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COLLEGE OF HEALTH SCIENCES

GENETIC VARIATIONS IN SCHISTOSOMA HAEMATOBIUM, DISEASE SEVERITY AND DRUG RESISTANCE/RE-INFECTION IN THE PRU DISTRICT OF GHANA

BY

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DECLARATION

I hereby declare that except for references to other people’s work, which I have duly acknowledged, this work is a result of my own research under the supervision of Dr. Patience Borkor Tetteh-Quarcoo and Rev. Prof. Patrick Ferdinand Ayeh-Kumi, both of the Department of Medical Microbiology, College of Health Sciences, University of Ghana. This work neither in whole nor in part had been submitted for another degree elsewhere.

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DEDICATION

This work is dedicated to my family, friends and the whole research fraternity.
ACKNOWLEDGEMENTS

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ABSTRACT

Background: Schistosomiasis is a neglected tropical disease caused by the genus Schistosoma. It has a worldwide distribution with more cases occurring in Africa. Urogenital schistosomiasis caused by Schistosoma haematobium is prevalent in several places within Ghana, particularly in areas where there are large water bodies. Persistent infection of S. haematobium leads to cytological changes of urothelial cells which in turn could lead to the development of bladder cancer. The persistence of this infection can be as a result of multiple re-infection or therapeutic failure to the drug of choice, Praziquantel (PZQ), and this has been reported in places like Egypt and Mali, but not much have been reported about the trends in Ghana. Genetic variation in schistosomes might influence disease severity and development of drug resistance; therefore, there is the need to investigate the situation in the Pru district of Ghana.

General Aim: The aim of this study was to investigate possible genetic variations in S. haematobium, assess disease severity and drug resistance/re-infection in the Pru district of Ghana which had over 50% prevalence in the year 2010, as reported by the world health organization.

Methodology: This was a longitudinal study involving baseline and follow-up sampling among basic school children living in schistosomiasis endemic communities, within the Pru district of the Brong Ahafo Region of Ghana. Urine samples were collected at baseline with consent/assent from the parents or guardians/children, and examined for S. haematobium ova by microscopy. Egg count and viability test (modified hatchability technique and vital stains) were used for assessment of resistance/re-infection, while urine cytology was used for disease severity. Children with positive S. haematobium eggs were treated with a single oral dose (40mg/kg) of PZQ after which egg count, viability test as well as cytological analyses were repeated weekly for comparison with baseline. Disease severity was assessed by identification of cytological changes (squamous cell metaplasia, inflammation and hyperkeratosis) in both baseline and post treatment urine samples. Molecular analysis involved PCR amplifications and visualization (on agarose gel) of chromosomal gene (ITS2), for confirmation of S. haematobium and the detection of possible genetic variants in the mitochondrial genes (NAD1 and COX1).

Results: A low prevalence (6.5%) of S. haematobium infection was observed in this study with a re-infection rate of 19.2%, but no drug resistance was detected after complementing results from egg count and viability tests. Cytological abnormalities such as squamous cell metaplasia, hyperkeratosis and inflammation were found with most of the S. haematobium samples, which reduced with the weekly follow up examinations. Disease severity recorded in the current study was 26.7%. All the cases identified by microscopy got amplified with S. haematobium species specific DNA marker (ITS2), however, some were found to vary in their ability to express the mitochondrial genes; NAD1, and or COX1.

Conclusion: Severe form of S. haematobium disease has been observed in the Pru District of Ghana with re-infection recorded among the study participants as well as variants of the mitochondrial genes, but no records of PZQ resistance. This finding will therefore add to knowledge on genetic variations in S. haematobium, disease severity and drug resistance or re-infection in Ghana. Similar studies at other locations in Ghana are therefore recommended.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Urogenital schistosomiasis is a parasitic disease caused by *Schistosoma haematobium*. It is prevalent in several parts of the world with most cases occurring in Sub-Saharan Africa, particularly, areas where there are large water bodies such as rivers, lakes and streams. In Ghana, *S. haematobium* is the predominant human schistosome species and it is widely distributed in the country (Onori *et al*., 1963; McCullough and Ali, 1965).

The disease manifests itself mainly as passage of blood in urine. In endemic communities in Ghana (even in the capital city), there is high prevalence of haematuria among school age children (Tetteh-Quarcoo *et al*., 2013). Other signs of *S. haematobium* infection include dysuria and proteinuria. The adult *S. haematobium* is found in the venous plexuses around the urinary bladder and the eggs the female release, passes through the wall of the bladder causing haematuria and fibrosis of the bladder; the bladder becomes calcified, and there is increased pressure on the ureters and the kidneys (Gower *et al*., 2011).

Disease severity refers to the extent of damage to cells, tissues and or organs as a result of an etiological agent marked with characteristic set of signs and symptoms. Disease caused by *S. haematobium* varies from mild to persistent urinary schistosomiasis which induces inflammation of the bladder that could develop into fibrosis and malignancy (Mostafa *et al*., 1999). Studies
have shown that persistent and chronic infection leads to the development of bladder cancer, mostly squamous cell type bladder cancer (Akinwale et al., 2008; Sacko et al., 2011).

Currently, the drug of choice for the treatment of schistosomiasis is Praziquantel (Midzi et al., 2008; Barakat and El Morshedy, 2011). It still remains the core of the current strategy against schistosomiasis control and it is highly effective against all the five schistosome species that infect humans (Seto et al., 2011). The safety and efficacy of Praziquantel have ensured its widespread usage. It is recommended that school aged children and high-risk groups of adults in communities with prevalence of between 10% and 50% use it once every two years. In communities with above 50% prevalence rate, both children and adults are required to be treated once a year (WHO, 2006). This drug of choice has been used extensively and successfully in many national control programmes. However, there are some reported cases of clinical relevant resistance developing (McManus and Loukas, 2008). Laboratory studies have shown that activities of Praziquantel are stage dependent with the drug acting principally against the adult worm stages, whilst immature schistosomes are less susceptible (Sabah et al., 1986).

Genetic variation among schistosomes is believed to have influence on their behavior and certain characteristics including; host parasite interaction, virulence and pathogenesis as well as susceptibility to drugs (Gasmelseed et al., 2014; Afifi et al., 2016). Genetic diversity of schistosomes can be affected by various factors such as different environmental conditions and interaction with both human host and snail intermediate host (Norton et al., 2010; Gower et al., 2011).

Most genetic studies have analyzed the mitochondrial NADH dehydrogenase 1 (nad1) and cytochrome sub unit gene (cox1) of schistosoma species, leading to geographical separation of S.
*mansoni* isolates into five lineages (Weber *et al.*, 1967; Morgan *et al.*, 2005; Rollinson *et al.*, 2009). However, cox1 analysis of *S. haematobium* revealed low levels of genetic diversity with two phylogenetic groups (Weber *et al.*, 1967). Internal transcribed spacer gene (ITS1 and ITS2) of schistosomes has also been demonstrated as a marker for genetic variation studies, with ITS specific for *S. haematobium* (Barber *et al.*, 2000; Quan *et al.*, 2015). These techniques could still be very helpful in investigating possible genetic variation in schistosome populations, which the current study sought to achieve.

1.2 Problem Statement

Over a decade ago, an estimated number of above 200 million people were reported to have been suffering from schistosomiasis worldwide, with about 85% of the reported cases occurring in sub-Saharan Africa (Van der Werf *et al.*, 2003; Southgate *et al.*, 2005). *Schistosoma haematobium* which causes urogenital schistosomiasis has been identified to be the most common human schistosome species in Ghana, and it can be found in many parts of the country (Onori *et al.*, 1963; McCullough and Ali, 1965). In the wake of the need to eradicate urogenital Schistosomiasis, various researchers have sought to bring to light the impact of urinary schistosomiasis on various age groups (Tetteh-Quarcoo *et al.*, 2013; Der *et al.*, 2015). In light of that, there has been studies regarding the impact of *S. haematobium* infection on the urinary tract pathology in Accra and elsewhere (Der *et al.*, 2015; Muscheck *et al.*, 2000), but not much can be said about the middle belt of Ghana.
Also, there have been reported cases of therapeutic failure of Praziquantel use in the treatment of schistosomiasis in Mali and Nigeria but there is no idea about such a situation in the Pru district of Ghana (Midzi et al., 2008; Barakat and El Morshedy, 2011).

Genetic variation of *S. haematobium* and *S. mansoni* have been established in Egypt, Mali and Senegal (Ezeh et al., 2015; Afifi et al., 2016), but there is limited information about what the situation is in Ghana, especially within the middle belt of the country where the Pru district is located. Genetic variation could lead to changes in the level of gene expression, host parasite interactions, disease transmission, evolution of new strains and possibly development of drug resistance, therefore there is the need for genetic variation studies at various locations (Muscheck et al., 2000; Gasmelseed et al., 2014).

### 1.3 Justification

Gaining enough knowledge about the effect of *S. haematobium* on the urothelial cells will provide information about cytological changes in the urine of children for better prognosis of disease caused by the parasite.

The use of Praziquantel as a mono therapy in the treatment of schistosomiasis and other helminthic diseases has called for continuous monitoring for possible development of resistance or re-infection. This study will provide information about the use of Praziquantel in the chosen study area with regard to resistance development.

Genetic variation studies of *S. haematobium* are limited as compared to *S. mansoni*. With high prevalence of *S. haematobium* in Ghana, there is the need to give more attention to genetic variation studies to know if there are variations or not and possible influence on disease severity.
and development of drug resistance. It will also provide useful information for better diagnosis, development of vaccines and other therapeutic control measures to enhance effective monitoring and prioritization of resources on prevention and control measures.

1.4 Aim

The aim of this study is to investigate possible genetic variations in *S. haematobium*, disease severity and drug resistance or re-infection in the Pru district of Ghana.

1.5 Specific Objectives

The specific objectives of the study are:

- To determine whether there are genetic variations in *S. haematobium* in the Pru district of Ghana.
- To assess cytological abnormalities and disease severity caused by *S. haematobium*.
- To determine if there is possible resistance of *S. haematobium* to Praziquantel or re-infection.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Schistosomiasis

Schistosomiasis is a parasitic disease caused by the genus *Schistosoma*. Schistosomes are blood dwelling parasitic flat worms, belonging to the family Schistosomatidae; order, digenea; class, Trematoda; and phylum, Platyhelminths. There are many species under the genus *Schistosoma* but only five are implicated in causing human schistosomiasis and these are *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. Intercalatum*, and *S. Mekongi* (WHO, 2010). *Schistosoma* species differ in their final location in the human host, the species of the snail intermediate host they use in their life cycle, the pathology they induce, as well as the number, size and shape of the eggs they produce and the position of the characteristic spine on the egg.

