have observed that the climatic changes impact on the parasite-snail systems thereby interfering with their interactions (Paull and Johnson, 2011; McCreesh and Booth, 2014).

The molecular assay of the snails on the other side revealed that in addition to the three genetic variants observed in human, some were infected with a fourth genotype of the parasite. Since this variant was not found in pupils' samples, it is either because humans are not susceptible to it or that it is a non-human parasite. Schistosomiasis is a disease of man and animal and it is not impossible that various genotypes the parasite share the same host. This agrees with Dabo *et al.*, (1997) who observed that multiple genotypes of *S. haematobium* can be found in the same *Bulinus*

species of snails. The overlapping contact sites caused by the movements of the intermediate and the definitive hosts of the parasite might be responsible for the co-existence of many genotypes in a given area, and the genetic interactions between resident and in-coming strains can give birth to new genotypes (Brouwer *et al.*, 2001; Quan *et al.*, 2015).

The morbidity indicators of the disease were associated with the genetic variations of the parasite and were reflected by haematuria (P = 0.04) and proteinuria (P = 0.001). These findings support the belief that the variance within parasite population influences the immune response and the clinical outcome of the disease (Brouwer *et al.*, 2001) and that although the species of *S. haematobium* are considered to be morphologically identical, some genetic variations of the parasite may direct some phenotypic expressions such as infectivity, virulence or fecundity and drug susceptibility (Imbert-Establet and Combes, 1986; Sire *et al.*, 1999; Curtis *etal.*, 2001; Gyseels *et al.*, 2006). It however disagrees with Gasmelseed *et al.*, (2014) who observed that the genetic diversity of *S. haematobium* is not associated with the severity of the disease. This study was carried out after a mass chemotherapy. The adaptation of the parasite to re-infection may not be unconnected to a lower severity of the disease (egg count) as observed this study in which case, a forecast on the phenotypic expression of the parasite with regards to their pathologies and disease outcome may not convincingly be supported at this time.

Another objective of this study was to determine the biodiversity of the *Bulinus* snails responsible for infection in the study area. Molecular studies identified *B. forskalii; B cameruniensis, B. truncatus* and *Bulinus globusus*. These species have

earlier been reported in three endemic countries in Africa (Cameroon, Senegal and Egypt) and *Bulinus* species with lower genetic diversity are predicted to have higher infection prevalence than those with greater diversity in host susceptibility (Zein-Eddine, *et al.*, 2014). *B. globusus* was the most prevalent snail (82%) specie in our study in the Northern Cross River State occurring in all the LGAs while the least occurring specie was *B. truncatus* found only in Ogoja LGA. The large discrepancy in the prevalence of these resident species may not be unconnected to the different ecosystems found in the senatorial zone. The highlands of Obudu and Obalinku on one side and the valleys around Bekwarra and Neibouring LGAs where there are pools of water that last for longer periods and creating swamps that are convenient microhabitats for the aquatic snails. Dagal *et al.*, (1986) observed that high temperatures have been associated with increased snail mortality. This was recently supported by Kalinda *et al.*, (2018) who proved that variations in temperature and others ecological factors directly interfere with the snail populations and that an increase in cercariae production at transmission sites may increase the risks of *Schistosoma* transmission. *B. globusus* has been described in the country earlier, in the neighboring Nassarawa State by Abe, (2015) and in Ogun State, South-western Nigeria as the specie representing more than half (17/30) of the snail population (Akinwale *et al.*, 2015) and having a paraphyletic relationship with *B. globosus* species from East Africa (Abe *et al.*, 2018). Kane *et al.*, (2008) reported the division between *B. globosus* from the two regions in Africa. Mkize *et al.*, (2016) reported this specie in South Africa with a moderate genetic diversity within populations using microsatellites. Knowledge of the genetic diversity of schistosome as well as snail

