Molecular Diagnosis of Schistosomiasis: Application of Real-time PCR for Pre- and Post- Treatment Evaluation

A Thesis submitted to the Department of Theoretical and Applied Biology, College of Science, Kwame Nkrumah University of Science and Technology in partial fulfilment of the requirements for the Award of Master of Philosophy Degree

By

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DECLARATION:

I hereby declare that expect for references to other people's work, which have duly been acknowledged this exercise is a result of my own research and this thesis neither in whole nor in part had been presented for another degree elsewhere.

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ABSTRACT

Schistosomiasis, a major human helminthic disease in terms of mortality and morbidity infects over 200 million people worldwide. It is a major health problem due to its high prevalence in affected communities and its ability to manifest into severe clinical forms. New diagnostics for detection of infections are essential considering that the present parasitological methods, though specific, are not very sensitive and not always able to judge accurately the performance of Praziquantel (PQZ) in terms of adult worm death but rather in terms of cessation of egg excretion. A highly sensitive real-time PCR (RT-PCR) developed for the detection of *Schistosoma sp.* DNA in both urine and faeces sample was compared with microscopy detection of schistosome eggs. Both urine and stool samples were collected from study participants aged between 3 and 20 years before and after (3 weeks and 8 weeks) treatment with PQZ. Study participants were found to have either single *(S. mansoni or S. haematobium)* or mixed *S. mansoni and S. haematobium* infections by microscopic examination of urine and stool samples. Utilising the two diagnostic methods, microscopy detected both *S. haematobium* and / or *S. mansoni* eggs in 93% (133) urine and/ or stool samples collected before treatment whereas RT-PCR amplified DNA in 97.2% (139) of same samples. A significant reduction in prevalence and intensity of infection was observed after PZQ treatment with no significant differences between sexes and age groups using both detection methods. Exhibiting a higher sensitivity, RT-PCR detected *Schistosoma* DNA in 67% and 69.3% urine and/ or stool samples tested at 3 and 8 weeks post treatment respectively whereas microscopy detected schistosome eggs in 25% and 30.7% at 3 and 8 weeks post treatment respectively. RT-PCR therefore provided a lower estimation of the cure rate than the parasitological technique (microscopy detection of eggs) since it detected more positive cases at all post-treatment surveys. RT-PCR amplified 35.4% (75/212) of urine egg negative samples and 67.1% (94/140) of the stool egg negative samples when comparing both microscopy and RT-PCR for the entire study population over all three time periods. However

RT-PCR failed to amplify schistosme DNA in 20 individuals with light egg intensity infection in urine and 3 individuals with light egg intensity in stool. Results of this study show RT-PCR to be significantly more sensitive than microscopy in detecting and evaluating infection prevalence, an important aspect of epidemiological studies. Most probably, RT-PCR for evaluation of treatment resulted in lower cure rate due to its ability to detect DNA from schistosome eggs (devitalised or containing a living miracidium), including cell-free DNA released from damaged eggs and any juvenile or adult worm DNA breakdown products in the faecal and urine samples even if the drug is killing the vast majority of worms. Thus, RT-PCR technique can be especially useful in circumstances of lower intensity or prevalence of infection, a condition for which the parasitological examination shows a well-documented limitation of its sensitivity.

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CHAPTER ONE

INTRODUCTION

Schistosomiasis occurs in many parts of the world, particularly Africa, South America and Asia, where more than 600 million people live in schistosomiasis transmission zones (Gryseels *et al.*, 2006). An estimated 207 million people are presently infected worldwide, and of these, 85% live in sub-Saharan Africa (King and Dangerfield-Cha, 2008). It is one of the most important human helminthes infections in terms of morbidity and considered as a major health problem due to its high prevalence in affected communities and its ability to manifest into severe clinical forms (Montresor *et al.*, 1997). While recognized as a debilitating disease causing considerable morbidity, it is also estimated that annual global mortality could be as high as 300,000 (Rollinson, 2009). In order to control the severe morbidity, regular chemotherapy with Praziquantel (PZQ) is required (Utzinger *et al.*, 2009). An estimated 128 million school-aged children require annual PZQ treatment for rudimentary control of schistosomiasis if the World Health Oorganization's (WHO) goal of treating at least 75% of all infections in school-aged children is to be realised (Utzinger *et al.*, 2009).

Schistosomiasis is also an emerging problem in non-endemic areas due to broader distribution of vector snails and increased immigration. In order to effectively control schistosomiasis, reliable, accurate, precise, specific and sensitive diagnostic methods are needed for proper identification of infection and evaluation of chemotherapy in field situations (Wang *et al.*, 2011). Most epidemiological assessments of the burden of schistosomiasis have relied on microscopic examination for both intestinal and urinary schistosomes. This method provides a relatively easy and cheap tool for detecting and estimating the concentration of schistosome eggs in faecal and urine samples in many poor countries endemic with schistosomiasis (Lengeler *et al.*, 2002). However, the difficulties in meeting the multiple sampling requirements for classical parasitological diagnosis often lead to suboptimal results (because

of the false-negative cases). Samples need to be collected at suitable or specific times for a good recovery of parasite eggs, and processed within 24-48 hours with careful examination to give high sensitivity particularly when infections are low (Gryseels and De Vlas, 1996). Consequently, direct parasitological detection techniques are most often associated with poor sensitivity which limits both the diagnosis of individuals with early or low level infections and evaluation of the efficacy of chemotherapy (Wang *et al.*, 2011).

Immunodiagnostic techniques, which are more sensitive and simpler to perform, have also become a common epidemiological tool for screening target populations in many schistosome-endemic areas. However, detection of the antibodies lacks specificity (Rabello *et al.*, 2002) and immunodiagnostic techniques such as circumoval precipitin test (COPT), indirect haemagglutination test (IHA) and enzyme -linked immunosorbent assay (ELISA) used for chemotherapy evaluation, are associated with high positive rates for detection of schistosomal antibodies over a long period of time (40.2%-41.2%, 1 to 2 years post-treatment and 4.26% - 17.5% after at least 3 years of treatment) (Wang *et al.*, 2011). This limits the application of immunodiagnostics for detection of new infection and evaluation of chemotherapy.

It has been reported that assays based on polymerase chain reaction (PCR) techniques, have shown high sensitivity and specificity for the detection of parasitic DNA, having been successfully used to reveal the presence of DNA from a broad range of parasites including schistosoma sp (Obeng *et al.*, 2008; ten Hove *et al.*, 2008). The ability of this technique to exponentially amplify a minute amount of DNA too small for direct analysis by many orders of magnitude to a larger volume that can be detected and even quantified makes it a better diagnostic tool for detection of infection. Its high sensitivity allows diagnosis of schistosomiasis when no eggs are detected by microscopy, which is the case during the acute phase of the infection when worms have not yet produced large numbers of eggs, during

active and chronic infections with low egg loads (Nicolls *et al.*, 2008; Clerinx and Van Gompel, 2011) and in cases with low egg excretions as seen in inmunocompromised persons (Mwinzi *et al.*, 2004). A significant improvement introduced by Real-time PCR (RT-PCR) is the relatively quick production of results for a large number of samples and the early detection of amplicon by use of sensitive fluorescence detection equipment (Nitsche *et al.*, 2000).

1.2 Rationale of study

Despite the fact that the focus of national schistosomiasis control programs is on morbidity reduction, monitoring and evaluation of these programs is largely dependent on measuring infection as proxy for morbidity, therefore, accurate and early case identification are critical for the effective execution of these control programs. Microscopic detection of *Schistosoma* eggs in stool or urine specimens is considered the gold standard for diagnosing schistosomiasis in patients, as well as for monitoring the effectiveness of the treatment. However, this method lacks the sensitivity in low prevalence and in post-treatment situations (Pontes *et al.*, 2003). Even when the disease is active, the analyzed samples might not contain eggs due to the random distribution and highly variable shedding of the eggs (Wang *et al.*, 2011). Furthermore, there is lack of sensitivity in the detection of circulating parasite antigens in low prevalence areas and in post-treatment cases whereas the detection of anti-*Schistosoma* antibodies lacks specificity (Lier *et al.*, 2006).

The morbidity associated with schistosomiasis has been controlled in some endemic areas through chemotherapy. However, it is difficult to eliminate the disease completely and the epidemiologic situation persists at a low level both in prevalence and the intensity of infection. In order to address this issue, research is now focused on identification of sensitive and specific diagnostic tests for early identification with schistosomiasis infection and post

treatment evaluation. Recently, several groups have employed more specific and sensitive diagnostic methods, mainly using the PCR techniques (Pontes *et al.*, 2002; Gobert *et al.*, 2005; Xia *et al.*, 2010). These PCR techniques are able to detect DNA from schistosome eggs (devitalised or containing a living miracidium), including cell-free DNA released from damaged eggs and any juvenile or adult worm DNA breakdown products in faecal, urine and serum samples (Enk *et al.*, 2012). Other studies have also explored the use of the RT-PCR format successfully (Obeng *et al.*, 2008; ten Hove *et al.*, 2008) and is preferred to conventional PCR methods as it avoids post-PCR handling and contamination, high sensitivity and a short turnover time (Cnops *et al.*, 2012). This work, therefore, sets out to use RT-PCR technique and the protocol described by Obeng *et al.* (2008) to monitor and evaluate treatment of schistosomiasis.

1.2.1 General Objective

This study aimed at using RT-PCR technique for the diagnosis of *Schistosoma sp.* infection in Tomefa before and after PZQ treatment, to evaluate treatment efficacy.

1.2.2 Specific Objectives

The specific objectives were to determine:

- prevalence and intensity of *Schistosoma sp*. infection by microscopic egg detection in participants before treatment
- prevalence of *Schistosoma* sp DNA by RT-PCR in participants before treatment
- prevalence of infection 3 and 8 weeks after treatment by microscopy detection of eggs and RT-PCR amplification of *Schistosoma sp*. DNA
- Compare cure rates at 3weeks and 8 weeks post-treatment as measured by RT-PCR and microscopy detection of infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Schistosomiasis

An estimated 200 million people in 74 countries have schistosomiasis; 85% of whom live in sub-Saharan Africa (Chitsulo *et al.*, 2000). It is reported as the second most socioeconomically devastating parasitic disease after malaria, most commonly found in Asia, Africa, and South America, particularly in areas where the water bodies contain numerous freshwater snails, which may carry the *Schistosoma* parasite (Mutapi, 2011). The disease is more prevalent in children who may acquire the disease by swimming or playing in infected water. Although it has a low mortality rate, schistosomiasis is often a chronic disease that can damage internal organs and, in children, impair growth and cognitive development (Utzinger *et al.*, 2009).