The parasite was first discovered in Egypt in the year 1851 by a German physician called Theodore Bilharz, which is why schistosomiasis is also called Bilharziasis (Nawal, 2010). After malaria, schistosomiasis is the second most devastating disease in the sub Saharan Africa with over 112 million people considered as at risk for infection (WHO, 2013a). Schistosomiasis occurs mostly in rural communities with water bodies such as Lakes, ponds, and Streams, but over the years, urban schistosomiasis is an increasing problem due to construction of man-made reservoirs, irrigation systems as well as rural urban migration and population growth (Mott *et al.*, 1990; Gryseels *et al.*, 2006). The risk groups of *Schistosoma* infection include occupational groups such as fishermen, farmers, irrigation workers, women and children that use water for domestic purposes and recreation (WHO, 2010). Besides the risk factor for the infection, other
predisposing conditions for schistosomiasis include; genetic conditions, house hold clustering, climate change, immune response of the host, and other infections such as hepatitis and HIV (Bethony et al., 2001; Quinell, 2003)

The most predominant schistosome in Africa is S. haematobium (Onori et al., 1963), which is the parasite of interest in this study, is the agent that causes urogenital schistosomiasis. Urogenital schistosomiasis is characterized with bloody urine, difficulty and painful urination. Chronic infection with S. haematobium could lead to frequent inflammation of the bladder, urothelial calcifications that could possibly lead to the development of bladder cancer. Urinary tract pathology is as a result of egg induced inflammatory response to the deposition of eggs trapped in the tissues of the walls of the bladder (Corachan, 2002).

2.2 Biology and life cycle of S. haematobium

The Adult male and female S. haematobium are found in the venous plexuses of the bladder where the female lays per day about 30 eggs, which are deposited in the inner walls or in the tissues of the bladder. The eggs can be differentiated from other Schistosoma species by its characteristic oval shape and a terminal spine. The ova containing ciliated embryos are discharged into the urine of the host during micturition. The egg hatches releasing the first larval form called miracidium in fresh water medium. Within the water, the free swimming miracidium penetrates the Bulinus snail intermediate host and develops into the first stage or mother sporocyst, followed by the second stage sporocyst or the daughter sporocyst. After 14 days the daughter sporocyst matures into radia, which in turn produces thousands of forked tailed cercaria larvae, which is the infective form to man. Within water, the cercariae infect the definitive host by skin penetration where they enter the blood stream and migrate to the liver through heart-lung
migration and mature into adult worm, which migrate and inhabit the vesical plexus of the bladder and lay eggs to repeat the life cycle (Figure 2.1). The average lifespan of the adult worm is 5 years; however it can survive up to 25 years within the definitive host (Wilkins, 1987; Gryseels and De Vlas, 1996).

Figure 2.1 The life cycle of *S. haematobium*; Source: www.cdc.org; 6/12/2017; 11:12am
2.3 Disease severity

Several terms are used in different literatures to indicate severity of schistosomiasis. Disease severity due to *S. haematobium* is mostly correlated with morbidities such as the degree of hematuria, proteinuria, and the cytological changes observed in the urinary bladder and ureters (Abdel-Salam and Ehsan, 1978; Hatz, *et al.*, 1992). Epidemiological evidence seems to correlate endemicity of urogenital schistosomiasis and bladder cancer. In the early 90’s, significant number of cancer cases reported by the Cairo Cancer Institute were associated with *S. haematobium* in Egypt, which is one of the highly endemic places in Africa (Aboul-Nasr *et al.*, 1986). At the same period countries such as Malawi, Kuwait, and Iraq (Lucas, 1982; Al-Shukri *et al.*, 1987, Al-Saleem *et al.*, 1990) where *S. haematobium* infection was the leading cause of schistosomiasis also recorded bladder cancers as the leading malignant diseases. Some studies have linked squamous cell type bladder cancer with persistent infection with *S. haematobium* and considerable day to day changes of egg morphology in urine (Muscheck *et al.*, 2000; Akinwale *et al.*, 2008). Exfoliated cells in urine of infected adults in a rural fishing community in Nigeria were prepared for cytological analysis. The results revealed severely dysplastic to malignant squamous cells supporting a similar study in Kenya that showed urinary tract hyperplasia. (Hodder *et al.*, 2000; Akinwale *et al.*, 2008).

In a recent study, moderate to high genetic differences between *S. haematobium* populations in Egypt, Zimbabwe and South Africa were reported (Afifi *et al.*, 2016). This may account for the degree of cytological abnormality of the urinary tract inflicted by *S. haematobium* in Egypt as compared to a mild pathology in countries of Sub-Saharan Africa (DeClerq *et al*, 1994, Edington, 1994). Clinical manifestations of urinary schitosomiasis as occurred in Egypt is usually caused by obstructive uropathy and sometimes metaplastic urethral changes which could lead to lower urinary tract infections, the risk of squamous cell carcinoma and adenocarcinoma.
(Afifi et al., 2016). A retrospective study of histopathologic review of tissue schistosomiasis study at the Korle-Bu Teaching Hospital between 2004 and 2011 found 98.7% of tissue schistosomiasis to be due to *S. haematobium* and also established significant association between urinary schistosomiasis and squamous cell abnormality (Der et al., 2015). Ultrasonography examination of the urinary tract was done on students with *S. haematobium* infection in endemic areas of Sudan. This revealed varied degrees of abnormalities including abnormal wall thickening, multiple nodulations and bladder wall calcifications which are similar to a related study in Mali that observed fibrosis and aplastic urothelial cells (Keita et al., 2005; Gasmelseed et al., 2014).

The mice model was used to investigate urothelium changes during *S. haematobium* infection in relation to the tumor suppressor gene P53 and its role in many cancers including schistosomal bladder cancers. It was established that *S. haematobium* eggs are highly inflammatory, resulting in granuloma formation, urothelial hyperplasia and squamous cell carcinoma (Fu et al., 2012; Hsieh et al., 2015)

### 2.4 Treatment success of Praziquantel

Prior to the use of Praziquantel, Metrifonate and Oxamnique were the drugs used for the treatment of human schistosomiasis in the 1960s (Abaza, 2013). These drugs were withdrawn due to high levels of toxicity, adverse effects such as mutagenicity and emergence of drug resistance (Batzinger and Bueding, 1977; Frédéric et al., 2016). Praziquantel remains the drug of choice for all human adult *Schistosoma* species. It is also effective on trematodes and cestodes. The mechanism of action of the drug is not well understood. However, it is known to act on the adult worm by disruption of voltage operated Ca2+ channels, causing intense muscular paralysis leading to death (Salvador-Recatalà and Greenberg, 2012; Abaza, 2013). The efficacy of
Praziquantel in the treatment of *S. haematobium* infection among school aged children in a rural community in Nigeria was considered satisfactory following post treatment cure rate of 85.5% and 100% for 8 and 12 weeks respectively (Ojurongbe *et al.*, 2014). Between 1984 and 1992, a substantial review of year to year cure rate (conversion from egg positive to negative) status on urine examination for schistosomiasis and treatment with both Praziquantel and Metrifonate was carried out in Kenya (Charles *et al.*, 2000). It was established that response to treatment with Metrifonate declined from 79% to 47% between 1984 and 1987. On the contrary, no consistent downward trend with Praziquantel response to treatment was observed. However, Charles *et al.* (2000), concluded that emergence of Praziquantel resistance should be anticipated within 10 to 20 years after the review and advised that research and development of other anti-schistosomal drugs should be a concern of public health.

### 2.5 Therapeutic failure and drug resistance

The World Health Organization defines drug resistance as the ability of a disease causing microorganism to withstand a drug that once stalled or killed it. Generally, drug resistance has been a major concern and threatens the ultimate goal of prevention and treatment of increasing rate of infections caused by microorganisms including bacteria, viruses, fungi and parasites. The most serious aspect is the cost due to treatment of resistant infections as compared to non-resistant ones (WHO, 2014).

In the mid-1970s and mid-2000s, Oxamniqueine (OXA) was used in the treatment of *S. mansoni* and *S. haematobium* infections in Brazil (Coura and Amaral, 2004). In 1973, resistance to OXA was identified in Brazil (Frédéric *et al.*, 2016). Molecular evaluation and genetic studies showed
that the gene, SmSULT – OR was responsible for resistance to OXA. SmSULT – OR gene code for an enzyme within the parasite for intracellular activation of the drug.

Praziquantel remains the drug of choice and currently used as a mono-therapy in various health care settings, as well as mass drug administration programmes in Africa and other parts of the world, in the treatment of schistosomiasis (Dye et al., 2013). It is very important to do resistance evaluation far ahead of time across the globe, most especially in the endemic areas. The first cases of suspected resistance with Praziquantel was reported from Senegal, where there was 18-39% low cure rate, adding up to similar evidence gathered from Egypt (Gryseels and De Vlas 1996; Ismail et al., 1999), however, preliminary studies on strains from this two geographical locations have not yet proven any genetic basis for resistance development (Doenhoff et al., 2009).

The cure rate of Praziquantel is variable and rarely reaches 100%; some infections persist even after repeated treatment. In addition, there is some evidence of experimentally induced resistance with varied concentrations of the drug (Ismail et al., 1999; Doenhoff et al., 2009). Moreover, Mendonça da Silva et al. (2005), reported therapeutic failure of Praziquantel in the treatment of S. haematobium infected among Brazilian soldiers returning from Africa; viable eggs were identified after 6 and 24 months of treatment.
2.6 Urogenital schistosomiasis and re-infection

Praziquantel has proven to be efficacious in the treatment of schistosomiasis; however re-infection has been reported even after achieving total egg clearance and cure rate few months after treatment (DeClerq et al., 2000; Webster et al., 2013a). Various factors including levels of schistosomiasis endemicity, lack of health education, ecological and seasonal variations, occupation and socio-economic activities, which frequently expose people to infected water bodies have been observed to influence re-infection (Mutapi et al., 1999; Saathoff et al., 2004). After achieving total egg clearance and zero egg count in a study conducted on school children in Ndumo area of uMkhanyakude district KwaZulu-Natal, South Africa, re-infection rate of 8.1% was recorded 28 weeks post treatment (Kubayaya et al., 2017). Similarly, Goran et al. (2001), recorded an alarming re-infection rate of 63%. This was a comparative evaluation on school based chemotherapy on urogenital schistosomiasis and re-infection patterns, 24 months post treatment with Praziquantel in highly endemic communities in Côte d'Ivoire. In a related study, the prevalence of infected Bulinus snails were proportional to the re-infection patterns based on the period of assessment, in addition to the prevailing ecological season (Goran et al., 2001; Tchuente et al., 2004).

2.7 Genetic variations

There has been a considerable interest in the area of population genetics with regards to the study of parasite population including schistosome species of clinical importance. Although studies of genetic diversity of natural schistosome population are complicated relating to the fact that the adult worm are inaccessible in the cardiovascular system of mammalian host, elucidating the
genetic features is very important in helping us understand many aspects of the disease epidemiology and transmission (Steinauer et al., 2009).

Within the last few years that we have obtained genome sequences and with the availability of DNA markers including microsatellite, Internal transcribed spacer (ITS1 and ITS2), cytochrome oxidase I in the mitochondrial genome (COX I), Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 1 (NAD1) etc, for Schistosoma species (Shrivastava et al., 2005; Gower et al., 2011, Young et al., 2012), it might be pertinent for predicting relevant intervention in response to evolutionary change in parasite population towards elimination rather than control measures.

Genetic surveillance is very relevant in predicting gene flow and genetic diversity which is a good tool to monitor and control schistosomiasis in endemic areas.