populations has been shown to play a key role in the monitoring and surveillance of disease, and the implementation of new molecular-based approaches will be of help to assess the impact of schistosomiasis control strategies (Rollinson, 2009). The phylogenetic relationship of *B. globosus* from Nasarawa State in Nigeria clusters with *B. globosus* sequence data from other West African countries such as Burkina Faso, Senegal and Niger forming a monophyletic lineage, but instead forms *africanus* has a close affinity with West African *B. globosus* species. The presence of other species reported in this study have been described in other geographic African region such as Egypt with *B truncatus* (Zein-Eddine, *et al.*, 2017). Phylogenetic inferences in a previous literature has clustered B. *forskalii*, *B. camerunensis* and *B. senegalensis* into the *B. forskalii* complex as the ITSl and ITS2 fragments failed to make a clear distinction between the three species (Brown's (1994). In addition to this, Zein-Eddine *et al.* (2014) also noted that ITS region was unable to separate clearly between *B. globosus* and *B. umbilicatus* in the *B. africanus* group and suggested that analyses of mitochondrial genes were more suitable for identification of *Bulinus* species. The presence of *B. truncatus* in the southern African country can be attributed to the favourable environmental factors and migration of snail population (Abe *et al.*,2018). Human activities have also increased the number of snail hosts which is of concern calling for the need to improve measures for effective snail control strategies. The presence and distribution of various *Bulinus* species in the studied areas poses a threat to the inhabitants who are engaged in activities involving water either for fishing, swimming or fetching for domestic chore (Abe *et al.*, 2018). The presence of other species reported in this study have been described in other geographic African region

such as Egypt with *B truncatus* (Zein-Eddine, *et al.*, 2017). The presence of *B. truncatus* in the southern African country can be attributed to the favourable environmental factors and migration of snail population (Abe *et al.*,2018).

The most infected snail specie with *S. haematobium* was *B. camerunensis* (100%). A previous experimental infection of *Bulinus* snails from eight different sources including the resident specie *B. africanus* with miracidia hatched from the eggs from the urine of infected school-children from the Nelspruit district of South Africa revealed has shown various degree of susceptibility (Joubert *et al.*, (1990). This specie, which was reported only in Ogoja LGA probably had conducive environmental conditions that enhanced its interactions with the resident strains of *S. haermatobium*. Human activities have also increased the number of snail hosts which is of concern calling for the need to improve measures for effective snail control strategies. Rollinson *et al.*, (2014) recommended a global commitment for the elimination of schistosomiasis in endemic countries, with emphasis on the genomic status of snail hosts in view to establish a reliable comprehensive genome identification database that is lacking across Africa.

The sensitivities of the different techniques were microscopy (57.3%), IgM Serology (80.9%) and haematuria (41.8%). While the two first were highly specific (100%), the latter was not (Table 14). These findings do not differ much from those of Ibironke *et al.*, (2012) who recorded sensitivities of 87%, 70%, and 100% respectively using the same techniques and primers. It agrees with Pontes *et al.*, (2002) who observed that in areas of endemicity, *Schistosoma*-specific PCR performed on DNA isolated from a small amount of sample is more sensitive than microscopic

examination. Theoretically, PCR is expected be more sensitive and specific as it is an upgrade to the other techniques. It is believed that molecular biology was considered to enhance the identification and characterization of the parasites and became progressively established and recognised as a diagnostic tool in parasitology (Jardim *et al.*, 2006). However, a contamination or an ongoing excretion of dead DNA might contribute to raising positive values and as such, clinical evaluation and laboratory findings should be put together for a meaningful and reliable interpretation of PCR (Vrweij, 2014). Clerinx and Van (2011) postulated that this method can be reliable only from six weeks after exposure since the symptoms generally appear only thereafter (Bottieau *et al.*, 2006; Jaureguyberry *et al.*, 2010) and the routine microscopy of the urine sample, which is considered as the standard shows less sensitivity.