Schistosomiasis is a waterborne disease caused by trematode blood fluke of the genus Schistosoma. The three major schistosome sp affecting man; *S. mansoni*, *S. haematobium* and *S. japonicum*, are distributed in various tropical and sub-tropical areas (Gryseels *et al.*, 2006). *Schistosoma mansoni* is prevalent in many countries of Africa, parts of the Middle East, South-America and in the Caribbean. *Schistosoma haematobium* is distributed in parts of Africa and in the Middle East, whereas *S. japonicum* is distributed in China, Indonesia and the Philippines. Other human sp have a more restricted distribution; *S. intercalatum* in the Democratic Republic of Congo, *S. guineensis* in Central-Africa and *S. mekongi* in Laos and Cambodia (Webster *et al.*, 2006).

Infection with schistosomiasis occurs when larval forms of the parasites, known as cercariae, are released from the intermediate hosts (aquatic snails belonging to the genera *Bulinus*, *Oncomelania*, *Biomphalaria* and *Neotricula*) and penetrate the human skin during water contact. The parasite eggs are deposited in several tissues, primarily the liver, the urinary

bladder and the urinary tract. Chronic infections result in periportal fibrosis of the liver, calcification of the urinary bladder and other infections including genital schistosomiasis which might increase the risk of HIV infection (Poggensee *et al.*, 2001).

2.1.1 Global estimates of infections

Schistosoma haematobium is the causative agent of most of the estimated cases of schistosomiasis that occur worldwide, most of which are reported in Sub-Saharan Africa (Mutapi, 2011). In 2002, World Health Organization expert committee estimates showed that 27,000 people die annually from schistosomiasis. van der Werf *et al.* (2003), on the other hand, using the limited data available from Africa, estimated the schistosomiasis mortality alone at 280,000 per year. This estimate included the prevalence of non-functioning kidneys associated with *S. haematobium* infection and hematemesis (vomiting of blood) from *S. mansoni* infection. In addition, it was estimated that urinary schistosomiasis in Africa results in approximately 18 million cases of bladder wall pathology and 20 million cases of hydronephrosis (distension and dilation of the renal pelvis), and African intestinal schistosomiasis results in approximately 8.5 million cases of hepatomegaly (condition of an enlarged liver) (van der Werf *et al.*, 2003). In a related study, it was also concluded that, in Africa the mortality attributable to urinary schistosomiasis could be as high as 150,000 per year, and the number dying as a result of intestinal schistosomiasis could be as high as 130,000 per year (Fenwick *et al.*, 2003). The burden of disease is disputed because original estimates did not consider symptoms, sequelae and the chronic nature of schistosomiasis. A WHO (2002) expert committee concluded that annual deaths could be as high as 200,000, compared with 15,000 as had been reported. An analysis of these discrepancies indicates that there is underestimation, making schistosomiasis second only to malaria among tropical diseases as a cause of morbidity. A strategy of morbidity control through chemotherapy has

resulted in successful control in Brazil, Northwest Africa, the Middle East, China and the Philippines (WHO, 2002). There is a threat of resurgent transmission in China due to ecological changes owing to the construction of the 'Three Gorges' dam, and in the Philippines owing to inadequate support for public health interventions (WHO, 2002). Economic development has virtually eliminated transmission of the disease from the Caribbean and Mauritius (WHO, 2002).

Most African countries and some countries in the Middle East are endemic for *S. haematobium* which causes urinary schistosomiasis. *Schistosoma mansoni* and *S. intercalatum* responsible for intestinal schistosomiasis, have been reported in some countries in Africa (Chitsulo *et al.*, 2000). *Schistosoma haematobium* afflicts an estimated 111 million persons in Africa and the Middle East resulting in minimal pathology, frequently occurring in the bladder (van der Werf *et al.*, 2003). Ultrasonography is the main method for the detection of pathology in the urinary system as it is a non-invasive and an innocuous technique (Hatz *et al.*, 1998). There is a significant proportion of *S. haematobium*-infected patients (25-50%) who experience moderate to severe morbidity (Chitsulo *et al.*, 2000). Population surveys in endemic areas indicate that haematuria, dysuria, anaemia, and inflammatory urinary tract pathology are the most common forms of *S. haematobium*-associated disease (Hatz *et al.*, 1998). Urinary schistosomiasis typically manifests as a lifelong process of tissue injury because, although individual *S. haematobium* worms survive only 4-5 years, multiple worm infections and re-infection are common in high-risk areas, so that parasite-related inflammation and fibrosis continue for decades. After 20 to 30 years of infection, the late complications of *S. haematobium* infection begin to emerge clinically. These more life-threatening, late disorders include kidney dysfunction, obstruction of the urethras or bladder outflow, and/or urothelial metaplasia and cancer formation (Hatz *et al.*, 1998). In intestinal schistosomiasis, individuals infected with *S. mansoni* can have pathologic changes in the

intestines, liver and sometimes the spleen. It can result in abdominal pain, diarrhoea, and blood in the stool. Liver enlargement is common in advanced cases, and is frequently associated with an accumulation of fluid in the peritoneal cavity, hypertension of the abdominal blood vessels and enlargement of the spleen (Danso-Appiah *et al.*, 2004).

2.1.2 Vector ecology and distribution

Each *Schistosoma* sp has its own aquatic snail sp as a vector for their transmission and the specificity of the interaction is primarily responsible for the geographical range of schistosomiasis; for example, *Biomphalaria* (*S. mansoni*), *Bulinus* (*S. haematobium, S. intercalatum*), *Oncomelania* (*S. japonicum*), *Tricula* (*S. mekongi*) (Woolhouse and Chandiwana, 1989). The snails that act as vectors for schistosomiasis in Africa belong to two genera (Biomphalaria and Bulinus). The genus Biomphalaria is medium sized, reaching 22 mm in diameter and more than 3 mm in height when fully grown. The whorls of the snail's shell are evenly convex, angular or carinate (i.e. with a strong spiral ridge or keel); in some sp the last whirl descends to produce a concave underside. The genus Bulinus has a sinistral (the aperture is on the left when the shell is held with its aperture towards the observer) shell with a spire which is highly varied in its shape and height relative to the aperture (Mandahl-Barth, 1962). *Biomphalaria pfeifferi* is the common host for *S. mansoni* in Ghana, Tanzania, Zimbabwe, Zambia, Mozambique and South Africa, while the hosts of *S. haematobium* are *Bulinus globosus* in Ghana, Angola, South Africa, Malawi, Mozambique, Zambia, Zimbabwe and Tanzania. *Bulinus africanus* is the intermediate host in Tanzania, Zambia and Mozambique while *B. nasustus* is a host in Tanzania (Woolhouse and Chandiwana, 1989).

These snails require well-defined ecological conditions to thrive and are often associated with man-made impoundments or small, seasonal streams or ponds used intensively by both humans and livestock (Hamburger *et al.*, 2001). Average temperature plays a role; for

example, the snails cannot proliferate when it is too cold whilst some snail sp resist periods of long-term drought which explains the occurrence of schistosomiasis in locations where there is only abundant water during the rainy season and no transmission during the dry season (Woolhouse and Chandiwana, 1989). When stranded on dry land by a falling water level the snails make little or no attempt to escape back to the water, thus being exposed to the risk of death by desiccation or predation. The snails are generally found in shallow waters near the shore. Under natural conditions it is rare to find them in depths exceeding 2metres. This is correlated with the availability of food and shelter for the snails, which are available only near the surface. When an individual snail is infected with one trematode and afterwards is re-infected with a trematode of another sp, biological antagonism between the parasites can occur, with high probability that one of the two will be eliminated (Woolhouse and Chandiwana, 1989).

2.2 Life cycle and transmission of Schistosomiasis

Schistosomes have a typical trematode-vertebrate-invertebrate lifecycle, with humans being the definitive host. An essential requirement for the transmission of schistosomiasis is human contact with contaminated water bearing the snail intermediate hosts of the genus *Bulinus*, *Biomphalaria* and *Oncomelania* (Jourdane and Cheng, 1987). The life cycles of all five human schistosomes are broadly similar (Figure 1): parasite eggs are released into the environment from infected individuals, rupturing on contact with fresh water to release the free-swimming miracidium that swims actively in the water by means of fine hairs (cilia) covering its body (Jourdane and Cheng, 1987). The miracidium survives for about 8-12 hours, during which time it infects fresh-water snails (by penetrating the snail's foot) in order to develop further.