ITS2 is a known molecular marker which is specific to S. haematobium. A study conducted in Mali and Sudan, using PCR amplification of ITS2, separated S. haematobium species from a group of closely related schistosomes known as S. haematobium complex (Quan et al., 2015; Moendeg et al. 2017). A similar study characterized geographic strain differentiation of S. japonicum using microsatellite markers and established high genetic diversity in the areas with high prevalence than places with low prevalence in the Philippines, as it occurs in China (Shrivastava et al., 2005; Moendeg et al., 2017). A comparative study of S. mansoni genetic variant strains from human host in two different locations in Brazil revealed 14 to 24 alleles and 5 to 27 alleles per locus in Melquiades and Virgem das Graças respectively using microsatellite markers that identifies up to 7 loci (Thiele et al., 2008).

Although S. haematobium is most prevalent in Africa with over 112 million cases (WHO, 2013b), little is known about its genetic variation as compared to S. mansoni. However, a study
conducted by Ezeh et al. (2015), demonstrated high level of genetic variation of *S. haematobium* in Mali and Nigeria by comparing the allelic composition in these two regions with 4 microsatellite loci.

In other genetic variation study, Brouwer et al. (2001), characterize the extend of genetic diversity of *S. haematobium* within and among its definitive host in east central Zimbabwe by whole genome scan with random amplified polymorphic DNA markers. It was established from the study that analysis of molecular variance was greater within rather than between the hosts.

DNA barcoding study was conducted on *S. mansoni* and *S. haematobium* in Yemen to gain insight into the genetic diversity among the two shistosome species. Mitochondrial gene cox1 was analysed and compared to previously published cox1 data and established the existence of nineteen unique haplotypes of *S. mansoni*, which were grouped into four lineages and nine haplotypes of *S. haematobium*, which were also grouped into two lineages (Sady et al., 2015).

### 2.8 Genetic variations in mitochondrial genes

Mitochondrial genes including; cytochrome oxidase subunits (cox1-6) and NADH dehydrogenase group (nad1-6 and nad4L), are known molecular markers used for species identification and detection of specific strains in the genus *Schistosoma* (Laila et al., 2012). In a study conducted to determine the occurrence of bidirectional hybridization of closely related schistosoma species known as *S haematobium* complex, cox1 rapid diagnostic multiplex PCR (RD-PCR), was done to distinguish *S. haematobium* strains having mitochondrial marker (cox1) before they were sequenced to know their gene identity (Webster et al., 2013b). Similarly, cox1 RD-PCR was used to discriminate closely related schistosoma species; *S. haematobium* and *S. bovis* that have *Bulimus* snail species as their intermediate host, which is mostly found in Africa.
by Webster et al. (2009). DNA barcoding studies done in Pemba Island using two mitochondrial genes nad1 and cox1 separated *S. haematobium* into two groups. Group 1 is characterized as mainland Africa strains and group 2 occur mostly in Zanzibar. Studies involving mitochondrial gene sequences are known to be suitable markers for phylogeny and genetic variations in *S. japonicum* strains in different geographical origins such as Philippines, Japan and China. This was revealed by examining sequence variation in the complete mitochondrial protein coding genes cytochrome b (cox b), NADH dehydrogenase subunits 2 and 6 (nad2 and nad6) (Fen, et al., 2015).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This was a longitudinal study which involved baseline (pretreatment samples) and weekly follow-ups (post treatment samples). Urine samples were collected and screened for *S. haematobium* ova by light microscopy. Urine cytology was done on the study participants that were identified with *S. haematobium* ova to investigate cytological damage to the squamous epithelial cells of the urinary tract. In addition, molecular analysis was done by PCR to look for possible genetic variants of *S. haematobium* using mitochondrial molecular markers; NAD1 and COX1 after confirmation with species specific marker ITS2. A single oral dose of Praziquantel (40 mg/kg), was given to the positive cases; post-treatment follow-up urine samples were collected on weekly basis up to the fourth week and the sixth week to re-assess the urine cytology and monitor egg reduction rate as a measure leading to possible resistance determination. After the twelfth week, all the cases collected at base-line were re-sampled to check for re-infection.

3.2 Study Site

Samples were collected from communities (along the stretch of the Volta Lake) in the Pru district which is located in the middle belt of Ghana. The Pru district can be found in the eastern part of the Brong Ahafo Region of Ghana and shares boundaries with the Sene district to the east, Atebubu Amantin district to the south and the Gonja east district (in the northern region) to the north (Figure 3.1). The district is located within the transition zone and experiences both tropical and savanna types of climate. Two rainy seasons occur annually with the major one
beginning in June whilst the minor season usually occurs in September or October. Yeji is the administrative capital of the Pru district; most communities in the district are situated along the Volta Lake, where fishing is the major occupation. People within these communities also use the river for farming, washing and other recreational activities. The district is one of the areas in Ghana with high prevalence of schistosomiasis. The Pru district recorded above 50% *S. haematobium* infection rate in 2010, as reported by the World Health organization (WHO, 2010), making this area a suitable site for screening people to investigate possible genetic variations, disease severity and to determine the possibility of resistance development to the drug of choice, Praziquantel or re-infection.
Figure 3.1 Geographical map showing the Pru District in the Brong Ahafo Region of Ghana (www.mapdata2018.google.com, 24 July 2018)

Figure 3.2: Ecological view of the Volta Lake in the Pru District
3.3 Subjects/study population

Inclusion criteria:

- Basic school children in the selected study communities who consented together with their guardians.

Exclusion criteria

- Children who had taken medication for schistosomiasis prior to or during sample collection were excluded.

3.4 Sample size

The minimum sample size was 330 based on the Prevalence (P) of Schistosomiasis, 30.7% obtained from a study by Tetteh-Quarcoo et al. (2013). With 95% confidence level and a corresponding Z-value of 1.96, Confidence interval (C) of 0.05, the samples size was calculated using the formula \( \frac{Z^2 \cdot P \cdot (1-P)}{C^2} \).

Sample size = \( \frac{(1.96^2 \cdot 0.307 \cdot 0.693)}{0.05^2} = 327 \sim 330 \). However, 520 samples were collected for the study.

3.5 Study Procedure

The study was carried out in the Pru district of the Brong Ahafo region of Ghana. Questionnaires were given to gather demographic and clinical information covering; age, sex, source of water and others, after informed consent from parents, guardians and teachers. A clean, dry, screw cap, wide mouth and leak proof urine containers were given out to the study participants to provide about 20 ml of urine, between the hours of 10:00 am and 1:00 pm to increase the chance of detecting ova. The samples were transported on ice immediately to the Laboratory unit of the St.
Mathias Hospital, Yeji for screening for *S. haematobium* ova and egg counting by microscopy. The positive samples were transported to the Parasitology and Pathology laboratories at the school of Biomedical and Allied Health Sciences, Korle Bu, for molecular and cytology analysis respectively to be performed.

### 3.6 Laboratory investigation

Laboratory procedures used for the sample analysis were; Light microscopy for ova detection, egg count and viability testing for possible resistance assessment, Cytological examination (Papanicolou staining method) for disease severity and molecular analysis with PCR for genetic variation (Figure 3.3).

![Flow chart for laboratory examinations.](http://ugspace.ug.edu.gh)

**Figure 3.3: Flow chart for laboratory examinations.**
3.6.1 Urine strip test

All samples were examined macroscopically for urine colour, appearance and the presence of blood in the urine (Figure 3.4A). Biochemical tests including protein (proteinuria), blood (microhaematuria) and leukocytes (pyuria) as well as specific gravity, nitrite, bilirubin and urobilinogen were also done, using urine strips (URIT 10V, URIT Medical Electronic Co., Ltd, China) on all the urine samples (Figure 3.4A).

3.6.2 Detection of ova and Egg Count (for possible resistance assessment)

For ova detection and egg count (Figure 3.4A and B), 10ml of each urine sample was centrifuged in a centrifuge tube for 5 minutes at 5000 rpm speed to sediment. The supernatant was discarded; leaving small amount of urine in the centrifuge tube which was used to re-suspend the deposit to make a total volume of 0.5 ml. fifty microlitres (50 µl), of the re-suspended urine deposit was pipetted on a microscope slide, with cover glass placed on it. This was done for S. haematobium ova detection with a light microscope. The egg count was determined by the number of eggs present / 10 ml of urine (Figure 3.4B) (Samie et al., 2010).

The count was used to calculate the Egg Reduction Rate (ERR), and monitor the effect of Praziquantel after treatment (WHO, 2013a). Weekly post treatment follow up was done for the first four weeks and then the sixth and after the twelfth weeks to determine possible resistance or re-infection (Figure 3.4C). The presence of viable eggs in the post treatment assessment up to the sixth week gives an indication of therapeutic failure or possible drug resistance (Figure 3.4C). On the contrary, non detection of ova (zero egg count) on the sixth week post treatment implies that there is no drug resistance (Figure 3.4C), whilst re-appearance of viable eggs after the twelfth week post analysis represent re-infection (Figure 3.4C).
Figure 3.4A: Flow chart for ova detection

Figure 3.4B: Flow chart for egg count and viability test
3.6.3 Viability test (for possible resistance assessment)

Two methods involving; Hatchability and vital staining with Neutral red and Trypan blue were employed (Figure 3.4B).

3.6.3.1 Hatchability test

Modified hatchability method (Figure 3.4B) was used in this study; about 10 ml of each urine sample was transferred into a centrifuge tube and spun for 5 minutes at a speed of 5000 rpm to sediment. Fifty microlitres (50 µl) of the sediment was mixed with 20 µl of distilled water on a microscope slide and a cover slide placed on it. The whole setup was incubated for about 5 minutes before microscopy using the x10 and x40 objectives. Maximum amount of light was
provided by moving the source of light close to the stage and widely opening the iris diaphragm of the microscope to look for movement of miracidia or flame cells. All ova showing movement (miracidia or flame cells) were counted against forms that showed no such movement, both at baseline and weekly post Praziquantel treatment, to assess the effect of the drug on egg viability. This method takes advantage of the organism’s phototropism since they have the tendency to swim towards light (Shimizu, 2006). It is generally expected that the baseline samples will produce more viable eggs than post treatment samples. This is because, besides the adult worm not surviving to excrete more active eggs, Praziquantel has been noted to have effect on *Schistosoma* eggs (Guidi et al., 2010). Thus, the presence of free swimming miracidia up to the sixth week gives an indication of therapeutic failure or suspected resistance.

### 3.6.3.2 Vital staining

Two dyes used for the vital staining were 0.4% Trypan blue dye and 1% Neutral red dye (Figure 3.4B). Ten milliliters (10 ml) of each urine sample was transferred into a conical centrifuge tube and centrifuged for 5 minutes at a speed of 5000 rpm. The supernatant was discharged leaving traces of the urine to re-suspend the deposit to make a volume of 0.5 ml. Two slides containing 50 µl of the re-suspended urine were prepared for each sample. Fifty microlitres (50 µl) of trypan blue dye was added to one preparation and 50 µl of neutral red dye was also added to the other slide preparation, a cover slide was placed on each of them and incubated for 5 minutes to allow dye penetration before microscopy was done (Figure 3.4B).