The serology proved to be more sensitive than the microscopy and data recorded in this study were higher than that of Kinkel *et al.*, (2012) who observed that the sensitivity of serological tests ranges from 41% to 78%. It supported previous literatures that have suggested that the antibody production is generally between four to eight weeks after infection although it may be delayed in some cases (Logan *et al.*, 2013; Martinez-Calle *et al.*, 2013); This could be due to the fact that seroconversion could occur before the egg production and that the study was carried out in an endemic region where the residual antibodies from a previous infection may still be in circulation. Because the study area is an endemic region to the disease, it may be possible that some of the positive reactions are due to an infection that has been treated before. Tsang & Wilkins, (1997) suggested that the presence of antibodies is indicative

of schistosomiasis at some time and cannot be correlated with clinical status, worm burden, egg count and prognosis. It is believed that a positive result may persist for years and as such not differentiating between a current and a previous infection (Young *et al.*, 2010).

IgG was of no diagnostic significance in this study. Although a recent study has revealed the early involvement of natural IgG in infection, and its contribution to pathogen clearance (Panda *et al.*, 2013), the involvement of this antibody is minimal in primary exposures and the presence of the immunoglobulin is not necessarily induced by the current sickness.

5.2 Conclusion

Infection with *S. haematobium* among pupils in Northern Cross River State had an overall prevalence of 9.4% and was not influenced by age ($P = 0.334$) but with gender ($P = 0.031$) and LGA of residence ($P = 0.00$). The intensity of the disease, unlike in previous literatures was low with a mean egg count of 16.97 ± 8.05.

No significant difference was observed in the antibodies profiles (IgG and IgM) of the study participants. The microsatellite marker (Dra1Sh) revealed that there are three genotypes of the parasite population within the human host while the snail population harboured a fourth genetic variant not detected in the infected pupils. Human infection with each susceptible genotype varied significantly ($P = 0.001$) from one LGA to another. Symptoms of haematuria and proteinuria were significantly associated with the genetic variants of the parasite. Four genotypes of *Bulinus* snails serve as vector of *S. haematobium* in Northern Cross River State, namely *B. truncatus*, *B. forksalii*, *B. globusus* and *B. cameruniensis*. *B globosus* is the most prevalent (82%)

- Further malacological surveys should be carried out to identify the susceptible and resistant snail species to the parasite. If these resistant strains are introduced in endemic areas, they can help in reducing the cercarial population by competing with the resident strains in which the parasite continues its life cycle.

5.4 Contribution to Knowledge

The following contributions were made:

(1) This study has unraveled three genotypes (namely A_1, A_2, and A_3) of *S. haematobium* causing urinary schistosomiasis in Northern CRS. A_1 is closely related to *S. haematobium* JQ397406 (ShITSi), A_2 is closely related to *S. haematobium* JQ397413 (ShITSn) while A_3 is closely related to FJ588860 (*S. haematobium*) as blasted against the known variants in the NCBI.

(2) The phylogenies of *Bulinus* snails in Northern Cross River State were traced to *B. globosus*, *B. forskalii*, *B. camerunensis*, and *B. truncatus*. *B. globosus* was the most prevalent snail (82%) and available in all LGA of the study area while *B camerunensis* was the most infected (100%) with S. *haematobium*.

(3) A significant difference was observed in the distribution of snails infected with the larval stages of *S. haematobium* across the study area

(4) The intensity of the disease (egg count) in the study area was not associated with the genetic variants of the parasites.

(5) The immune profiles of pupils against *S. haematobium* in the senatorial zone was found not to be associated with age and gender.

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APPENDICES

Appendix 1: Ethical approval from Cross River State Ministry of Health

GOVERNMENT OF CROSS RIVER STATE OF NIGERIA
MINISTRY OF HEALTH, CALABAR
HEALTH RESEARCH ETHICS COMMITTEE
E-mail: crsmohresearchethics@yahoo.com
+234 08034047926

CRS/MH/HREC/016/Vol V/058 21st November, 2016

Ajioyep, Noundo Armand Claude
University of Calabar
Calabar

CERTIFICATE OF ETHICAL APPROVAL

The Cross River State Health Research Ethics Committee (CRS-HREC) having reviewed your application for Ethical Approval of the Research titled "Toll-like Receptors Gene Polymorphism Associated with Urinary Schistosomiasis in Pupils of Northern Cross River State, Nigeria" with REC No. RP/REC/2016/425 has granted FULL ETHICAL APPROVAL.