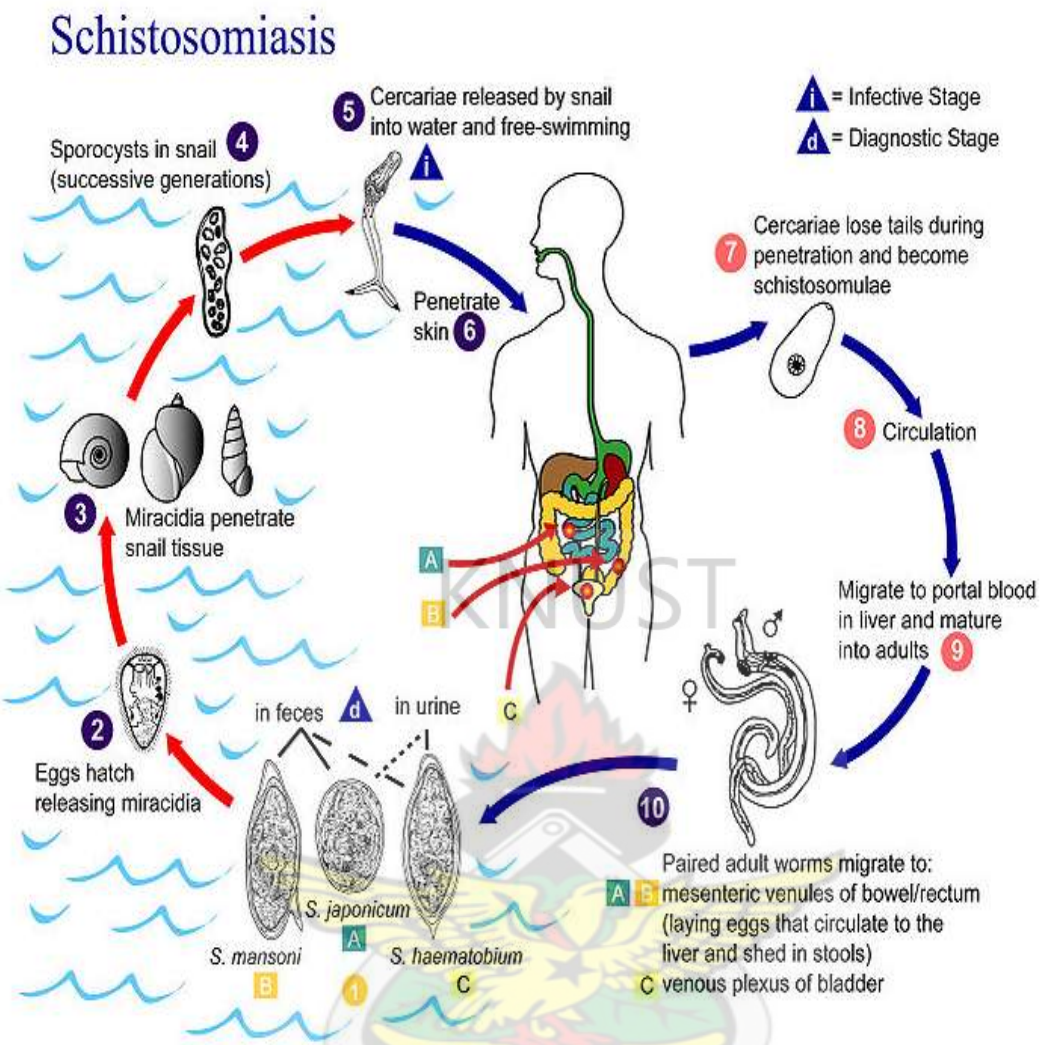


Figure 1: Life cycle of Schistosoma species (Source: http:// www.dpd.cdc.gov/dpdx)

After infection, the miracidium transforms into a primary (mother) sporocyst close to the site of penetration. Germ cells within the primary sporocyst divide to produce secondary (daughter) sporocysts, which migrate to the snail's hepatopancreas. Once at the hepatopancreas, germ cells within the secondary sporocyst begin to divide again, this time producing thousands of new parasites, known as cercariae, which are the larvae capable of infecting mammals (Wu and Halim, 2000). Cercariae emerge daily from the snail host in a circadian rhythm, dependent on ambient temperature and light. Young cercariae are highly motile, alternating between vigorous upward movements and sinking to maintain their position in the water (Jourdane and Cheng, 1987). Cercarial activity is particularly stimulated by water turbulence, shadows and human skin chemicals. Penetration of the human skin occurs after the cercariae have attached to and explored the skin. The parasite secretes enzymes that break down the skin's protein to enable penetration of the cercarial head through the skin. As the cercariae penetrates the skin it transforms into a migrating schistosomulum stage which may remain in the skin for 1-2 days before locating a post-capillary venule from where it travels to the lungs to undergo further developmental changes necessary for subsequent migration to the liver (Sturrock, 2001).

Eight to ten days after penetration of the skin, the parasite migrates to the liver sinusoid; juvenile worms develop an oral sucker and it is during this period that the parasite begins to feed on red blood cells (Sturrock, 2001). The nearly-mature worms pair up with the longer female worm residing in the gynaecophoric channel of the male, wholly depending upon the male for nutrition. Worm pairs relocate to the mesenteric or rectal veins with *S. haematobium* ultimately migrating from the liver to the perivesical venous plexus through the haemorrhoidal plexus. Once they have fully matured, adult schistosomes are unable to undergo any further migration through the body (Abdel-Wahab *et al.*, 1992).

Parasites reach maturity in 6-8 weeks, at which time they begin to produce eggs. Adult *S. mansoni* pairs residing in the mesenteric vessels may produce up to 300 eggs per day during their reproductive lives. *Schistosoma japonicum* may produce up to 3000 eggs per day (Corachan, 2002). Many of the eggs pass through the walls of the blood vessels, and through the intestinal wall, passing out of the body in faeces whereas *S. haematobium* eggs pass through the bladder wall and into the urine. Up to half the eggs released by the worm pairs become trapped in the mesenteric veins, or will be washed back into the liver, where they will become lodged (Shebel *et al.*, 2012). Worm pairs can live in the body for up to five years. Trapped eggs mature normally, secreting antigens that elicit a vigorous immune response (Corachan, 2002). The eggs themselves do not damage the body but rather the cellular infiltration resultant from the immune response that causes the pathology classically associated with schistosomiasis.

2.3 Diagnosis of Schistosomiasis

Diagnosis plays a vital role in the control of schistosomiasis. Though there are limitations, all diagnostic techniques produce results that influence decisions on individual and community treatment, estimations of prognosis and assessment of morbidity, evaluation of chemotherapy and other control measures (Feldmeier and Poggensee, 1993).

2.3.1 Parasitological Methods

2.3.1.1 Direct Methods

Schistosome ova in urine, stool or the rectal mucosa, or schistosomula, adult worms or eggs in tissue biopsies may be detected using direct (Microscopy) methods. Although tedious and time consuming, microscopy still remains a standard method for detecting schistosome eggs

in urine and faeces (Feldmeier and Poggensee, 1993). The eggs of *S. haematobium* are ellipsoidal with a terminal spine, *S. mansoni* eggs are also ellipsoidal but with a lateral spine, and *S. japonicum* eggs are spheroidal with a small knob (Shebel *et al.*, 2012).

A syringe filtration technique using filter paper, polycarbonate or nylon filters is recommended for diagnosis of urinary schistosomes quantitatively. This technique allows urinary egg counts to be performed. 10ml of urine is usually filtered, but filtrations of volumes up to 3000ml using a suction pump have been reported (Feldmeier and Poggensee, 1993). In the centrifugation method, the urine sample is centrifuged and the pellets observed under the microscope. The sensitivity of centrifugation compares favourably with that of the usual filtration of 10ml in low sensitivity infection (Feldmeier and Poggensee, 1993).

Microscopic diagnosis of stool samples for intestinal schistosomiasis may involve methods such as the Kato-Katz technique, direct smear examination, and the formalin-ethyl acetate method. Primarily, the standard microscopical diagnostic method has inadequate sensitivity due to great fluctuations of egg output in urine or stool, hence some of the eggs are undetected (Engels *et al.*, 1996).

However, sample preparation for direct observation is time-consuming, labour intensive, and proper diagnosis depends on qualified laboratory technicians. In the case of slide reading, a second independent reading is preferable, but not always required for accurate diagnosis. If need be, divided readings are resolved by a third reader (Feldmeier and Poggensee, 1993). In endemic regions, where resources are limited, this has proved to be difficult and misdiagnosis can significantly impact patient care. In reality, all major intestinal helminth infections are still solely dependent on microscopy for diagnosis and for other parasite infections; many are confirmed by the use of microscopy in conjunction to other methods of diagnosis including serology-based assays and more recently molecular-based assays (Ndao, 2009)

Diagnosis by microscopy is further complicated by interactions between *S. haematobium* and *S. mansoni in* hyperendemic areas where multiple infections call for more sensitive differential diagnosis, especially in places where the efficacy of PZQ is low (Feldmeier and Poggensee, 1993).

2.3.1.2 Indirect Methods

A number of indirect indicators exist for both intestinal and urinary schistosomiasis, but those for urinary schistosomiasis are more specific (Feldmeier and Poggensee, 1993). The reason is that whereas pathology for urinary schistosomiasis is limited to the lower urinary tract, those for intestinal schistosomiasis are more generalized. At present, no single indirect indicator of pathology for both infections possesses sufficient sensitivity and specificity to merit the substitution for parasitological methods (Feldmeier and Poggensee, 1993).

Haematuria (blood in urine), proteinuria and leucocyturia forms a common indirect sign of infection and may be detected using paper strips soaked in a reactive agent,. In order to improve the sensitivity and specificity of the technique, it is recommended that all three parameters are determined together (Feldmeier and Poggensee, 1993). The simultaneous reading of all three parameters gives a better correlation to egg output than each parameter alon . A major disadvantage of microhaematuria is that it can lead to false-positive results for child-bearing women when menstruation blood contaminates the urine (Feldmeier and Poggensee, 1993). For intestinal schistosomiasis, the repeated presence of blood (or occult blood) in stool is indicative of a high intensity of infection.

2.3.2 Serological methods

Methods employed include techniques that detect host's antibodies or circulating antigens secreted by different life cycle stages of the schistosomes. Feldmeier, (1993) reported that antigens may also be present in the hatching fluid of viable eggs. Some of the immunological methods include Indirect Immunoflourescent (IIF), Immunoblot assay, Enzyme-linked immunosorbent assay (ELISA), Cercarial-Huller reaction (CHR) and the recently developed dipstick ELISA technique which is a rapid and specific method and has proven to be more sensitive than the standard microscopic method (Bosompem *et al.*, 1997). It is cost-effective and very easy to apply on the field. It is, however, not a conventional method and has to be applied alongside microscopy. Another limitation is that it sometimes fails to detect some microscopically confirmed infections (Bosompem *et al.*, 1997).

2.3.3 Molecular Methods

The use of molecular methods for schistosome differentiation dates back to 1984 when McCutchan *et al.* (1984) showed the utility of rRNA probes for the analysis of restricted fragment length polymorphisms (RFLPs). Polymerase chain reaction (PCR) has shown its usefulness in the clinical approach of a wide variety of pathogenic infections, such as human immunodeficiency virus (HIV), *Legionella pneumophila, Plasmodium falciparum,* and *Trypanosoma cruzi* (Pontes *et al.*, 2002). In the study of *Schistosoma* sp, it has also been successfully used for sex determination of the cercariae, for the cloning and sequencing of specific genes, in the determination of genetic variability and population structure of *Schistosoma* strains and species, and in the development and application of new techniques to generate expressed sequence tags (Pontes *et al.*, 2002). Molecular-based approaches such as loop-mediated isothermal amplification (LAMP), RT-PCR polymerase chain reaction, and Luminex have shown a high potential for use in parasite diagnosis with increased specificity

and sensitivity over the existing diagnostic tests (Ndao, 2009). They permit the detection of infections from very low parasitized samples including those from asymptomatic patient's samples (Ndao, 2009). Moreover, multiplexed PCR allows for the detection of multiple sequences in the same reaction tube proving useful in the diagnosis of several parasitic infections simultaneously (Verweij *et al.*, 2007).