Nuclei of living cells absorb neutral red dye, thus eggs containing active miracidia absorb and retain the neutral red dye leaving the dead ones unstained. On the contrary, dead cells absorbed and retained the trypan blue stain leaving active cells intact without the stain (Sarvel et al.,...
2006). As indicated with the hatchability method, it is also expected that the two vital stains will detect more viable eggs at baseline with more non viable eggs occurring weeks after treatment so that re-appearance of viable eggs after the twelfth week post treatment assessment will be seen as re-infection provided no egg was detected after the sixth week post treatment analysis (Figure 3.4C).

3.6.4 Cytological examination of exfoliated cells in urine (for disease severity)

About 4 ml of each urine sample was centrifuged at a speed of 1500 rpm for 10 minutes. The supernatant was poured away leaving up to 1 ml of the urine that was used to re-suspend the deposit. Between 4-5 drops of the re-suspended deposit was used to make a smear on a microscope slide and fixed with 95% ethyl alcohol for a minimum of 15 minutes, before staining with the Papanicolaou staining method that employs Haematoxylin, Orange G (OG) and Eosin alcohol (EA) stains (Figure 3.5A). The stained slides were examined microscopically for cytological changes such as; squamous cell metaplasia (epithelial cells with nuclear enlargement or increase nuclear/cytoplasmic ratio), inflammation, hyperkeratosis (orange coloured urothelial cells as a results of increased keratin content) and red blood cells (Figure 3.5B) by an experienced microscopist. The cytological examination was done on baseline and post treatment samples on weekly basis up to the eighth week to monitor the reversal of cytological damage to urothelial cells caused by *S. haematobium* infection, weeks after treatment. Disease severity was defined as the occurrence of squamous cell metaplasia (SCM) with inflammatory cells with or without the other abnormalities (Figure 3.5B), (Hodder *et al.*, 2000). Squamous cells found within the severe group were graded based on the extent of nuclear enlargement as mild, moderate or marked (Figure 3.5B), (Hodder *et al.*, 2000).
Figure 3.5A: Flow chart for cytological analysis

Figure 3.5B Flow chart of cytological abnormalities and disease severity
3.6.5 Molecular analysis (for genetic variations)

Procedures used under molecular analysis were DNA extraction and amplification of ITS2 by PCR for confirmation of *S. haematobium* ova before PCR amplification of mitochondrial DNA markers; NAD 1 and COX 1, as determinants of genetic variations were done.

3.6.5.1 DNA extraction.

Genomic DNA was extracted from *S. haematobium* eggs using Zymo DNA mini kit, according to the manufacturer’s instructions (Appendix IV).

3.6.5.2 Amplification and visualization of the ITS2 region by PCR.

Amplification of the ITS2 region by PCR was performed in a 25 μl volume, which included 6 μl of genomic DNA from each sample, 12.5 μl of master mix (which includes TaKaRa Ex Taq DNA Polymerase, TaKaRa Ex Taq buffer, dNTP mixture), 2.5 μl each of primers ITS2F (5’-GAA TTA ATG TGA ACT GCA TAC TGC TT-3’) and ITS2R (5’-TTC CTC CGC TTA TTG ATA TGC TT-3’) and 1.5 μl nuclease free water using PCR Thermal Cycler (Takara Bio Inc.). All the PCR assays were performed with an initial denaturation step of 94˚C for 30 sec, followed by 40 cycles of denaturation at 98˚C for 10 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec, followed by 1 cycle at 72˚C for 7 min and a final hold at 4˚C. Agarose gel electrophoresis (2%) with ethidium bromide staining was used to visualize the ITS2-PCR amplicons (Figure 3.6A). DNA ladders 100bp and 50bp were used to locate the ITS2 region with a band size of 468bp. ITS2 is *S. haematobium* species specific DNA marker, thus its amplification is a confirmatory test for the detection methods such as microscopy (Figure 3.6B).

3.6.5.3 Amplification and visualization of mitochondrial genes (NAD1 and COX1)

Mitochondrial subunits, cytochrome oxidase (cox1) and NADH dehydrogenase 1 (nad1) of *S. haematobium* were amplified with the following primers; *nad1F* 5’-GGC TGA TGT TCG TGA TTA TGC TT-3’ and *nad1R* 5’-GGC TGA TGT TCG TGA TTA TGC TT-3’.
TCA AA-3’ and *nad1R* 5’-CGA AGT CGA GAA AAT GAA CCA-3’; *cox1F* 5’-AAA AGC TGT GGG TCT CGT GT-3’, *cox1R* 5’-AAT GAA GAA GCG GAG AAA GC-3’. The PCR assays were performed with an initial denaturation step of 94°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 10 sec, annealing at 58°C for 30 sec, and extension at 72°C for 60 sec, followed by 1 cycle at 72°C for 10 min and a final hold at 4°C. Mitochondrial genes; NAD1 and COX1 have been used in genetic variation studies for speciation and strain identification (Webster *et al.*, 2009; Fen, *et al.*, 2015). Expression (NAD1, COX1 or both NAD1 and COX1) or non expression (Figure 3.6B) determines variant forms of *S. haematobium* circulating within the population.

**Figure 3.6A: Flow chart for molecular analysis**
3.7: Data handling and statistical analysis

All study participants involved in the study were number coded for identification purpose, instead of using names for security and confidentiality.

The data were entered into Microsoft Excel 2013 and SPSS version 22. Line and bar graphs were generated for various variables whilst t-test was used to compare the significance of egg viability at baseline and post Praziquantel treatment using P-values < 0.05.
3.8 Research clearance

Approval for the study was given by the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana (Appendix XII). Urine samples were collected with consent from study participants and their parents (Appendices IX and X).
CHAPTER FOUR

4.0 RESULTS

4.1 General view of *S. haematobium* infection

Out of 520 participants, 34 were found with *S. haematobium* infection representing 6.5% infection rate. Among these infected individuals; 10/137 (7.3%) were found in Emmanuel Presbyterian (EP), 8/86 (9.3%) in Al Iman Islamic, 4/87 (4.6%) in Vutideke D/A, 7/107 (6.5%) in Prang D/A and 5/102 (4.9%) in Royal Education Complex basic schools respectively. The infection rate in all the five schools involved in the study was found to be below 10%.

4.1.1 Morphological forms of *S. haematobium*

Morphological variations in terms of shape and spine position of *Schistosoma haematobium* ova were observed (Fig.4.1)
**Figure 4.1: Morphological forms of *S. haematobium* ova.** The black arrows indicate the spine, red arrows indicate the embryo. All eggs represented as A, B, C, D, E, F, G and H, have the usual characteristic oval shape and terminal spine. However, B has blunt spine, D appears to be oval to round shaped with slightly curved spine. E has a short spine. F looks elongated and has slightly curved spine. H shows cluster of ova in one field.

**4.2 Clinical morbidity indicators associated with *S. haematobium* infection**

Clinical indicators usually associated with *S. haematobium* infection such as haematuria (blood in urine), proteinuria (protein in urine) and leukocyturia (pus cells in urine) were detected both at baseline and post-treatment among the infected study subjects. Haematuria was the highest parameter (91%) detected among the infected participants at baseline (Figure 4.2). However, there was gradual decline in haematuria among the post-treatment study subjects with 68.2% at week 1, 36.4% at week 2, 22.7% at week 3 and 18.5% at week 4. With proteinuria, 53% was detected at baseline and 22.7%, 13.6% and 4.5% were recorded during follow-up at weeks 1, 2, and 3 respectively whilst at week 4, no form of proteinuria was detected (0%) (Figure 4.2). Leukocyturia was the lowest parameter that was detected among the infected participants. The percentage leukocyturia at baseline was 11.8% which declined to 9.1% and 4.5% during the
follow-ups at week 1 and week 2 respectively, whiles week 3 had 2.0% and week 4 recorded 0% (Figure 4.2). Proteinuria, haematuria and pyuria were not detected during the sixth week for the post treatment assessment (Figure 4.2).

Figure 4.2: S. haematobium infection morbidity indicators at baseline and post treatment

4.3 Drug (Praziquantel) resistance or re-infection assessment

Following Praziquantel treatment, there was gradual reduction in egg detection as S. haematobium positive and the overall egg count as follows; week 1, 14 positives, week 2, 5 positives, week 3, 3 positives and week 4, zero (0) (Figure 4.3). Further follow up to the sixth week showed no evidence of infection among all the thirty four (34) participants that were infected prior to Praziquantel treatment indicating that, there was no drug resistance (Figure 4.3). However, there was re-infection (Figure 4.3). Out of the 34 S. haematobium cases recorded at base-line, 26 were available for the re-infection assessment. Five out of these number were found
to have ova in their urine by light microscopy giving re-infection rate of 19.2% (5/26) (Figure 4.3).

Fig. 4.3: Summary of drug resistance or re-infection assessment

4.3.1 Egg Reduction and Clearance Rates at Post treatment Weekly Follow ups.

The mean egg count among the 34 infected participants at baseline was 101.2 (Figure 4.4A). However, there was drastic reduction in mean egg count after the post-treatment weekly assessment. Week one post-treatment assessment recorded mean egg count of 34.3 (Figure 4.4A) among 14 infected participants giving egg reduction rate (ERR) and clearance rate (CR) of 65% and 58.8% respectively (Figure 4.4B). Two weeks after Praziquantel treatment, five (5) participants were positive with *S. haematobium* infection with mean egg count of 32.5 whilst week three recorded mean egg count of 15.0 among three participants with *S. haematobium* ova.
in their urine (Figure 4.4B). Clearance rate of 85% and egg reduction rate of 68% were recorded in week 2 (Figure 4.4B). The highest clearance rate was found in week 3 at 91.2% whiles the rate of egg reduction was 85% (Figure 4.4B). No ova were detected in week four and up to the sixth week post-treatment assessment (mean count was 0.0; 100% ERR) (Figure 4.4B).

Figure 4.4A: Egg count at baseline and weekly post treatment
4.3.2 Egg Viability assessment

The percentage of live eggs involving all the viability methods at baseline were higher than dead eggs as opposed to the post treatment follow up assessment which detected more dead than live eggs (Figure 4.5). Mean percentage viability of live eggs at baseline (pre-treatment) was 95.4% whiles that of dead egg was 4.6% (Table 4.1). On the contrary, mean percentage viability at post-treatment recorded 33.3% live eggs and 66.7% of dead eggs (Table 4.1). At 95% confidence level and a corresponding Z-value of 1.96, Confidence interval (C) of 0.05, the statistical difference between live and dead ova post Praziquantel treatment was significant ($P<0.01$).
Table 4.1 *S. haematobium* Egg viability at Baseline and Post-treatment.

<table>
<thead>
<tr>
<th>Viability Method</th>
<th>Baseline (pre-treatment)</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live eggs (%)</td>
<td>Dead egg (%)</td>
</tr>
<tr>
<td>Hatchability</td>
<td>93.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Neutral red</td>
<td>97.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>96.3</td>
<td>3.7</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>95.4</strong></td>
<td><strong>4.6</strong></td>
</tr>
</tbody>
</table>

Figure 4.5: Egg viability at baseline and post treatment
4.3.2.1 Modified Hatchability Test

Movement of flame cells or the embryo within the egg shell and swimming of the miracidia as it hatches out of the shell were observed during this procedure and this was the determining factor for live eggs (Figure 4.6). Eggs were considered dead when no such movements were observed after a period of time. Figure 4.6 gives the picture and direction of movement of miracidia leaving the shell as the ovum matures and hatches in the presence of optimum conditions such as water and light.