This approval is valid for ONE YEAR from the date of its issuance.

You may proceed with your study in accordance with the protocol. You are requested to abide by every professional and ethical code for the conduct of this research including advising the CRS-HREC of any changes to your protocol in advance.

The CR-HREC reserves the right to request an audit of this research at any time during or post implementation. A copy of the completed research (Results) should be submitted to the Department of Clinical Governance, SERVICOM and E-Health for policy and decision making in the State Ministry of Health.

Yours sincerely,

Dr. Bassey Ikpeme
Ag. Chairman CR-HREC

Appendix 2: Questionnaire

Questionnaire

(For Disease Outcome)

Community Name .....

Community Code .....
Name of the closest water stream/river .....

Serial number .....

Name of ESP .....

Age: 4 – 6[ ], 7 - 9 [ ], 10 – 12[ ], 13 and above [ ]

Sex: Male [ ]

Parent's Occupation Civil Servant [ ], Farmer / fishing [ ], Trader [ ], Artisans [ ] Driver [ ]

Guardian level of education Primary [ ], Secondary [ ], Tertiary [ ]

Do you swim in or bathe in the river Yes [ ], No [ ], not sure [ ]

Health facility Clinic [ ], Primary health Care [ ], Chemist [ ]

How long have you been in the community? .....

When last was Praziquantel distributed?

Do you know what is schistosomiasis? Yes [ ], No [ ]

What is the local name ?.....

How do you think it is contacted?.....

What symptoms /signs make you believe you have it?.....

Do you experience any of these symptoms?.....

- Abdominal painYes [ ], No [ ]
- Cloudy urineYes [ ], No [ ]
- Blood stained urineYes [ ], No [ ]
- Painful urination Yes [ ], No [ ]

Screening Result:

Proteinuria (Using the strip) .....

Microscopy: .....

- RBCs .....
- Ova of *S. haematobium* ... ...