2.4 Prevention and Control

Control of schistosomiasis is targeted at reducing morbidity in communities to a level that will no longer be of public health importance, preventing people from contaminating water with their urine and faeces, as well as reducing contact with such contaminated waters. Researchers are using several control measures in attempts to eradicate or control the spread of schistosomiasis in endemic areas. These include the application of chemotherapy, health education and the provision of safe drinking water and sanitation facilities (King, 2010).

Chemotherapy plays a very important role in the control of schistosomiasis, as in all other helminthic diseases (Fenwick *et al.*, 2003). PQZ and oxamniquine are the safe and effective drugs used against schistosomiasis in Africa and the Americas (Ferrari *et al.*, 2003). Treatment of schistosomiasis has shown great advances with the introduction of PQZ into the therapeutic arsenal (Stothard *et al.*, 2013). Advantages of this drug include its easy administration (40 mg/kg body weight, a single dose by the oral route), low toxicity, and low intensity of side effects and have also become significantly less expensive (Rey, 2001). These important factors have contributed to the tolerance and easy application of individualized and mass treatments. Chemotherapy, apart from being costly needs to be repeated during treatment at relatively short intervals which varies between endemic areas according to the prevalence and intensity of infection prior to treatment (Stothard *et al.*, 2013).

With the introduction of safe drugs for the treatment of schistosomiasis and the improvements in water supply and sanitation in many places, snail control is employed less often as a means to control the disease (WHO, 2002). However, it still remains an important and effective measure of control especially where transmission occurs to a significant extent through children playing in water. Snail control can be achieved through environmental, biological and chemical means. Environmental control is a matter of concern for both the definitive and the intermediate hosts (Wu and Halim, 2000).

The principal objective in the case of the definitive host is prevention of human contact with snail infested water, while changes in the ecology of snail habitats help eliminate the snails and hinders their breeding (WHO, 1985). The snail intermediate hosts are aquatic and prefer to attach to plants in waters with low velocities. The plants provide the snails with food, shelter and serve as substrates for their eggs. In irrigated fields, periodic draining of canals and ditches will reduce snail populations and rapid complete drainage reduces the amount of vegetation and kills the snails by desiccation (WHO, 1985). In natural breeding sites, alternatives to drainage include filling, increasing velocity of flow, stream straightening, deepening of marginal areas, removal of vegetation and prevention of pollution (Chitsulo *et al.*, 2000).

Biological control utilizes diseases and predators of snails such as sciomyzid larvae (Insecta; Diptera), birds that may eat snails or pathogenic bacteria (Chitsulo *et al.*, 2000). The introduction of non-vector snails that compete with the snail vectors is an important biological control measure. For example, *Marisa connuarietis* (Ampullaridae), which was introduced into Puerto Rico, was found nearly to eliminate *Biomphalaria glabrata,* the vector of *S. mansoni* (WHO, 1985).

The use of molluscicides for snail control has always had a role in integrated control programmes in the Philippines, Egypt, and Ghana (Chu *et al.*, 1981a). In the past, molluscicides were often applied on an area-wide basis. However, this costly and environmentally harmful method has been replaced by focal application were studies are first carried out to identify sites and seasons of transmission to allow for the periodic application of the chemicals (Chu *et al.*, 1981b). Chemical control of schistosomiasis may have some disadvantages, some of which include repeated long term application by highly skilled personnel, expensive chemicals, adverse effects on non-target organisms particularly fish, and ability of snails to temporarily leave the water to escape the chemicals (Pointier, 2001).

Health education ranks as the highest priority in schistosomiasis control programmes and requires long-term community commitment (Wang *et al.*, 2013). It aims at helping people to understand their role in the transmission of the infection through teaching of the life cycle of the parasite so they can stop indiscriminate defecation and urination. Children and adolescents who are at the greatest risk of infection are educated mainly through school education programmes. School authorities are encouraged to provide adequate sanitary facilities such as latrines for school children. Adult education is mainly targeted at women and high-risk occupational groups who have frequent contact with water. Women are encouraged to use soap during washing and the washing done in a basin on the lake or riverbank rather than in the main lake or river. Education through radio and television services which are now widespread in most developing countries are also very effective and are to be used to maximize control efforts (Wang *et al.*, 2013).

The installation of safe water supply for drinking, bathing and washing of clothes is the most cost-effective measure of control in most areas (WHO, 1985). The provision of adequate water supply also goes a long way to reduce the rates of infant diarrhoea and various intestinal helminthes as well as improve the general health and productivity of the people .Good

community water supplies through pumps and pipes or pit wells encourage people to stay away from infested fresh water sources (Kosinski *et al.*, 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Tomefa, one of the clusters of villages situated along the Densu Lake, formed as a result of the damming of the Densu River to abstract water for treatment. The Tomefa village is endemic for both urinary and intestinal schistosomiasis with prevalence rates of 70% and 78%, respectively (Danso- Appiah *et al.,* 2004). The general vegetation is the coastal savannah type with short grass and scattered bushes. Along the shores of the lake are aquatic plants (*Ceratophyllum demersum*, *Pistia stratiotes* and *Nymphaea odorata*), which harbour the schistosome host snails (*Bulinus truncatus rohlfsi* and *Biomphalaria pfeifferi*), making the lake the main source of infection. The inhabitants fetch water from the Densu Lake for drinking, bathing and for other domestic purposes, whilst others, particularly children, swim in it. Tomefa is inhabited mostly by migrant fisher folks and their families from other fishing communities in Ghana.

The village has only one basic school (Peace Chapel School) for the approximately 200 children that live in the community. Permission to conduct the study was sought from the chief, opinion leaders, the local administration of the school and parents of the school children. No community-wide mass drug administration has been undertaken in the area except one that was done in 2011 by the Noguchi Memorial Institute for Medical Research (NMIMR) which treated schistosomiasis egg positive school children with PZQ after conducting a school survey.

3.2 Study design

The study consisted of (i) parasitological baseline screening, (ii) treatment of children with a single oral dose of PZQ (40 mg/kg) (iii) 3 weeks and 8 weeks follow-up survey of treated participants especially those infected at baseline study. (iv) DNA extraction and PCR amplification of *Schistosoma* sp. DNA of all samples collected at each time point to compare methods and evaluate treatment efficacy.

3.2.1 Sample collection

After consents were sought from community leaders and the headmaster of the school, parasitological screening which included registration and collection of urine and stool samples was carried out. All children (n=143) present in school were registered with an identification number after they had provided parasitological record including sex, age, and class, aided by their teachers. Urine and stool samples were collected from all (143 urine and 138 stool samples) registered children on two consecutive days. Each child was given a plastic container labelled with a unique identification number and was asked to provide minimal volume of stool sample and at least 10 ml of urine (collected mid-morning) on the same day. Children who could not provide stool samples on the same day were asked to keep the containers for early morning stool to be collected by the field team the next morning. Samples were transported to the laboratory of the Noguchi Memorial Institute for Medical Research within two hours of collection and processed using the Kato-Katz and filtration techniques for stool and urine samples, respectively, to ascertain the schistosomiasis infection status of the participants before treatment.

3.2.2 Treatment of school children

On treatment day, all children (185) present in school were treated with a single dose of 40 mg/kg of PZQ (PZQ B. P. 600mg-ERNEST CHEMIST LIMITED, Ghana) as recommended by WHO treatment strategy for schistosomiasis (WHO, 2011) for prevalence greater than 50% of the study cohort. Only 113 (79%) of children sampled at baseline were part of the treatment group.

3.2.3 Post-treatment sampling

After the drug administration, 3 weeks and 8 weeks follow- up parasitological sampling was done. Urine and stool samples were collected from 93 participants who were sampled at baseline and treated on two consecutive days for each follow-up time point. In the laboratory, 10ml aliquots of urine were processed for microscopy analysis after urine chemistries (Glucose, Protein, pH and blood levels) had been done, and 2ml kept frozen at -20 °C for molecular analysis. About 0.5g of the stool samples were analysed by Kato Katz method and an aliquot of about 1g of each first sample from each person were kept frozen in cryo- tubes at -20ºC for molecular analysis. DNA was extracted from all samples; both urine and stool and PCRs subsequently performed on these extracted DNA samples.

3.3 Sample preparation and Laboratory Analysis

3.3.1 Microscopy analysis

3.3.1.1 Stool Sample

Stool specimens collected from each individual was prepared for microscopy examination using the Kato-Katz method. Briefly, 0.5g of stool was taken with a wooden spatula and scraped through a plastic mesh sieve of pore size 105 µm to remove particulate and fibrous material. A hole in a plastic template set on a glass microscope slide was filled with the

specimen. The template, when filled, contained approximately 41.5 mg of faeces. The template was lifted carefully off the slide, and the stool specimen cast on the slide was covered with a 25x35mm cellophane cover slip impregnated with 50% (v/v) glycerol in water containing 3% malachite green. The slide was turned upside down on a flat surface and pressed gently, but firmly, to spread the stool specimen evenly under the cellophane. The slide was left at room temperature for 30 minutes to clear before it was examined under the microscope for eggs of *Schistosoma* sp and any soil-transmitted helminthes present.

Two slides were prepared for every sample and examined by two microscopists. The average egg count from the two slides was recorded as egg count per that sample and the egg counted over the two days for each participant was also averaged and expressed as egg per gram (EPG= egg count x 24). With this, egg counts were categorised into egg intensity classes: negative, light intensity (1-100 EPG), moderate intensity (101-400 EPG) and heavy intensity (> 400 EPG).

3.3.1.2 Urine analysis

Each urine sample was tested for hematuria, proteinuria, glucose and pH using Urine Reagent Strips for Urinalysis (URS-10). The presence and quantification of *S. haematobium* ova was measured using the Nucleopore syringe filtration. Briefly, the tubes containing urine were shaken to resuspend the eggs and 10 ml was withdrawn into a syringe and filtered through 25 mm Millipore filter membrane of pore size 12 µm secured in a Swinnex support chamber. The schistosome eggs were trapped onto the filter membrane. With the aid of a pair of forceps, each membrane was carefully removed from the chamber and the side on which the eggs were trapped was inverted and placed onto a microscope slide. The slides were analyzed microscopically for schistosome eggs and average egg count per participant over the two days

calculated and expressed as egg per 10 ml of urine which is then categorised into egg intensity classes: negative, light intensity (1-49 eggs/10ml) and heavy intensity (≥50 eggs/10ml).