Figure 4.6: Microscopic view of miracidia and egg shells. The black arrows in A, B, C, D, E and F indicate miracidium, the white arrows shows the egg shell
4.3.2.2 Vital Staining (Trypan blue and Neutral Red)

Live eggs absorbed and retained the neutral red dye and they were looking red microscopically (Figure 4.7). The live eggs were distinguished from the dead ones because dead ova were not able to absorb and retain the neutral red dye after the incubation period. In the case of the trypan blue stain, the dead eggs absorbed and retained the stain after the required incubation period (Figure 4.7). Due to the inability of the viable eggs to pick up the trypan blue dye, they were easily distinguished from the dead eggs (Figure 4.7).
Figure 4.7: Microscopic view of live and dead eggs by vital staining (0.4% Trypan blue and 1% Neutral red). A in lane 1 represents live eggs showing non retention of 0.4% trypan blue dye. B in lane 2 indicates dead ova with retention of the trypan blue dye. C in lane 3 indicates live eggs with permanent retention of the 1% neutral red stain. D in lane 4 is showing dead eggs which failed to pick up the neutral red stain.
4.4 Disease severity

4.4.1 Cytological abnormalities

Cytological abnormalities such as squamous cell metaplasia, (cells with nuclear enlargement or increased nuclear cytoplasmic ratio), inflammation (shown with the presence of white blood cells), red blood cells and hyperkeratosis (increased keratin content of cells), (Figure 4.8A) were recorded and classified as severe or non-severe. Out of the 30 participants subjected to the cytological analysis, 8 (26.7%) were classified as severe based on the presence of squamous cell metaplasia with inflammation. The remaining 22 (73.3%) were categorized as non-severe (Figure 4.8B).

Figure 4.8A urine cytology showing normal and abnormal urothelial cells. Papanicolaou stained urine deposit smears showing: A; normal squamous urothelial cells from non infected participant; B; anucleated hyperkeratotic clustered cells; C, red arrows pointing at squamous cell metaplasia; D, black arrows pointing at inflammatory cells, green arrows pointing at red blood cells.
4.4.2 Cytological abnormalities at base line

Generally, squamous cell metaplasia was recorded in 8 (26.7%) samples out of the 30 *S. haematobium* positive cases subjected to the cytological analysis whiles inflammation was seen in 22(70.3%) cases (Table 4.2). However, both inflammation and squamous cell metaplasia were identified in 8 cases (26.7%) and such cases were classified as severe (Figure 4.8B). Red blood cells were identified in 60% (18/30) of both severe and the non-severe groups. Also, hyperkeratosis was recorded in 46.7% (14/30) both in the severe and the non-severe categories (Table 4.2).
Table 4.2: Cytological abnormalities revealed by urine cytology at base-line in the severe and the non severe groups

<table>
<thead>
<tr>
<th>Cytological abnormality</th>
<th>Severe (N= 8)</th>
<th>Non-severe (N = 22)</th>
<th>Total (N = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell metaplasia</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>8 (100%)</td>
<td>13 (59.1%)</td>
<td>21 (70%)</td>
</tr>
<tr>
<td>Red blood cell</td>
<td>8 (100%)</td>
<td>10 (45.5%)</td>
<td>18 (60%)</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>6 (75%)</td>
<td>8 (36.4%)</td>
<td>14 (46.7%)</td>
</tr>
</tbody>
</table>

4.4.3 Grading of squamous cell metaplasia within the severe group

Grading of squamous cell metaplasia (which is a key determinant of disease severity) showed that fifty percent (50%) had slight nuclear enlargement and were therefore graded as mild (Figure 4.9) whiles 25% in each case were graded as moderate and marked with regards to nuclear cytoplasmic ratio (Figure 4.9).
4.4.4: Cytological changes in the post-treatment weekly follow ups.

There was improvement in the cytological aberrations and other parameters in the severe cases during the post treatment urine cytology from week one up to the eighth week as compared to the base line study (Figure 5.0).

Squamous cell metaplasia persisted in all the severe groups in week 1 (100%), week 2 (100%) and week 3 (100%) post Praziquantel treatment (Figure 5.0). However, at week 4 and week 8 squamous cell metaplasia was found in 62.5% cases (Figure 5.0). Inflammation reduced from
100% at baseline to 62.5% and 37.5% at weeks 1 and 2 respectively whilst at week 3, week 4 and week 8, no inflammatory cells were found (0%) in each case (Figure 5.0). After recording 75% at baseline, hyperkeratosis surprisingly occurred in all cases (100%) at weeks 1 and week 2. It however reduced to 62.5% at week 3 and week 4 and further reduced to 37.5% at week 8 post treatment assessment (Figure 5.0). Red blood cells were found in all severe cases (100%) at baseline. Weeks 1 and 2 had red blood cells persisting in 62.5% and 37.5% cases respectively (Figure 5.0). However there were no red blood cells at weeks 3, week 4 and week 8 Post-treatment (Figure 5.0).

**Figure 5.0 Cytological changes at baseline and post treatment**
4.4.5 Case representation of disease severity at base line and reduction of urothelial cell abnormalities in the weekly post-treatment analysis

Pictorial view of two severe cases (case 1 and case 2), represents the occurrence of urothelial cell changes at baseline and post Praziquantel treatment (Figure 5.1). Generally, there were more urothelial cells excreted in the urine of *S. haematobium* infected participants at base line with more abnormalities (A1 and A2, Figure 5.1). Squamous cell metaplasia, hyperkeratosis with inflammation and red blood cells markedly occurred in all the two cases at base line(A1 and A2, Figure 5.1). Week 1 post treatment (B1 and B2, Figure 5.1), was not looking different from the baseline assessment since all the abnormalities were still seen at this stage in both cases. At post treatment week 2 (C1 and C2, Figure 5.1), both hyperkeratosis and squamous cell metaplasia persisted in both cases but case 1 saw more SCM with mild and moderate grades whilst case two had few SCM with mild grades. Few red blood cells and inflammatory cells occurred at week two (C1 and C2, Figure 5.1). Weeks 3 (D1 and D2, Figure 5.1), week 4 (E1 and E2, Figure 5.1) and week 8 (F1 and F2, Figure 5.1), had no inflammation and red blood cells. Hyperkeratosis looked scanty at week 3 (D1 and D2, Figure 5.1), week 4 (E1 and E2, Figure 5.1) and week 8(F1 and F2, Figure 5.1). Few urothelial cell showed SCM with mild nuclear enlargement at week 3 for case 2 whilst case 1 had SCM with both mild and moderate forms (D1 and D2, Figure 5.1). At week 4(E1 and E2, Figure 5.1), all cells had no forms of SCM in case 2, but case 1 still had few cell showing SCM. The eighth week assessment found most cells with normal morphology in both cases, but few isolated cells in case 1 had mild SCM (F1 and F2, Figure 5.1).
Figure 5.1: Papanicolaou stained smear showing urothelial cell changes at baseline and post PZQ treatment: A1: A lane showing cells at baseline for case 1. A2: A lane showing cells at baseline for case 2. B1: A lane showing cell at post treatment week 1 for case 1. B2: A lane showing cell at post treatment week 1 for case 2. C1: A lane showing cell at post treatment week 2 for case 1. C2: A lane showing cell at post treatment week 2 for case 2. D1: A lane showing cell at post treatment week 3 for case 1. D2: A lane showing cell at post treatment week 3 for case 2. E1: A lane showing cell at post treatment week 4 for case 1. E2: A lane showing cell at post treatment week 4 for case 2. F1: A lane showing cell at post treatment week 8 for case 1. F2: A lane showing cell at post treatment week 8 for case 2. Red arrows indicate SCM, black arrows indicate hyperkeratosis, blue arrows shows RBC’s, and green arrows indicate inflammatory cells.
4.4.6 Haematuria recorded with urine test strip and Papanicolaou stain at baseline and post treatment weekly analysis

Haematuria recorded at baseline with urine test strip were 8(100%), out of 8 severe and 14 (63.6%), out of 22 non severe groups respectively whilst Papanicolaou stain detected RBC’S in 6(75%) and 12 (54.5%) respectively in the severe and the non-severe groups. Urine test strip recorded 8 (100%) at week 1, 7 (87.5%) at week 2, 5 (62.5%) at week 3 and 2 (25%) at week 4 post treatment study for the severe group. For the non-severe group, urine test strip detected 12 (54.5%) at week 1, 10 (45.5%) at week 2, 4 (18.2%) at week 3 and zero (0%) at week 4. In the post treatment weekly follow up, Papanicolaou stain detected 4 (50%) and 2 (25%) at week 1 and week 2 respectively for the severe group. The non severe group had 7 (31.8%) at week 1 and 2 (9.1%) at week 2 with Papanicolaou stain. No red blood cells were detected from the urine cytology at weeks 3 and 4. At week 8 blood was not detected with both the urine test strip and Papanicolaou stain (Table 4.3).
Table 4.3 Haematuria at baseline and post treatment with urine chemistry and urine cytology

<table>
<thead>
<tr>
<th>Haematuria</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe</td>
<td>8(100%)</td>
<td>14(63.6%)</td>
<td>8(100%)</td>
<td>7(87.5%)</td>
<td>10(45.5%)</td>
<td>5(62.5%)</td>
</tr>
<tr>
<td>Non severe</td>
<td>12(54.5%)</td>
<td>4(50%)</td>
<td>2(25%)</td>
<td>2(9.1%)</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Urine chemistry

Cytology

4.5 Genetic variation

4.5.1 Amplification and visualization of Internal Transcribed Spacer 2 (ITS2) DNA marker

All the 34 *S. haematobium* cases revealed by light microscopy got amplified in the 468bp ITS2 gene, following PCR amplification. The PCR amplicons are shown on 2% agarose gel between 500bp and 400bp of both 50bp and 100bp ladder (Figure 5.2A).

4.5.2 Amplification of mitochondrial DNA markers; NAD 1 and COX 1

Out of the 34 *S. haematobium* cases that were confirmed by ITS 2 with PCR, 6 (17.6%) got amplified in NAD 1. NAD 1 is 431bp sized DNA found in between 500bp and 400bp ladder in the gel labeled as B (Figure 5.2). With COX 1 (110bp), only 2 (6%) out of the 34 amplified, as shown in gel C (Figure 5.2). One case that was found in the severe group got amplified in both NAD 1 and COX 1.
Figure 5.2: Two percent agarose gel showing ITS 2, NAD 1 and COX 1 amplicons: A is showing ITS 2 amplicons (1-7: 468bp ITS 2 region of S. haematobium; L: ladder; N: Neg Control). B is agarose gel showing NAD 1 PCR products (1, 2&8: 431bp NAD 1 gene of S. haematobium; L1- 50bp ladder; L2- 100bp ladder; N- Neg Control). C is agarose gel products of COX 1 (3&4: 110 bp COX 1 gene of S. haematobium; L1- 50bp ladder; L2- 100bp ladder; N- Neg control).
4.6 Genetic variations and disease severity

A total of 6 (20%) cases out of the 30 participants involved in the cytological analysis expressed NAD 1 gene. Out of this number, 2 were found in the severe category whilst the remaining 4 were within the non severe group (Table 4.4). For COX 1, 2 (6.7%) got amplified, with 1 from the severe and the remaining 1 from the non severe group (Table 4.4). A total of 22 (73.3%) participants (5 from the severe group and 17 from the non severe group) expressed neither NAD 1 nor COX 1 (Table 4.4).