Appendix 3: Preparation of buffer for the preservation of urine deposits

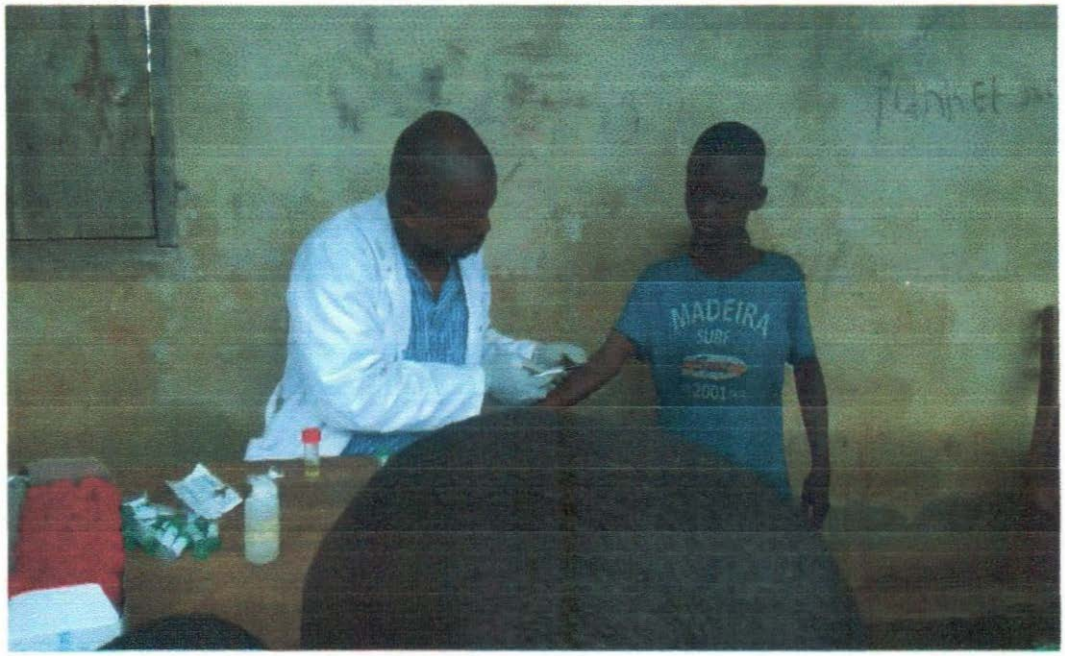
Ingredients

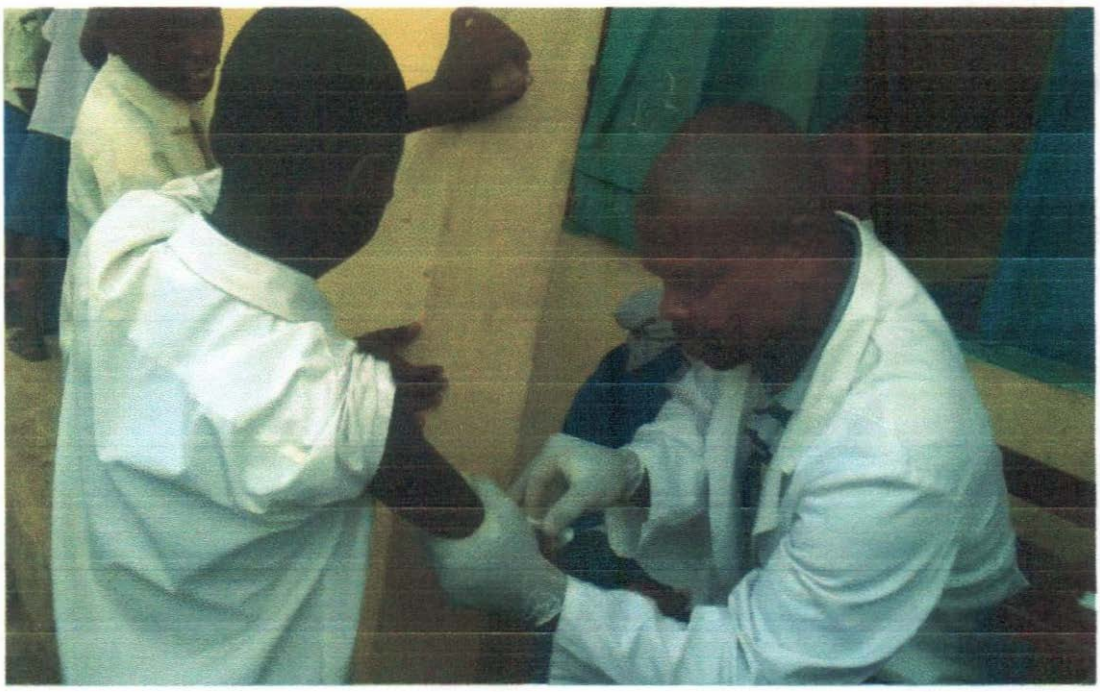
NaCl	8g
Na2HPO4	1.42g
KH2PO4	0.24g
Distilled water	1l

Preparation:

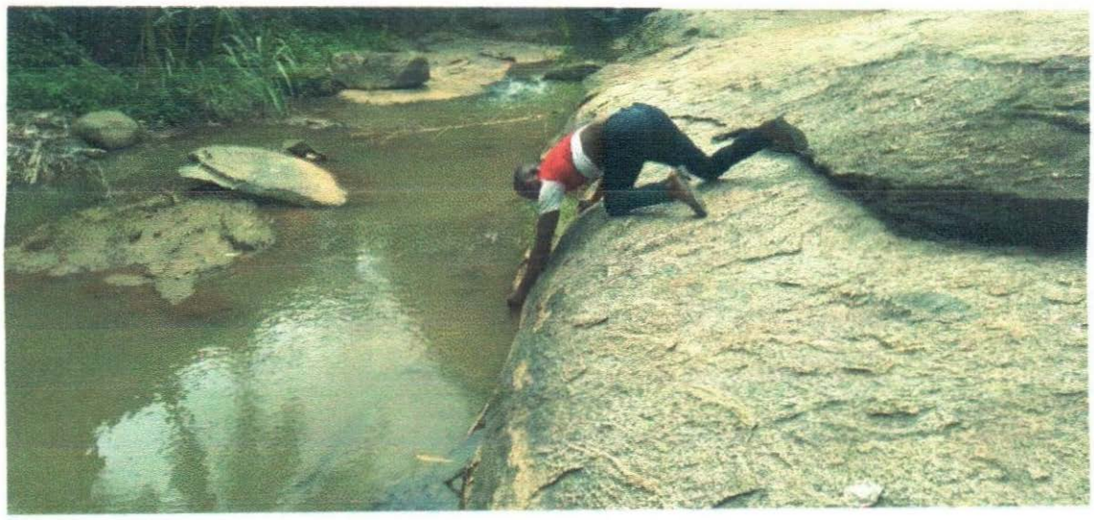
Weigh the salts and dissolve in 1l of distilled water.