3.3.2 Molecular Analysis

3.3.2.1 Primer Selection

Schistosoma genus-specific primers amplifying a 77-bp fragment of the internal transcribed-spacer-2 (ITS2) sub unit (Obeng *et al.,* 2008), consisting of a forward primer Ssp48F (5'-GGT CTA GAT GAC TTG ATY GAG ATG CT-3'), reverse primer Ssp124R (5'-TCC CGA GCG YGT ATA ATG TCA TTA-3') and a detection probe, Ssp78T [FAM-5'-TGG GTT GTG CTC GAG TCG TGGC-3'-Black Hole Quencher (Biolegio)], were selected for amplification of any *Schistosoma sp* DNA present in the sample (urine and/ or stool).

3.3.2.2 DNA Isolation

DNA was isolated from all samples (329 urine and 282 stool) stored using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following manufacturer's protocols. Before extraction with the stool samples, 0.1 g of each stool sample was emulsified in 2% pvpp in PBS and 200ul subsample of each urine sample was heated for 10 min at 100^{o}C before treatment with sodium dodecyl sulphate and proteinase K for 2 h at 55^{o}C (Verweij *et al.,* 2004). As an internal control, phocin herpes virus 1 (PhHV-1) was added to the lysis buffer (at 1000 plaque-forming units/ ml).

3.3.2.3 Real-time PCR amplification

Amplification of each DNA sample was performed in a 25ul reaction mixture containing PCR buffer (HotstarTaq mastermix; QIAGEN), 5mM $MgCl_2$, 12.5pmol of each *Schistosoma*

genus-specific primer, 15pmol of each PhHV-1-specific primer, 2.5pmol each of the *Schistosoma* genus-specific and PhHV-1-specific double-labelled probes and 5ul of the DNA sample. The thermo cycler used was set to give 15 minutes at 95^{o}C, followed by 50 cycles, each of 15 seconds at 95^{o}C, 60 seconds at 60^{o}C and 30s at 72. Amplification, amplicon detection and the related data analysis were performed with the ABI 7500 RT-PCR detection system (Applied Biosystems). The PCR output from this system consisted of a cycle-threshold (C_t) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, indicating the parasite-specific DNA load in the urine sample tested. A test was considered positive when the C_t is less than 50 and greater than 15 taking the nature of the amplification curve into consideration.

3.4 Data handling, analysis and presentation

Data was tabulated in Excel 2003 and imported into SPSS version 16.0 (SPSS Inc.; Chicago, IL) for statistical analysis. A participant was defined positive if *Schistosoma* sp eggs were found in urine and/ or stool and negative when there were no eggs in both samples. Data analysis was performed on a population size of 143 and for all; tests were performed with a significance level of 0.005.

CHAPTER FOUR

RESULTS

4.1 Study population characteristics

A total of 143 schoolchildren made up of 73 (51%) females and 70 (49%) males with ages ranging from 3 to 20 years (a mean age of 11.64 years) were registered to participate in the study. The largest number of children registered was between the ages 10-13 years (Table 1). Urine and stool samples were received from all 143 registered children except 5 of them who could not provide their stool samples on both days of the pre-treatment survey. Out of the number sampled at baseline, 113 (79%) were available for treatment with PZQ a week afterwards. From the number treated, 93 submitted their urine samples and 72 of these submitted stool samples three and eight weeks after treatment for re-examinations (Table 1).

Table 1: Characteristics of participants and their samples tested

		Number (n)	%
Number Sampled			
	Male	70	49
	Female	73	51
	Total	143	100
Age group			
	3-5 years	9	6.3
	6-9 years	30	21.0
	10-13 years	60	42.0
	14-16 years	31	21.7
	17-20 years	13	9.1
Number treated		113	79.0
Urine samples tested			
	Before treatment	143	43.4
	3 weeks post-treatment	93	28.3
	8 weeks post -treatment	93	28.3
	Total	329	100
Stool samples tested			
	Before treatment	138	49.0
	3 weeks post-treatment	72	25.5
	8 weeks post -treatment	72	25.5
	Total	282	100

4.2 Microscopic examination

4.2.1 Schistosome infection status before treatment

Out of the 143 individuals registered and sampled, 133 (93%) had schistosoma eggs (*S. haematobium* and /or *S. mansoni*) present in either their urine or stool samples or both (Table 2). There were no significant differences between sexes (Pearson Chi Square (χ^2) = 0.345 and p = 0.557) and among ages groups (χ^2= 5.654 and p = 0.226) of infected participants even though age group 10-13 years recorded the highest prevalence (Figure 2).

Table 2: Schistosomiasis infection status of participants before treatment

	Number (%)		
	Male	**Female**	**Total**
Number sampled	70 (49)	73 (51)	143 (100)
Number infected	66 (46.2)	67 (46.8)	133 (93)

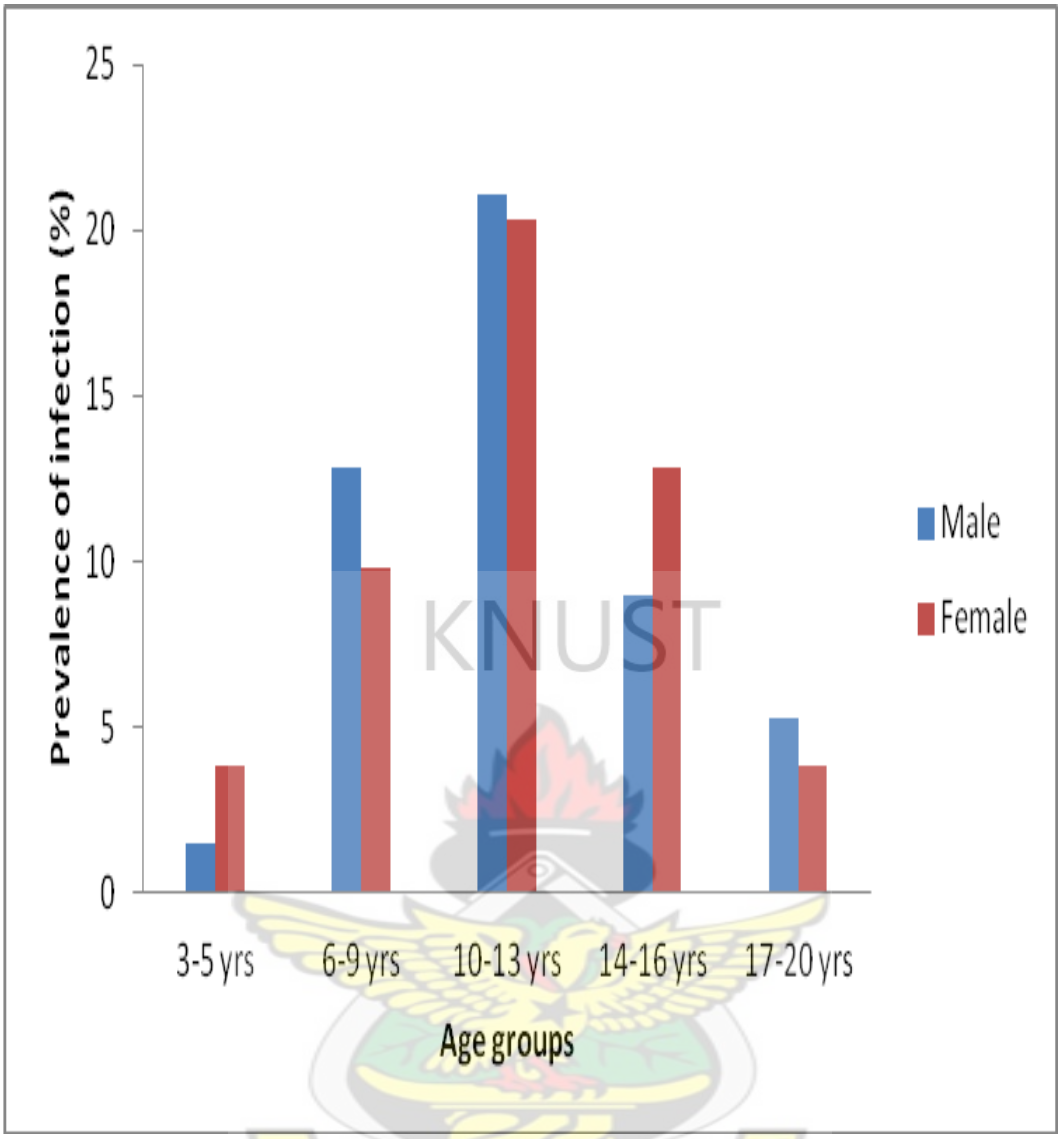


Figure 2: Schistosome eggs prevalence among age groups and within sexes

4.2.1.1 Prevalence and intensity of schistosome eggs in urine samples before treatment

Schistosome eggs were found in 86 (60.1%) of the urine samples (Table 3) with no significant differences between sexes (Pearson Chi Square (χ^2) = 1.778 and p = 0.182) and between age groups (χ^2 = 5.179 and p = 0.269) although age group 10-13 years had the largest number of positives (Figure 3). Intensity of infection was high in twenty-three (26.7%) of the infected children with a mean egg count of 125.4 eggs with no significant differences between sexes (χ^2 2.859 with p> 0.05). Both *S. haematobium* and *S. mansoni* eggs were found in 34 (39.5%) of the infected samples, 18 (52.9%) of which were from females. Two (2.3%) of the infected children, both females, were found positive with only *S. mansoni* eggs in the urine (Table 3).