Table 4.4 Expression of mitochondrial genes (NAD 1 and COX 1) and disease severity

<table>
<thead>
<tr>
<th>Mitochondrial genes</th>
<th>Severe (N=8)</th>
<th>Non-severe (N=22)</th>
<th>Total(N=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD 1</td>
<td>2(25%)</td>
<td>4(18.2%)</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>COX 1</td>
<td>1(12.5%)</td>
<td>1(4.5%)</td>
<td>2(6.7%)</td>
</tr>
<tr>
<td>Non expression of both NAD1 and COX 1</td>
<td>*6(62.5%)</td>
<td>17(77.3%)</td>
<td>22(73.3%)</td>
</tr>
</tbody>
</table>

*6 (The total number of non expression of both NAD1 and COX1 is 6 but not 5 because, the one that expressed COX1 also expressed for NAD1 in the severe group).
CHAPTER FIVE

5.0 DISCUSSION

5.1 General view of Schistosoma haematobium infection.

*Schistosoma haematobium* which causes urogenital schistosomiasis is the dominant and widely distributed schistosome species in various communities that live around water bodies in Ghana (McCullough and Ali, 1965, Aryeetey *et al.*, 2000), with the severity of disease varying from mild to severe, possibly influenced by the occurrence of genetically variant strain (Mostafa *et al.*, 1999; Afifi *et al.*, 2016). The prevalence (6.5%) of *S. haematobium* infection recorded in the current study using sedimentation technique (at the Pru district of Ghana) is lower than the over 50% prevalence reported by the World Health Organization in the year 2010 (WHO, 2010) using filtration technique. This difference (apart from the techniques used) could also be attributed to the annual distribution of Praziquantel to school children living in the endemic communities over the eight years period (2010 to 2018), as a measure for controlling schistosomiasis and other platyhelminthic diseases (WHO, 2013b). In addition, construction of bore holes and pipe borne water supply as well as health education on schistosomiasis might have contributed to the reduction of the infection in the Pru district.

5.2 *Schistosoma haematobium* infection and morbidity indicators

The detection of haematuria, proteinuria and leukocyturia at baseline that gradually reduced per weekly analysis after Praziquanuel treatment agrees with previous studies reported in 2012 (Bogoch *et al.*, 2012; Stete *et al.*, 2012). The high occurrence of haematuria (91%) and
proteinuria (53%) due to *S. haematobium* detected with urine test strip at baseline is in congruence with a similar study by Wilkins *et al.*, (1979) and a related study which asserted that, haematuria and proteinuria are associated with urogenital schistosomiasis (Sacko *et al.*, 2011). The attachment of the spine of *S. haematobium* ova to the inner walls of the bladder might have left some lesions or scar leading to the presence of small bleeds or the presence of blood in urine of the infected participant, even after termination of egg output (Sacko *et al.*, 2011; Stete *et al.*, 2012). On the other hand, proteinuria has been attributed to glomerular damage due to infections and immune complexes (Neale *et al.*, 1994). However, other researchers have associated protein in urine and with intensity of infection, suggesting that the protein is likely to originate from the lesion in the bladder and ureters created by the deposition of *S. haematobium* eggs (Wilkins *et al.*, 1979; Fu *et al.*, 2012). Haematuria, proteinuria and leukocyturia have been reported to decrease after treatment with praziquantel (Midzi *et al.*, 2008; Stete *et al.*, 2012). The weekly reduction in the occurrence of haematuria, proteinuria and leukocyturia, which persisted up to the third week after treatment in the current study agrees with the findings by Wami *et al.* (2016), who attributed the reduction in these clinical morbidity indicators, to the gradual healing of lesions and wounds created by egg deposited in the walls of the bladder. The outcome of the post treatment analysis in this study is in accordance with a similar study conducted by Stete *et al.* (2012), that have asserted that reduction in morbidity indicators (haematuria, proteinuria and leukocyturia) serve as additional parameters for assessment of Praziquantel efficacy.
5.3 Drug resistance and re-infection assessment (Egg reduction, clearance rates and viability)

Praziquantel is the drug of choice in the treatment and preventive chemotherapy of schistosomiasis and other platyhelminthic diseases (Abaza, 2013). Due to the large scale administration of this drug against schistosomiasis, it is very important to monitor its performance, so that early sign of resistance, delay clearance or drug failure can be detected. (Doenhoff et al., 2009; Utzinger et al., 2011).

Since there was no suspected drug resistance in this study, it can be said that when it comes to treatment of schistosomiasis caused by *S. haematobium*, Praziquantel continues to be potent and very useful in the Pru district. Results from some studies have suspected that Praziquantel treatment have sometimes been less effective (Mendonça da Silva et al., 2005; Alonso et al., 2006), which is slightly different from the observation made in this study. Meanwhile, another findings made in relation to Praziquantel efficacy is similar to the current observation, which indicated no resistance (Ojurongbe et al., 2014)

The re-infection rate of 19.2% observed 32 weeks post Praziquantel treatment implies that after treatment they could still get infected if they have continuous contact with the infested water bodies. Factors including socio-economic activities and occupation in the Pru district are possible reasons for this outcome. Re-visit of children to the lake for bathing, washing and assisting their parents in fishing (which is the major occupation in the area) might have contributed to the high re-infection rate recorded in the current study. Re-infection observed in this study was higher than what was recorded in a similar study conducted in the Ndumo area of uMkhanyakude district, KwaZulu-Natal, South Africa, which recorded re-infection rate of 8.1%
after 28 weeks post treatment (Kabuyaya et al., 2017). Another observation made in a related study recorded re-infection of 63%, 24 months post treatment (Goran et al., 2001), suggesting that re-infection pattern vary from one place to another due to differences in ecological factors, as well as the time interval for the post treatment assessment (Tchuente et al., 2004).

5.3.1 Egg reduction rate and clearance rate

The total egg clearance and reduction rate (ERR=100%), recorded six week post treatment in this study was in agreement with the recommended ERR range of ≥90% by the WHO’s 2013 protocolon Praziquantel efficacy assessment (WHO, 2013a; Ebai et al., 2017), within which the drug is said to be efficacious without any doubt of suspected resistance in the treatment of schistosomiasis. This observation compares favourably with similar studies conducted in Zimbabwe in basic school children that recorded egg reduction rate of 99% six weeks post Praziquantel treatment (Mutapi et al., 2011), adding up to the findings of Van Etten et al. (1997), in Gabon that recorded ERR above 90% 35 weeks after treatment. On the contrary, results obtained from the IkataLikoko area of southwest Cameroon had a reduced therapeutic effect (ERR, 80.3%) after a single follow up on the sixth week post Praziquantel treatment.
5.3.2 Egg viability assessment

The observation that there is agreement between the three viability methods (hatchability test, 1% neutral red and 0.4% trypan blue staining), used in the current study, at both baseline and post Praziquantel treatment, suggest that each method can be used as a tool for Schistosoma egg viability assessment.

The hatchability method which has been described as a very good viability method due the movement of miracidium, as it hatches out of its shell or movement of the flame cells within the shell (unhatched), in response to water and light (Shimizu, 2006), was observed in the current study. The absorption and non absorption of the vital stains (1% neutral red and 0.4% trypan blue stains) by viable and non viable S. haematobium ova observed in this study is consistent with previous studies using neutral red and trypan blue dyes (Sarvel et al., 2006; Elfaki et al., 2015).

The statistically significant (P value < 0.01) difference observed between mean percentage dead eggs recorded at baseline relative to that of post treatment, is in agreement with a similar study conducted on the Pemba Island to determine the efficacy of Praziquantel and long term appraisal of schistosomiasis control where more dead eggs were detected among children who continued to excrete eggs up to the seventh week post Praziquantel treatment, suggesting that the drug have some effect on Schistosoma egg viability (Guidi et al., 2010).
5.4 Cytological abnormalities and disease severity

Cytological changes including; squamous cell metaplasia (SCM) and hyperkeratosis with inflammation and red blood cells found in this survey among participants infected with *S. haematobium* suggest a possible link of urothelial cell changes with urogenital schistosomiasis. Similar studies conducted in Nigeria and Kenya related *S. haematobium* infection with squamous cell abnormalities such as severely dysplastic squamous cells and urinary tract hyperplasia which are precursors for bladder cancer development (Hodder *et al.*, 2000; Akinwale *et al.*, 2008). In Ghana, work done on tissue schistosomiasis at the Korle-Bu Teaching Hospital (located at the southern part of the country), by Der *et al.* (2015), found a possible link between *S. haematobium* infection and squamous cell carcinoma. This was a retrospective study conducted from 2004 to 2011, where 98.7% of the tissue schistosomiasis cases were attributed to *S. haematobium* infection, adding that, 93.6% of the affected organ (having malignant squamous cell abnormalities) was the urinary bladder.

Metaplastic squamous cell is epithelial cell change that occurs as a result of chronic inflammation of the urothelium in response to stimulating factors such as *S. haematobium* ova trapped in the bladder tissues (Botelho *et al.*, 2009; Inyang-Etoh *et al.*, 2015). This indicates the relationship between inflammation and development of squamous cell metaplasia which represent disease severity in this study (Hodder *et al.*, 2000). Disease severity (26.3%) recorded in this study possibly suggests malignant transformation which could lead to squamous cell carcinoma. Similar studies have reported the occurrence of metaplastic urothelial cells as having a strong link with *S. haematobium* infection and the development of bladder carcinoma (Khurana *et al.*, 2005).
5.4.1 Grading of Squamous Cell Metaplasia and cancer development

The current study recording of 50% mild, 25% moderate, and another 25% marked squamous cells, is of high clinical as well as public health concern. This is because, severe cases with mild metaplasia are categorized as having low grade urothelial lesion (LGUL) whilst those found with moderate and mild metaplasia grouped as high grade urothelial lesion (HGUL) (Papanicolaou and Marshall, 1945). Thus the chance of HGUL group developing cancer is high as compared to the LGUL group, which is more likely to revert back to normal cells, if immediate interventions such as early detection and treatment are met (Hodder et al., 2000). Therefore, the occurrence of HGUL in this study may reflect proliferative cell development and malignant transformation, which could lead to carcinoma formation. Studies involving mutations and schistosomal associated cancers in Egypt have shown that the risk of cancer development is high when chronic inflammation leading to mataplasia is combined with urinary carcinogens, particularly, modification of tumor suppressor genes such as p53 during chromosomal breakage (Warren et al., 1995).

5.4.2: Cytological changes following Praziquantel treatment

Squamous cell changes observed during the first three weeks and on the eighth week post treatment, relative to the-base line survey, indicate that these transitional metaplastic squamous cells, might have reversed back to their normal forms, but occurred several weeks after treatment. Studies have shown that squamous cell metaplasia is reversible, however, persistent exposure to factors that causes metaplasia may induce malignant transformation and development of cancer (Slack, 2007).