Appendix 4: Collection of Urine and blood samples at schools

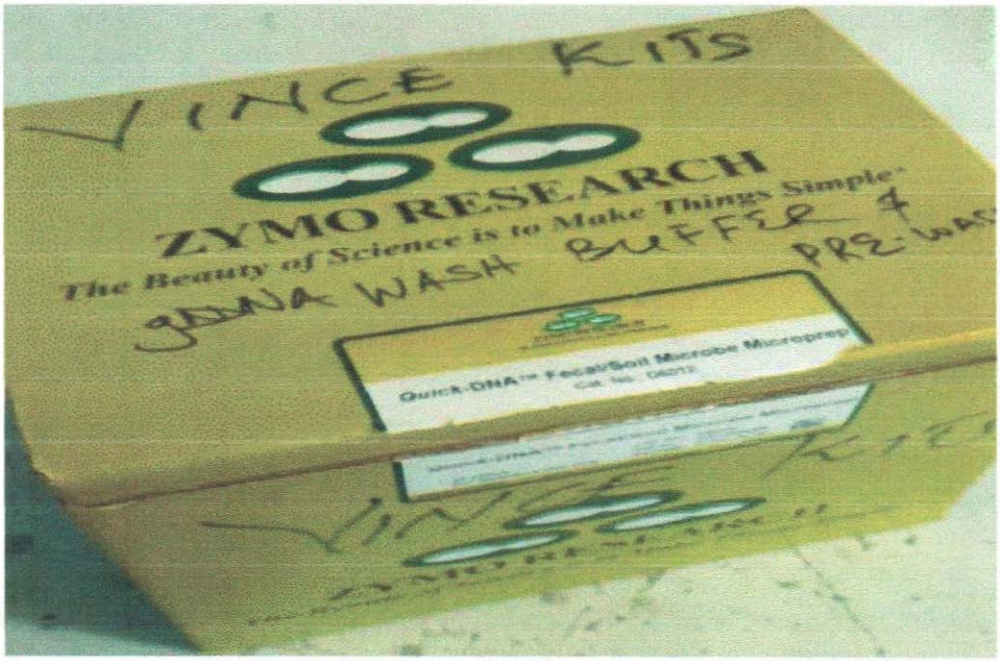




Appendix 5: Field Sampling of snails



Appendix 7: Extraction kits



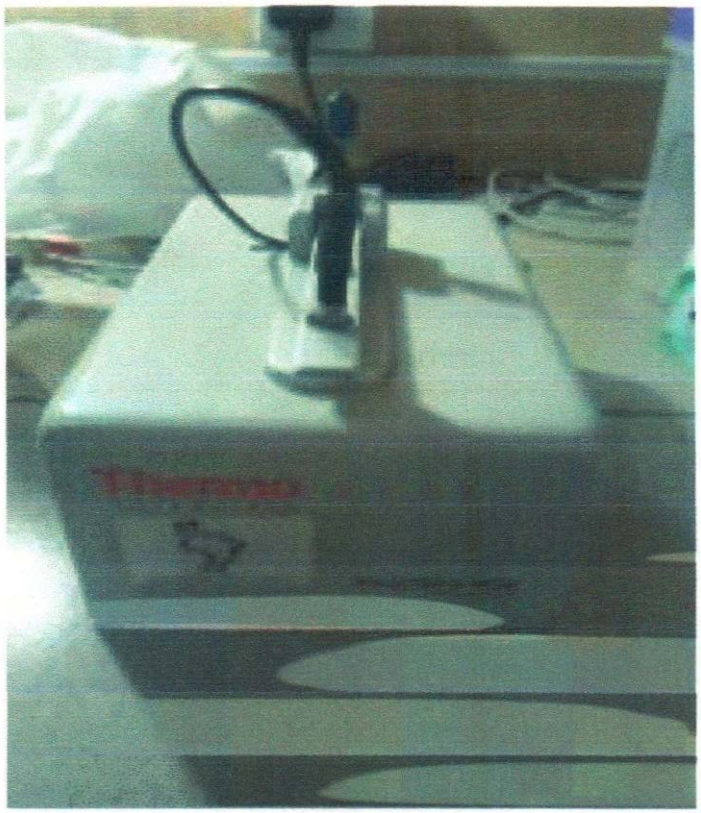
Appendix 8: Calculations for DraSh1 PCR products

Samples	Per component	Stock concentration	Per concentration	Per volume(µl)	Number of samples	Cocktail	Per conditions
	Master mix	2X	1X	15		225	
	Forward Primer	25	0.4	0.48		7.2	**ID: 95° C - 5'**
Urine deposits	**Reverse Primer**	25	0.4	0.48	15	7.2	**D: 95° C – 1'**
							A: 53°C1'30''
	Template			6			**IE: 72° C – 1'**
	Water			8.04		120.6	**FE: 60° C – 5'**
	Final Volume			30			

Appendix 10: Calculations for ETTSh PCR products

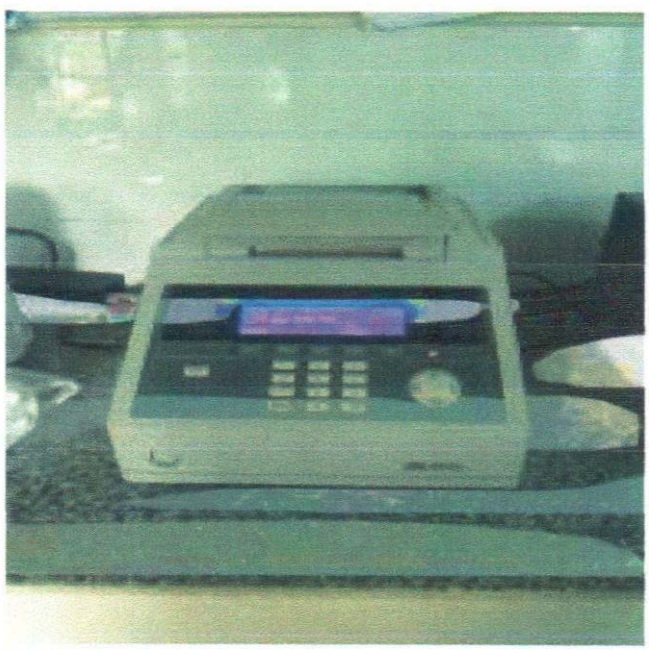
Samples	Pcr component	Stock concentration	Pcr concentration	Pcr volume(µl)	Number of samples	Cocktail	Pcr conditions
	Master mix	2X	1X	12.5		212.5	**Touch-down PCR**
Snail (Foot part)	**Forward Primer**	25	0.5	0.5		8.5	**ID 94°C – 5'**
	Reverse Primer	25	0.5	0.5	17	8.5	**FD 94°C – 40"**
	Template			1			**Annealing 51°C decreased by 0.5°C – 48°C for 35 cycles**
	Water			9.5		161.5	**IE 72°C – 45"**
	Final Volume			25			**FE 72°C – 5'**

Appendix 11: Scientific Nano-drop



Appendix 12: Thermocyclers

1- BioRAD



2- Biosystems

Appendix 13: Agarose of gel electrophoretic tank

Appendix 14: Trans-illuminator

Appendix 15: Microtitre plate after Sandwich ELISA test to *S. haematobium* IgG antibodies.