Table 3: Urine Microscopy results of participants before treatment

	Number (%)		
	Male	**Female**	**Total**
Number sampled	70 (49)	73 (51)	143 (100)
Number infected	46 (32.1)	40 (28)	86 (60.1)
Intensity of infection			
Light infection: 1-49 egg/10ml	31 (36.0)	32 (37.3)	63 (73.3)
Heavy infection: ≥ 50 eggs/ 10ml	15 (17.4)	8 (9.3)	23 (26.7)
Types of Schistosome egg found			
S. haematobium only	30 (34.9)	20 (23.3)	50 (58.2)
S. mansoni only	0	2 (2.3)	2 (2.3)
S. haematobium and *S. mansoni*	16 (18.6)	18 (20.9)	34 (39.5)

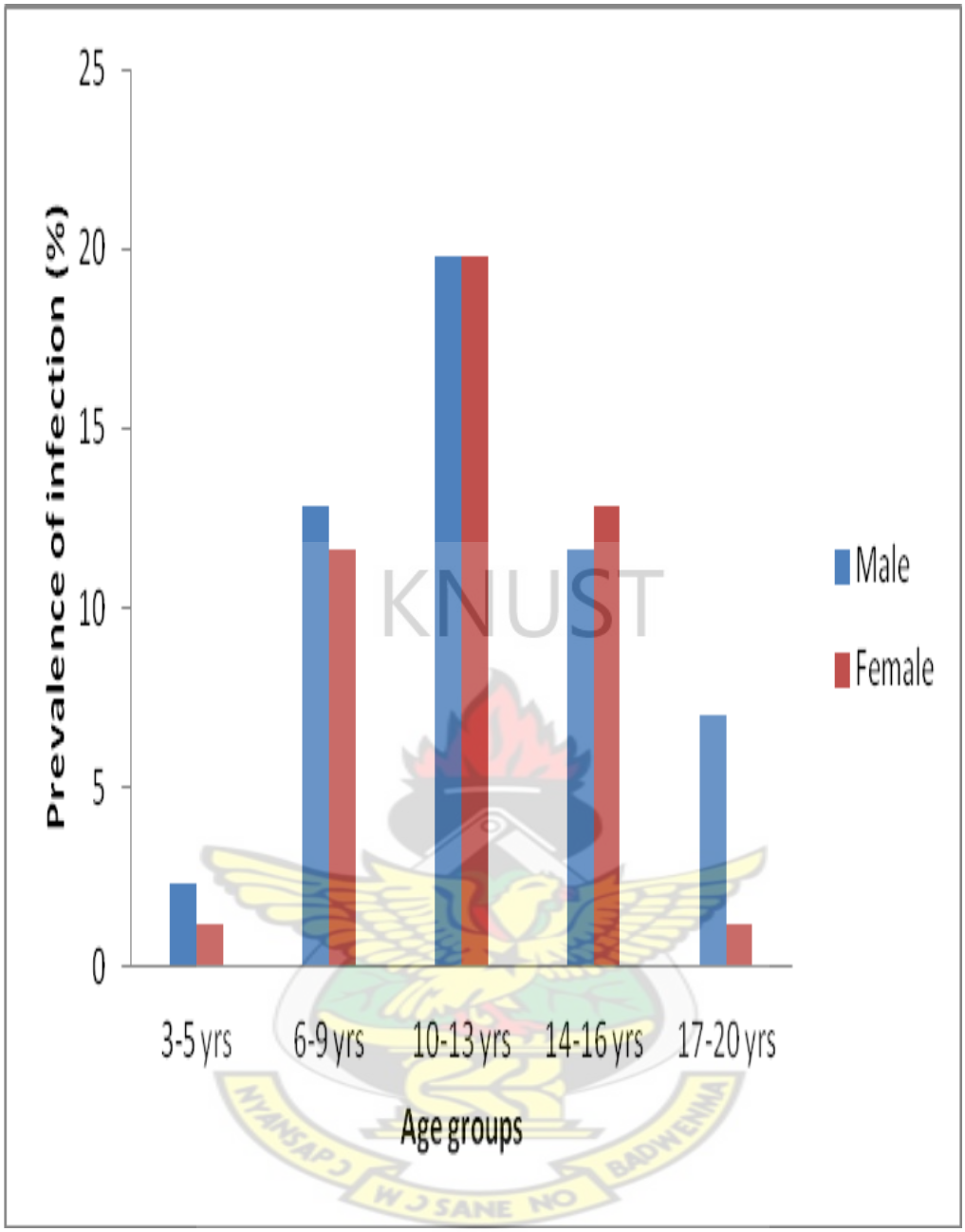


Figure 3: Schistosome eggs prevalence among age groups and within sexes

4.2.1.2 Prevalence and intensity of infections in stool samples before treatment

Out of the 143 participants, 138 (96.5%) provided stool samples for testing before treatment. As shown in Table 4, one hundred and fifteen (115) representing 83.3% were found infected with schistosome eggs (*S. mansoni* and/ or *S. heamatobium*) with no significant differences between sexes (χ^2 = 0.006 and p= 0.939). Heavy egg intensities were found in 48 (41.7%), moderate egg intensities in 44 (38.3%) with 23 (20%) recording light egg intensities. Mixed *S. mansoni* and *S. haematobium* eggs were found in 10 (8.7%) children, out of which 8 (80%) were males (Table 4). Age group 10-13 years recorded the highest infection prevalence (Figure 4) with no significant differences between age groups (χ^2 = 7.207 and p = 0.125).

Table 4: Stool Microscopy result of participants before treatment

	Number (%)		
	Male	**Female**	**Total**
Number sampled	67(48.6)	71 (51.4)	138 (100)
Number infected	56 (40.6)	59 (42.7)	115 (83.3)
Intensity of infection			
Light infection	8 (14.3)	15 (25)	23 (20)
Moderate infection	22 (39.3)	22 (37.3)	44 (38.3)
Heavy infection	26 (46.4)	22 (35.3)	48 (41.7)
Types of eggs found			
S. haematobium only	0 (0)	0 (0)	0 (0)
S. mansoni only	48 (45.7)	57 (54.3)	105 (91.3)
S. haematobium and *S. mansoni*	8 (80)	2 (20)	10 (8.7)

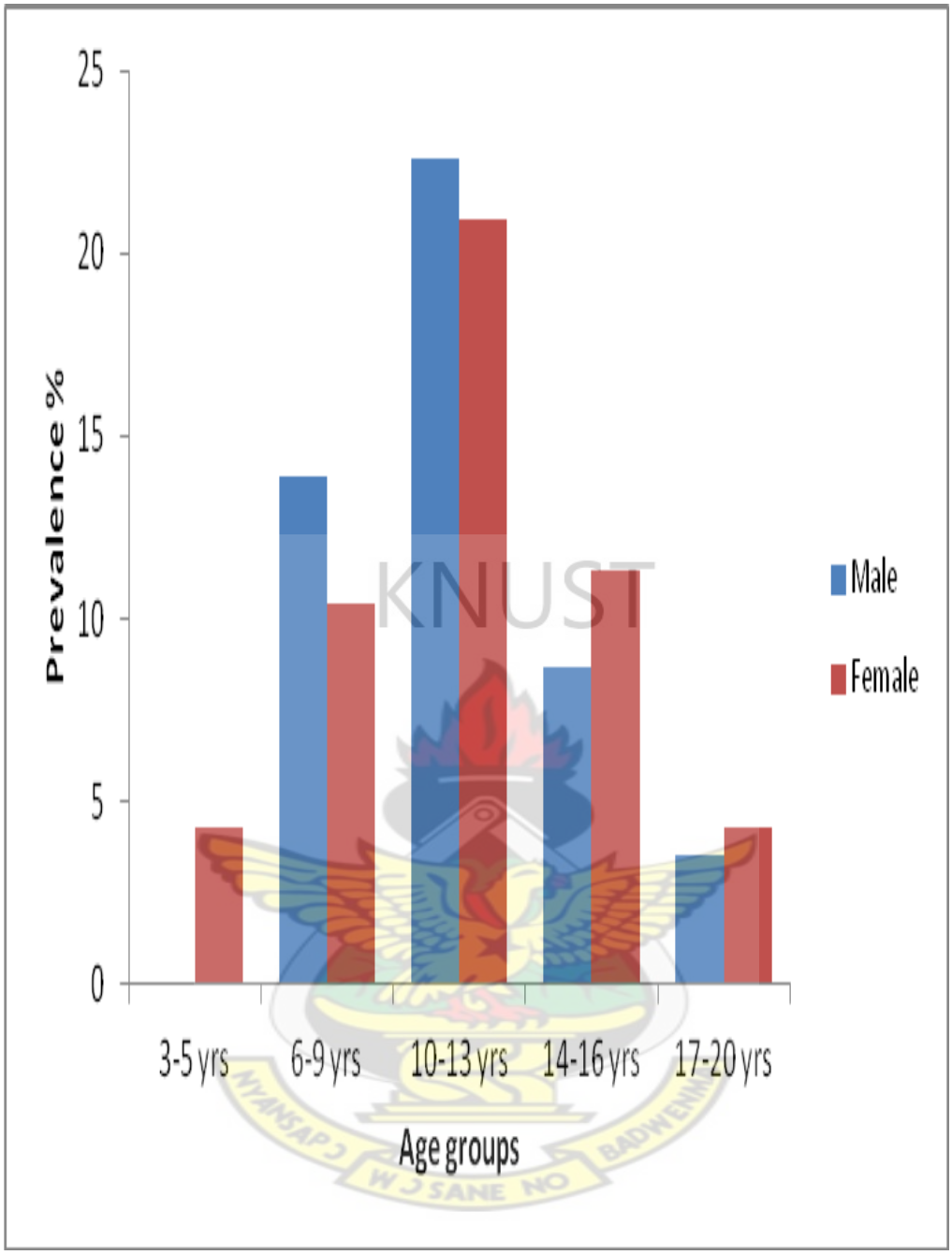


Figure 4: Schistosome eggs prevalence among age groups and within sexes

4.2.2 Treatment of participants

Of the 143 participants sampled at baseline, 113 (79.0%) made of 57 males and 56 females were available for treatment. Of these treated participants, 106 (93.8%) were infected with schistosoma eggs (*S. haematobium* and / or *S.mansoni*) in the urine and/ or stool samples before treatment (Table 5).