Reduction in the occurrence of inflammatory cells was observed to be consistent with reduction in egg count after treatment, this agrees with the assertion that, continuous egg deposition in the
urothelium has a link with chronic inflammation leading to squamous cell abnormalities and development of fibrosis (Gower et al., 2011). Hyperkeratosis was not used as a criterion for assessing disease severity in the current study, however, cells showing keratinizing metaplasia with inflammation were found to have a strong association with cancer development (Hodder et al., 2000). Hyperkeratosis was noted to be distributed both in the severe and the non severe groups at base-line and at post treatment. Keratinization usually occurs in response to changes such as pressure and other forms of local irritation within cellular environment. (Khurana et al., 2005).

5.5 Haematuria at baseline and post treatment with urine chemistry and urine cytology

Detection of haematuria both at baseline and after Praziquantel treatment with urine dipstick test strip and urine cytology (Papanicolaou staining) in the current study have added up to previous findings that have suggested haematuria as an indicator for diagnosing *S. haematobium* infection and assessing chemotherapeutic effect after treatment with Praziquantel (Wilkins et al., 1979; Murare and Taylor, 1987). While the results of this study confirm that, both dipstick and Papanicolaou stain showed complementing results in detecting haematuria, the slight difference in sensitivity of the dipstick to detect haematuria over the Papanicolaou stained microscopy, could be attributed to its ability to detect both haemolysed and intact red blood cells whiles Papanicolaou stain mainly demonstrate intact cells and not lysed cells (Papanicolaou and Marshall, 1945; Taylor, 1987).
5.6 Genetic variations

5.6.1 Amplification of Internal Transcribed Spacer 2 (ITS2) DNA marker

Studies involving genetic variations in schistosomes are very important since occurrence of genetic variant strains, induced by acquisition of new genes from related species or modification of contact sites to host (Ezeh et al., 2015), could influence a number of factors such as host parasite relationship, virulence, pathogenesis and disease manifestation as well as susceptibility to drugs (Dye et al., 2013; Quan et al., 2015).

All the S. haematobium ova detected by light microscopy got amplified in ITS2 by PCR. This serves as confirmation that, eggs seen in the light microscopy were actually S. haematobium even though morphologically, they might have had slight differences. This result agrees with a similar study conducted by Quan et al. (2015), in which all strains detected by microscopy in five different areas of Sudan got amplified in ITS2 before they were subjected to restriction fragment length polymorphic DNA analysis (RFLP). Again, in a study comparing the sensitivities of microscopy and serology (circulating antigen test (CCA) with PCR, all the ITS 2 region of S. haematobium in all the samples were amplified by PCR (Obeng et al., 2008).

5.6.2 Amplification of mitochondrial DNA markers; NAD 1 and COX 1

Mitochondrial genes NADH dehydrogenase subunit 1 (NAD1) and cytochrome oxidase subunit 1 (COX1), of S. haematobium have been used in genetic variation and strain identification studies (Morgan et al., 2005; Webster et al., 2012). In this study, the 20% amplification of NAD1, the 6.7% COX1 and the 2.9% amplification of both NAD1 and COX1, suggest the
possibility of genetic variations that influenced the ability or inability to express these mitochondrial genes. Even though it is known that the absence of the target gene, low amount of DNA, as well as differences in cycling conditions, can affect amplification in PCR (Webster et al., 2012). The last two reasons cannot be the cause of the non amplification of the mitochondrial genes seen in some of the samples in the current study. This is so because, the same extracted DNA from the samples used for the successful ITS2 amplification, were also the ones used for the mitochondrion genes amplification. This implies that low amount of DNA is not the reason for non amplification of the target gene. Similarly, difference in PCR cycling conditions cannot be the reason since, at least, some of the samples had amplification for the two mitochondrial genes, under the same conditions. These observations suggest that the remaining samples that did not have the mitochondrion gene amplified, are variant strains of *S. haematobium* that are non-producers of the NAD1 and COX1 genes or might have lost the genes due to mutation. A study conducted in the Pemba Island separated *S. haematobium* into two groups, based on NAD 1 and COX1 amplification (Webster et al., 2012). Another study using rapid diagnostic multiplex PCR (RD-PCR) was done to distinguish a group of *Schistosoma* species known as *S. haematobium* complex using COX 1, where strains that got amplified with COX1 were grouped as the true species of *S. haematobium* (Webster et al., 2013b).

5.7 Disease severity and expression of NAD1 and COX1 genes

Mitochondrial genes NAD1 and COX1, that produces electron transporting system and proteins, catalyzes the respiratory chain involved in energy production for cellular activities. Thus occurrence of these genes may confer high energy activities (Dingley et al., 2014). The
occurrence of variant forms of *S. haematobium* as mitochondrial gene producers (COX1 only, NAD1only and both COX1 and NAD1) and non mitochondrial gene producers has been observed in this study. However, no significant association was observed between disease severity and expression of NAD 1 or COX 1 genes (P-value = 0.5918). This finding from the current study seems to have deviated slightly from the observation made in the study conducted in Egypt, which indicated that genetic diversity is implicated in the pathology of the urothelium (Afifi *et al.*, 2016).
6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From this study, it was observed that, egg positivity of \textit{S. haematobium} was 6.5\% among the basic school children in the Pru district of Ghana. There was no therapeutic failure or drug resistance, however, re-infection rate of 19.2\% was observed among the participants. This outcome indicate that although Praziquantel remains potent in the treatment of urogenital schistosomiasis, people living around infested water bodies can be re-infected even after successful treatment.

Cytological abnormalities including; squamous cell metaplasia, hyperkeratosis, inflammation and red blood cells were found with \textit{S. haematobium} infection. Disease severity rate of 26.7\% based on the presence of squamous cell metaplasia with inflammation was recorded. Within the severe group, were cases categorized as having high grade urothelial lesion (HGUL), that are considered as having high risk of transforming into squamous cell carcinoma, indicating a link between \textit{S. haematobium} disease and the possibility of cancer development.

All the cases identified by light microscopy got amplified with \textit{S. haematobium} species specific DNA marker (ITS2), suggesting that they were true species of \textit{S. haematobium}. However, variant strains were identified as having expressed mitochondrial genes; NAD1 and COX1.
6.2 Recommendations

The outcome of this study will add to knowledge on genetic variations in *S. haematobium*, cytological abnormalities associated with urogenital schistosomiasis which could lead to bladder cancers as well as the therapeutic effect of Praziquantel in the treatment of schistosomiasis. It is however recommended that;

1. Similar studies in the remaining parts of the country be conducted to assess the situation in those places.
2. This cytological investigation is conducted also with the adult population within the same study area to determine what is happening among them.
REFERENCE


**Quinnell, R. J.** (2003). Genetics of susceptibility to human helminth infection. *Int J Parasitol,* **33**: 1219–1231


World Health Organization. (2010). Schistosomiasis Fact Sheet No. 115


www.cdc.org

www.mapdata2018.google.com

APPENDICES

Appendix I

Preparation of vital stains

a. **Neutral red dye (1%)**: Exactly 10 g of neutral red powder was weighed on a piece of clean paper and transferred into a bottle of 1 litre capacity. About 250 ml of distilled water was added to the bottle to completely dissolve the powder before topping it up with distilled water to reach the 1 litre mark. It was shook well to mix completely. The bottle was labeled and stored at room temperature.

b. **Trypan blue dye (0.4%)**: Four grams (4 g) of trypan blue powder was weighed on a piece of clean paper and transferred into a bottle of 1 litre capacity. About 250 ml of distilled water was added to the bottle to completely dissolve the powder before topping it up with distilled water to reach the 1 litre mark and shook well to mix completely. The bottle was labeled and stored at room temperature.
Appendix II

Papanicolaou staining

<table>
<thead>
<tr>
<th>Staining step</th>
<th>Reagents involved</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>95% ethanol</td>
<td>15 minutes or more</td>
</tr>
<tr>
<td>Prior to staining</td>
<td>Tap water</td>
<td>2 minutes</td>
</tr>
<tr>
<td><strong>Step 1 (nuclear staining)</strong></td>
<td>Haematoxylin stain</td>
<td>7 – 10 minutes</td>
</tr>
<tr>
<td></td>
<td>Slow running tap water</td>
<td>10 minutes</td>
</tr>
<tr>
<td></td>
<td>60% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>95% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td><strong>Step 2 (cytoplasmic staining)</strong></td>
<td>Orange G – 6 stain</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>95% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>95% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>Eosin Alcohol stain (EA)</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>95% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>Absolute ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td><strong>Step 3 (clearing)</strong></td>
<td>Xylene</td>
<td>1 minute</td>
</tr>
<tr>
<td><strong>Step 4 (mounting)</strong></td>
<td>DPX mountant</td>
<td></td>
</tr>
</tbody>
</table>
Appendix III

Primer suspension

The primers were lyophilized by adding appropriate volumes of nuclease free water to obtain a concentration of one hundred micro-molars (100 μM), as represented in the table below.

<table>
<thead>
<tr>
<th>Primers (S. haematobium)</th>
<th>Amount of substance (n mol)</th>
<th>Volume of nuclease free water (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS 2 F</td>
<td>25.24</td>
<td>252.35</td>
</tr>
<tr>
<td>ITS 2 R</td>
<td>36.5</td>
<td>365.02</td>
</tr>
<tr>
<td>NAD 1 F</td>
<td>52.62</td>
<td>526.25</td>
</tr>
<tr>
<td>NAD 1 R</td>
<td>45.72</td>
<td>457.23</td>
</tr>
<tr>
<td>COX 1 F</td>
<td>55.15</td>
<td>551.5</td>
</tr>
<tr>
<td>COX 1 R</td>
<td>36.5</td>
<td>365.02</td>
</tr>
</tbody>
</table>

The stock primers were reconstituted as working solutions by adding 90 μl of nuclease free water to 10 μl of the 100M stock solution to obtain 10M working solution.
Appendix IV

DNA extraction

Exactly 400 μl of genetic lysed buffer was added to 100 μl urine deposit to lyse the urine sample. The mixture was incubated at room temperature for 10 minutes after vortexing for 5 seconds. The mixture was then transferred into a Zymo-spin column suspended in a collection tube and centrifuged for one minute at 800 rpm. The Zymo-spin column was transferred into a new collection tube, 200 μl of DNA pre-wash buffer was added, and centrifuged for one minute at 800 rpm after which 500 μl of g-DNA wash buffer was added and centrifuged again at the same speed and time. After the last wash, the spin column was transferred into a clean micro centrifuge tube and 50 μl or more DNA elution buffer was added and kept at room temperature for 5 minutes. After the incubation period, it was centrifuged at 12000 rpm for one minute to elute the extracted DNA into the micro centrifuge tube.

Appendix V

Preparation of 1X TAE buffer from 50X stock solution

Twenty milliliters (20 ml) of the stock (50x TAE) was measured and added to 980 ml of dilled water to obtain 1 litre of 1X TAE buffer.
Appendix VI

Preparation of 1.5 % Agarose Gel

The agarose gel was prepared by adding 1.5 g of agarose powder to 100 ml of 1X TAE buffer. The suspension was heated to boil to enable the agarose to dissolve completely. Two microliters (2 μl) of ethidium bromide was added to the gel after allowing the temperature to drop to about 45°C and mixed to uniformly dissolve. The gel was poured into the gel tray with a fixed comb. The tray was allowed to stand undisturbed on a bench to cool at room temperature before the comb was removed.

Appendix VII

A table showing mean weekly egg viability post treatment in involving hatchability, and vital staining.

<table>
<thead>
<tr>
<th>Post treatment weeks</th>
<th>Hatchability</th>
<th>Neutral red</th>
<th>Trypan blue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live eggs (%)</td>
<td>Dead eggs (%)</td>
<td>Live eggs (%)</td>
</tr>
<tr>
<td>Week 1</td>
<td>81.6</td>
<td>18.4</td>
<td>95.7</td>
</tr>
<tr>
<td>Week 2</td>
<td>13.8</td>
<td>86.2</td>
<td>13.8</td>
</tr>
<tr>
<td>Week 3</td>
<td>0.0</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean</td>
<td>31.8</td>
<td>68.2</td>
<td>36.5</td>
</tr>
</tbody>
</table>
Appendix VIII

Other cytology pictures showing urothelial cell changes
Appendix IX

Consent form

PARTICIPANT’S PARENT/GUARDIAN IN A RESEARCH OF GENETIC VARIATIONS IN SCHISTOSOMA HAEMATOBIUM, DISEASE SEVERITY AND DRUG RESISTANCE/RE-INFECTION

Information: (To be read or translated to parents/guardians in their own mother tongue)

Dear Sir/Madam:

We kindly ask you to enroll your child (Children) into this study, which I will proceed to describe. This study is concentrating on children from age 5 and above, in order to find out whether the child has *Schistosoma haematobium* infection.

I would like to start by stressing that this study is strictly voluntary. Should you decide not to participate, it will have no consequences for you. Should you, at any point during the study, decide that you do not wish to participate any further, you are free to do so with immediate effect. Any such decision will be respected without any further discussion.

Summary of the study

*Schistosoma haematobium* infection is caused by a worm. A person becomes infected by getting into contact with a flowing or stagnant water body contaminated by the worm. The infection is common among people living along streams, rivers, waterways and irrigation ditches in Africa and some parts of the Middle East. The infection manifests itself as pain during urination and
blood in urine. The purpose of the study is to find out the type of *S. haematobium* infection in the area, damage they cause and the possible response to the drug of choice, Praziquantel.

If your child/ward participates in the study, I will take 20 ml of urine for laboratory diagnosis. All information gathered would be treated confidential.

**Risks**

Urine sampling is not an invasive procedure which might cause pain to the participants. However, there could be some inconveniences in producing the required amount of urine for the study.

**Benefits**

All the study participants found to be positive with *S. haematobium* would be treated with Praziquantel. Any complications with regards to the urine cytology would be reported to a physician for immediate attention.

You are allowed to ask questions. Thank you.

Yours sincerely,

Asamoah Ampong

(Investigator)

**Name of Participant:** .................................................................
PARTICIPANT STATEMENT

I have read the foregoing information. My concerns about this study have been duly addressed. I have been given an opportunity to have my questions about the study answered to my satisfaction. By signing or thumb printing it indicates that I now voluntarily agree to participate in this study knowing that I have the right to withdraw from the study at any time without it affecting my ability to access healthcare. I am also aware that I will be given a copy of the form after it has been signed.

........................................... ...........................................

Signature or Thumb print of Participant: Date:

Name of witness: .................................................................

WITNESS STATEMENT

I declare that I was present while the benefits, risks and procedures were read to the participant and all questions were answered. The participant has agreed to take part in the study. I confirm that, the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.
INVESTIGATOR STATEMENT

I certify that the participant has been given ample time to read and learn about the study. The benefits, risks, right to withdraw and procedures have been well explained and all questions and clarifications raised by the participant have been addressed.

 Signature of Researcher Date

GUARDIAN OR PARENT STATEMENT

I certify that I everything that my child and I needed to know have been duly explained to us and I give my consent for my child to take part in the study. I confirm that I have not been coerced into agreeing, and this consent has been given freely and voluntarily.

 Name of Researcher or Principal investigator: Asamoah Ampong

 Name of Guardian / Parent or legally Authorized representative

 University of Ghana  http://ugspace.ug.edu.gh
Name of Child: ……………………………………………………………………………

CHILD STATEMENT (Below eighteen (18) years)

I consent voluntarily to participate as a subject in this study and I understand benefits, risks, right to withdraw and procedures of the study. No one including my parent or guardian forced me into agreeing to participate in the study. This consent has been given freely and voluntarily.

Signature or Thumb print of Child Date

Appendix X

Child assent form

(This form is to be filled by clients below eighteen (18) years of age).

My name is Asamoah Ampong. I am a student of the University of Ghana, School of Biomedical and Allied Health Sciences; Korle-Bu. I am conducting a study on genetic variations in Schistosoma haematobium, disease severity and drug resistance in the Pru district of Ghana. I would be grateful if you participate in this research.
General Information

If you agree to participate in the study, you will be asked to provide demographic information as well as your urine samples will be collected.

Possible Benefits

All the study participants found to be positive with *S. haematobium* would be treated with Praziquantel. Any complications with regards to the urine cytology would be reported to a physician for immediate attention. Your participation in this study could also help in identifying any possible variations in *S. haematobium* strains circulating in Ghana.

Possible Risks and Discomforts

Urine sampling is not an invasive procedure which might cause pain to the participants. However, there could be some inconveniences in producing the required amount of urine for the study.

Voluntary Participation and Right to withdraw from the Research

Participation in this study is voluntary. You are allowed to answer any individual question or all the questions. You can withdraw from the study at any time. However, you are encouraged to fully participate in the study, since your samples and the information you would provide could go a long way to help the Nation as a whole.

Confidentiality

The information obtained in this study will be kept confidential and will not be accessed by unauthorized persons.
Contacts for Additional Information

If you have any questions please, contact the principal investigator, Asamoah Ampong. (Tel: 0249312515 or email: ampong.asamoah@yahoo.com). You may also contact his supervisor Dr. Patience B. Tetteh-Quarcoo (phone number 0244633251 or e-mail patborket2002@yahoo.com).

Please discuss this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before enrolling you into the study. Even if your parents say “yes” you can still decide not to participate.

Before taking the Consent

Do you have any concerns about the study that you wish to be addressed?

[ ] Yes [ ] No

If yes, please indicate your concerns below.

........................................................................................................................................................................
........................................................................................................................................................................
........................................................................................................................................................................

Name of Participant: ........................................................................................................................................

PARTICIPANT STATEMENT

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any question I have asked have been answered to my satisfaction. I
consent voluntarily to participate as a subject in this study and understand that I have the right to withdraw from the study at any time.

.............................................. ..............................................

Signature or Thumb print of Participant: Date:

**Name of witness:** .................................................................

**WITNESS STATEMENT**

I declare that I was present while the benefits, risks and procedures were read to the participant and all questions were answered. The participant has agreed to take part in the study. I confirm that, the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

.............................................. ..............................................

Signature or Thumb print of witness: Date

**Name of Researcher or Principal investigator:** Asamoah Ampong
INVESTIGATOR STATEMENT

I certify that the participant has been given ample time to read and learn about the study. The benefits, risks, right to withdraw and procedures have been well explained and all questions and clarifications raised by the participant have been addressed.

............................................  ............................................

Signature of Researcher            Date

Guardian or Parent Statement

I certify that I everything that my child and I needed to know have been duly explained to us and I give my consent for my child to take part in the study. I confirm that I have not been coerced into agreeing, and this consent has been given freely and voluntarily.

............................................  ............................................

Signature or Thumb print of Guardian or Parent            Date

Name of Child: ..............................................................................
CHILD STATEMENT (Below eighteen (18) years)

I consent voluntarily to participate as a subject in this study and I understand benefits, risks, right to withdraw and procedures of the study. No one including my parent or guardian forced me into agreeing to participate in the study. This consent has been given freely and voluntarily.

………………………………..………………………………

Signature or Thumb print of Child Date
Appendix XI

Questionnaire

GENETIC VARIATIONS IN SCHISTOSOMA HAEMATOBIUM, DISEASE SEVERITY AND DRUG RESISTANCE/RE-INFECTION IN THE PRU DISTRICT OF GHANA

I.D of Participant …………………………………………………………………………………………………………

Age of the Participant ……………….. Sex of the Participant (Male/ Female)

Class of Participant…………………………

Occupation of Parent/Guardian ………………………………………………………………………………….

Ever visited the lake site?   Yes ( )   No ( ).  How many times, if yes……………….?  

Ever urinated blood?       Yes ( )       No ( ) How long, if yes ………………………

Duration of residence in the community ………………………………………………………………………

Distance of house to water body …………………………………………………………………………………

Ever received treatment?   Yes ( )   No ( )     How many times, if yes……………….

96
Appendix XII

Ethical clearance

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.: CHS/EPRC/APRIL/2018

Asamoah Ampong
Department of Medical Microbiology
School of Biomedical and Applied Health Sciences
Korle- Bu

April 30, 2018

ETHICAL CLEARANCE

Protocol Identification Number: CHS-Et/M.8 – P2.11/2017-2018

The College of Health Sciences Ethical and Protocol Review Committee on April 26, 2018 reviewed and unanimously approved your research proposal.

Title of Protocol: “Genetic Variations in Schistosoma Haematobium, Disease Severity and Drug Resistance in the Middle Belt of Ghana”

Principal Investigator: Asamoah Ampong

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee’s duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till April 30, 2019.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: ........................................
Professor Andrew Anthony Adjei
Chair, Ethical and Protocol Review Committee

Cc: Provost, CHS
Dean, SBAHS
Head, Dept. of Medical Microbiology

University of Ghana  http://ugspace.ug.edu.gh
Appendix XIII

Letter of permission to embark on a research project with school children in the Pru district

GHANA EDUCATION SERVICE
PRU DISTRICT

Pru District Education Office
Post Office Box 6
Yeji – B/A

January 31, 2018

The Circuit Supervisors
Yeji North, East, Prang North & South
Yeji-B/A

LETTER OF PERMISSION TO EMBARK ON RESEARCH PROJECT ON SCHOOL PUPILS

The directorate as per the attached, grants and introduces to you the bearer of this letter in the person of Mr. Asamoah Amond of the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, College of Health Sciences, Korle Bu Circuit Supervisors, Headteachers/Headmasters, teachers as well as students/pupils of the concerned circuits are hereby entreated to accord him all the necessary supports he so requests

Circuit Supervisors, Headteachers/Headmasters must note that the researcher must commence duty effective February 15, 2018 and ends by April 6, 2018. Any extension must require further permission from the directorate.

[Signature]

SETH OPOKU BOATENG (Mr)
DEPUTY DIRECTOR F&A

FOR DISTRICT DIRECTOR
FINANCE ADMIN. CONTROLS
GHANA EDUCATION SERVICE
PRU DISTRICT

cc
1. The Headteachers/Headmasters Concerned
2. All First Line Directors