Table 5: Summary of infected participants treated among age groups and sexes

	Number treated			Number infected at baseline		
Age groups	Male	Female	Total	Male	Female	Total (%)
3-5 years	1	1	2	1	1	2 (1.8)
6-9 years	14	8	22	14	8	22 (19.5)
10-13 years	27	28	55	26	25	51 (45.1)
14-16 years	9	16	25	8	15	23 (20.4)
17-20 years	5	4	9	4	4	8 (7.8)
Total	56	57	113	53	53	106 (93.8)

4.2.3 Infection status three (3) weeks post-treatment

Out of the 113 participants (106 infected and 9 not infected) treated (Table 5), only 93 participants (88 infected and 5 not infected) sumbitted urine and/ or stool samples for follow-up re-examinations. Of the 88 participants who were found infected with schistosome eggs (*S. hamatobium* and/ *S. mansoni*) in either one or both samples before treatment, 22 were found with *Schitosoma* eggs (*S. haematobium* and / or *S.mansoni*) in either one or both samples three weeks after treatment, giving an overall cure rate of 75% (Table 6). Both males and females showed similar cure rates ($\chi^2 = 1.915$, $p = 0.166$) and age groups also showed no significant difference ($\chi^2 = 1.534$, p 0.821). The least cure rate (66.7%) was observed within

age group 6-9 years (Table 6). No infection was recorded in the 5 participants who were negative before treatment.

Table 6: Infections in urine and /or stool samples three (3) weeks post treatment.

	3 weeks after treatment								
	Number treated and re-examined			Number infected			Cure rates		
Age groups	Male	Female	Total	Male	Female	Total	Male	Female	Total (%)
3-5	1	0	1	0	0	0	1	0	1 (100)
6-9	10	8	18	4	2	6	6	6	12 (66.7)
10-13	22	24	46	7	4	11	15	20	35 (76.1)
14-16	6	13	19	1	3	4	5	10	15 (78.9)
17-20	2	2	4	1	0	1	1	2	3(75)
Total	41	47	88	13	9	22	28	38	66 (75)

4.2.3.1 Urine infection prevalence and intensity 3 weeks post treatment

Out of the 69 individuals with urinary schistosomiasis who were treated, 58 submitted urine samples for re-examination 3 weeks after treatment. From these 58, 16 (27.6%) had Schistosoma eggs present in their urine. An overall cure rate of 72.4% with a higher rate among females 77.4% than males 66.7% (Table 7) was recorded. A higher cure rate of 84.1% was recorded among lightly infected participants than those with heavy egg intensities (Table 7).

Table 7: Prevalence and intensity of eggs in urine three (3) weeks post treatment

Egg intensity	Number re-examined			Number infected			Cure rate		
	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
1-49 eggs	18	26	44	4	3	7 (16.9)	14	23	37 (84.1)
≥ 50 eggs	9	5	14	5	4	9 (64.3)	4	1	5 (35.7)
Total	27	31	58	9	7	16 (27.6)	18	24	42 (72.4)

4.2.3.2 Stool infection prevalence and intensity 3 weeks post treatment

Out of the 94 participants with stool infection who were treated, 62 (66%) submitted their stools for re-examination 3 weeks after treatment. From these 62 individuals, 9 (14.5%) were found having *S. mansoni* eggs in their stool samples, giving a cure rate of 85.5% (Table 8). Females recorded a higher cure rate of 93.3% as against 78.1% in males. There was a 100% cure rate for all those with light intensity infection, with children having moderate and heavy infections recording cure rates of 88.5% and 72.7%, respectively

Table 8: Prevalence and intensity of eggs in stool three (3) weeks post-treatment.

Egg count intensity	Number re- examined			Number infected			Cure rate		
	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
1- 100 eggs	5	9	14	0	0	0	5	9	14 (100)
101- 400 eggs	15	11	26	2	1	3 (11.5)	13	10	23 (88.5)
> 400 eggs	12	10	22	5	1	6 (27.3)	7	9	16 (72.7)
Total	32	30	62	7	2	9 (14.5)	25	28	53 (85.5)

4.2.4 Infection status Eight (8) weeks Post- treatment

With all the 88 infected but treated individuals who provided samples (stool and urine) at 3 weeks post treatment still available and providing their samples by eight (8) weeks after treatment, re-examination revealed 27 (30.7 %) participants were still excreting either *S. haematobium* or *S. mansoni* eggs in the urine or stool respectively. A cure rate of 69.3% with much higher rates of 76.1 % in females than males (63.4%) was recorded (Table 9). Age group 10-13 years recorded the highest cure rate of 84.8% whereas no cure was recorded within age group 3-5 years (Table 9).

Table 9: Infections in urine and /or stool samples eight (8) weeks after treatment

	Number treated and re- examined			Number infected			Cure rates		
Age groups	Male	Female	Total	Male	Female	Total	Male	Female	Total (%)
3-5	1	0	1	1	0	1	0	0	0
6-9	10	8	18	6	4	10	4	4	8 (44.4)
10-13	22	24	46	4	3	7	18	21	39 (84.8)
14-16	6	13	19	3	4	7	3	9	12 (63.2)
17-20	2	2	4	1	1	2	1	1	2 (50)
Total	41	47	88	15	12	27	26	35	61 (69.3)

4.2.4.1 Urine infection intensity and prevalence 8 weeks post treatment

On re- examination at eight weeks follow-up survey, 12 made up of 7 males and 5 females of the 58 urine infected participants at baseline were found to be still excreting schistosoma eggs in their urine samples giving a cure rate of 79.3% (Table 10). A higher cure rate was observed in light egg infected participants than heavy egg infected participants (Table 10).

Table 10: Prevalence and intensity of eggs in urine eight (8) weeks post treatment

Egg count intensity	Number re- examined			Number infected			Cure rate		
	Male	Female	Total	Male	Female	Total	Male	Female	Total (%)
< 50 eggs	18	26	44	2	2	4	16	24	40 (90.9)
≥ 50 eggs	9	5	14	5	3	8	4	2	6 (42.9)
Total	27	31	58	7	5	12	20	26	46 (79.3)

4.2.4.2 Stool infection intensity and prevalence

The 62 participants whose stools were re-examined at 3 weeks post treatment submitted stool samples again for re- examination at eight weeks follow-up, 15 (24.2%) of which were found still infected with *S. mansoni* eggs giving a cure rate of 75.8%, with males recording a lower cure rate than females (Table 11). Participants with light egg intensity recorded the highest cure rate of 78.6% whereas those with moderate and heavy egg intensities recorded cure rates of 73.1% and 77.3% respectively.

Table 11: Prevalence and intensity of eggs in stool eight (8) weeks post treatment

Egg count intensity	Number re- examined			Number infected			Cure rate		
	Male	Female	Total	Male	Female	Total	Male	Female	Total (%)
< 100 eggs	5	9	14	2	1	3	3	8	11 (78.6)
101- 400 eggs	15	12	26	5	2	7	10	10	20 (73.1)
> 400 eggs	12	9	22	2	3	5	10	6	16 (77.3)
Total	32	30	62	9	6	15	23	24	47 (75.8)

4.3 Molecular results

4.3.1 Real-time PCR amplification prevalence before treatment

Out of the 143 individual samples at baseline tested by RT-PCR, 139 (97.2%) had *Schistosoma* sp. DNA (*S. haematobium* and /or *S. mansoni*) present in either their urine and/ or stool samples (Table 12). There were no significant differences between sexes (χ^2 = 1.117, p = 0.290) and among ages groups (χ^2 = 2.352, p = 0.671) of infected participants. *Schistosoma* sp DNA was amplified in 109 (76.3 %) of 143 urine samples tested (Table 13), with no significant difference between sexes (χ^2 = 0.299, *p* = 0.492) as well as between age groups (χ^2 = 3.638, *p* = 0.457). A total of 131 (94.8 %) stool sample were found to contain *Schistosoma* sp. DNA by RT-PCR (Table 14) with no significant differences within sexes (χ^2 = 0.096, *p* = 0.532) or age groups (χ^2 = 3.019, *p* = 0.555). Age group 10-13 years showed the highest prevalence of *Schistosoma* DNA in both urine and stool samples.

Table 12: RT-PCR amplification prevalence in urine and / stool samples tested

	Number tested			Number positive		
Age groups	Male	Female	Total	Male	Female	Total(%)
3-5 years	3	6	9	3	6	9
6-9 years	17	13	30	17	13	30
10-13 years	29	31	60	28	30	58
14-16 years	13	18	31	12	18	30
17-20 years	8	5	13	7	5	12
Total	70	73	143	67	72	139

Table 13: RT-PCR amplification prevalence in urine samples tested

	Number tested			Number positive		
Age groups	Male	Female	Total	Male	Female	Total(%)
3-5 years	3	6	9	1	4	5 (3.5)
6-9 years	17	13	30	15	9	24 (16.8)
10-13 years	29	31	60	22	22	44 (30.8)
14-16 years	13	18	31	12	14	26 (18.2)
17-20 years	8	5	13	6	4	10 (7)
Total	70	73	143	56	53	109 (76.3)

Table 14: RT-PCR amplification prevalence in stool samples tested

	Number tested			Number Positive		
Age groups	Male	Female	Total	Male	Female	Total (%)
3-5 years	3	6	9	3	6	9 (6.5)
6-9 years	17	13	30	17	13	30 (21.7)
10-13 years	29	31	60	28	28	56 (40.6)
14-16 years	12	16	28	11	15	26 (18.8)
17-20 years	6	5	11	5	5	10 (7.2)
Total	67	71	138	64	67	131 (94.8)

4.3.2 Cure rate as measured by RT-PCR analysis

Considering the 88 treated egg positive participants who were re-examined after treatment, RT-PCR detected *Schistosoma* DNA in 59 (67%) urine and/ or stool samples tested at three weeks post treatment giving a cure rate of 33%. At eight weeks re-examinations, RT-PCR detected DNA amplification in 69.3% (61/88) of the samples a cure rate of 30.7%.

Table 15: Cure rates measured by RT-PCR.

	Number treated and re-examined			Number negative at 3 weeks Post treatment			Number negative at 8 weeks post treatment		
Age groups	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
3-5	1	0	1	1	0	1 (100)	0	0	0
6-9	10	8	18	1	2	3 (16.7)	1	1	2 (11.1)
10-13	22	24	46	6	11	17 (37)	5	8	13 (28.3)
14-16	6	13	19	2	4	6 (31.6)	2	8	10 (52.6)
17-20	2	2	4	1	1	2 (50)	1	1	2 (50)
Total	41	47	88	11	18	29 (33)	9	18	27 (30.70)

4.3.2.1 Urine samples

Out of the 58 treated urine egg positive participants re-examined, *Schistosoma* sp. DNA were amplified in 37 (63.7%) at 3 weeks post- treatment and 9 (15.5%) at 8 weeks post- treatment giving a cure rate of 36.3% and 84.5% respectively (Table 16).

Table 16: Cure rate as measured by RT-PCR of urine samples

	Number treated and re-examined			Number negative at 3 weeks post treatment			Number negative at 8 weeks post treatment		
Egg intensity	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
1-49 eggs	18	26	44	9	10	19 (43.2)	17	24	41(93.2)
≥ 50 eggs	9	5	14	1	1	2 (14.3)	6	2	8 (57.1)
Total	27	31	58	10	11	21 (36.2)	23	26	49 (84.5)

4.3.2.2 Stool samples

At 3 weeks post-treatment re-examinations, RT-PCR detected *Schistosoma* sp. DNA in 35 (56.5 %) of the 62 treated stool egg positive participants, a cure rate of 43.5%. Schistosoma sp. DNA was detected in 51 (82.3%) stools at 8 weeks post- treatment giving a cure rate of 17.7% (Table 17).

Table 17: Cure rate as measured by RT-PCR of stool samples.

	Number treated and re- examined			Number negative at 3 weeks post treatment			Number negative at 8 weeks Post treatment		
Egg count intensity	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
1- 100 eggs	5	9	14	1	3	4 (28.6)	1	3	4 (28.6)
101- 400 eggs	15	11	26	5	8	13 (50)	2	1	3 (11.5)
> 400 eggs	12	10	22	5	5	10 (45.5)	2	2	4 (18.2)
Total	32	30	62	11	16	27 (43.5)	5	6	11 (17.7)

4.4 Comparing microscopy and RT-PCR detection of Schistosoma infection

4.4.1 Urine samples

For all urine samples (n=329) collected at all three time periods and analysed as a single group, samples that showed a high egg intensity using microscopy were better detected by RT-PCR than low. As high as 19 (67.9 %) of the high egg counts (>50 eggs per 10ml urine) showed low C_t values (C_t < 30), indicating a higher amount of schistosome DNA in the samples. Only 2 (7.1 %) samples had relatively high C_t values (above 35 cycles). None of the high intensity samples tested negative for RT-PCR, indicating 100% sensitivity of high infections (>50 eggs per 10ml urine) to RT-PCR detection. Out of the 212 negative individual samples captured by microscopy egg count, 75 of them representing 35.4 % tested positive for schistosome DNA. Out of the 89 individuals that were detected to be of low intensity infections (<50 eggs per 10ml urine) microscopically, 20 tested negative for RT-PCR.

Table 18: Intensity of infection by microscopy eggs count and RT-PCR Ct values

Microscopic egg count	RT-PCR Class				
	Negative	Ct > 35	30< Ct < 35	Ct < 30	Total
Negative	137	27	27	21	212
1-49 eggs/10ml	20	9	22	38	89
50< eggs/10ml	0	2	7	19	28
Total	157	38	56	78	329

4.4.2 Stool samples

RT-PCR achieved 100% detection for all moderate (101- 400 EPG) and heavy (>400 EPG) egg infected stool samples. Most of the high egg counts (>100 EPG) also showed low C_t values, indicating a larger amount of schistosome DNA in the samples (Table 19). Ninety-four (67.1 %) samples negative by microscopy egg count were positive by RT-PCR, with

most of the samples (58.5 %) exhibiting relatively low C_t values (C_t <30). Majority (78.9 %) of the low intensity infected (1-100 EPG) samples tested positive by RT-PCR with C_t <30, however, 3 (7.8 %) samples in this egg class were negative by RT-PCR analysis.

Table 19: Eggs per gram stool and RT-PCR Ct values presented in classes

	RT-PCR Class				
Microscopic egg count	Negative	Ct > 35	30< Ct < 35	Ct < 30	Total
Negative	46	21	18	55	140
1-100 EPG	3	1	4	30	38
101-400 EPG	0	1	3	48	52
>400 EPG	0	1	2	49	52
Total	49	24	27	182	282

4.4.3 Repeat samples (urine and stool) at all three time points

The results for microscopy and RT-PCR measuring schistosome DNA in urine and stool samples before treatment, 3 weeks and 8 weeks post-treatment, are summarised in Table 20 below. The means shown are the calculated arithmetic means and the ranges are that of positives samples. RT-PCR showed larger number of positives than microscopy at all time periods in both urine and stool samples tested.

Table 20. Result from microscopy egg count and RT-PCR based on repeat sampling at all time points

	Before Treatment	3 weeks post treatment	8 weeks post treatment
Urine samples			
Eggs per 10ml			
Number	93	93	93
Positive	58	17	14
Range *	1-347	1-328	1-247
Mean *	44.86	29.38	30.0
RT-PCR Ct value			
Number	93	93	93
Positive	68	49	14
Range *	18.7- 39.9	20.3-39.1	22.5- 41.2
Mean *	29.7	30.5	32.8
Stool samples			
Eggs per 10ml			
Number	72	72	72
Positive	62	9	18
Range *	24- 4080	24- 3024	24- 3096
Mean *	651	391	292
RT-PCR Ct value			
Number	72	72	72
Positive	68	40	61
Range *	16.2- 38.7	18.1- 38.4	16- 49.1
Mean *	22.03	28.63	27.06

* Range and Mean of positive samples

Figure 5 and 6 displays scatters plotting egg counts versus RT-PCR for urine and stool as well as the Spearman correlation coefficients for each of the three time periods. C_t values of 50 and egg counts below one are negative samples. The differences in these methods were significant ($p< 0.05$) at all time points for both urine and stool samples except at 8weeks post treatment sampling of stool samples were p-value was greater than 0.05 ($p= 0.621$).

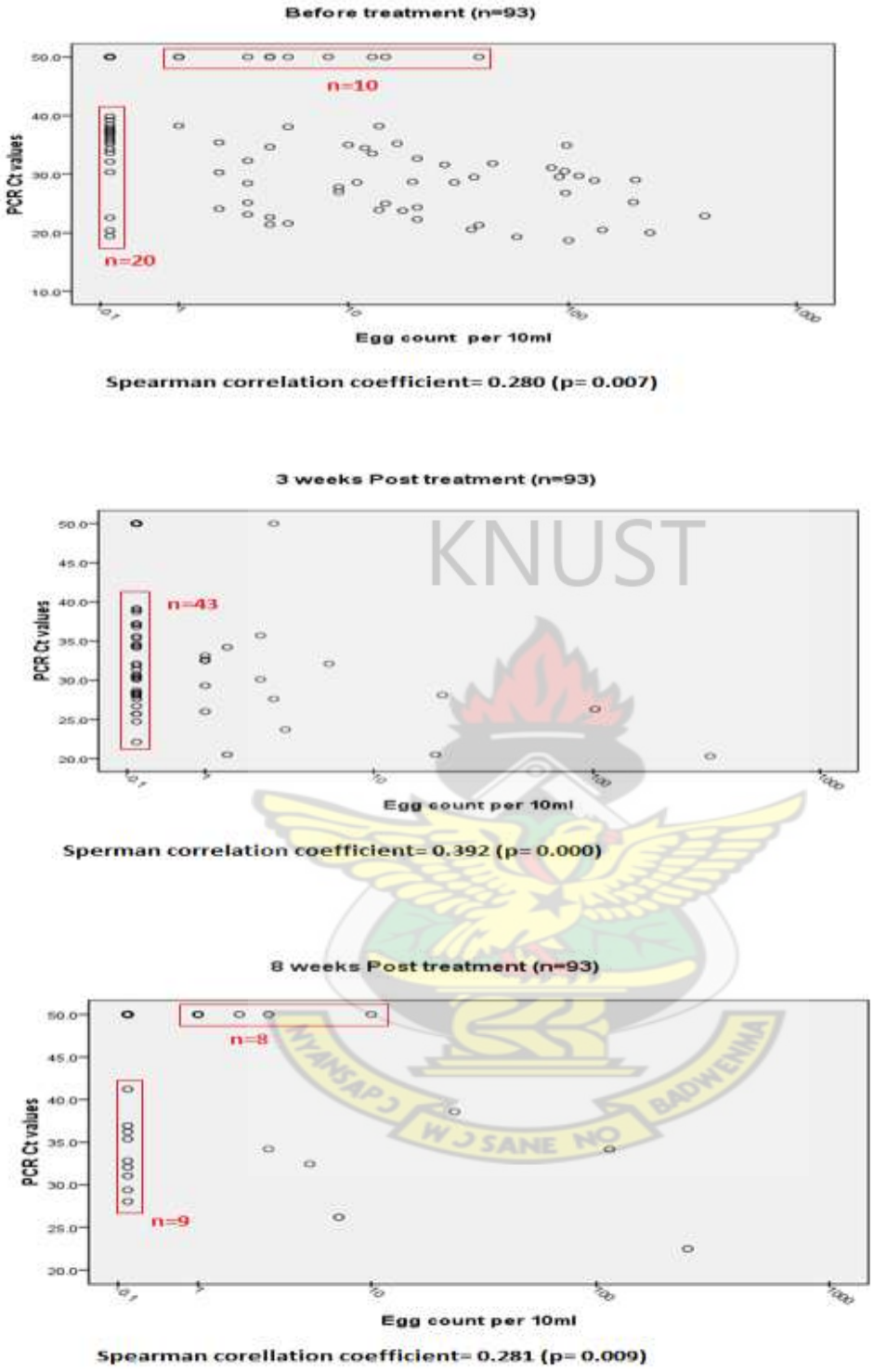
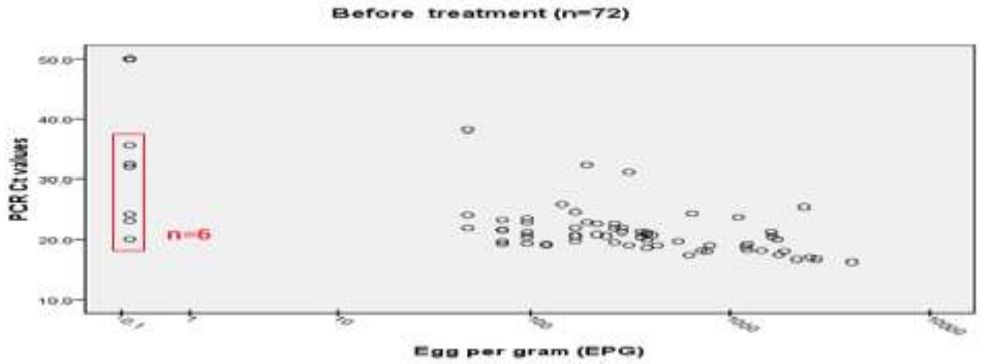
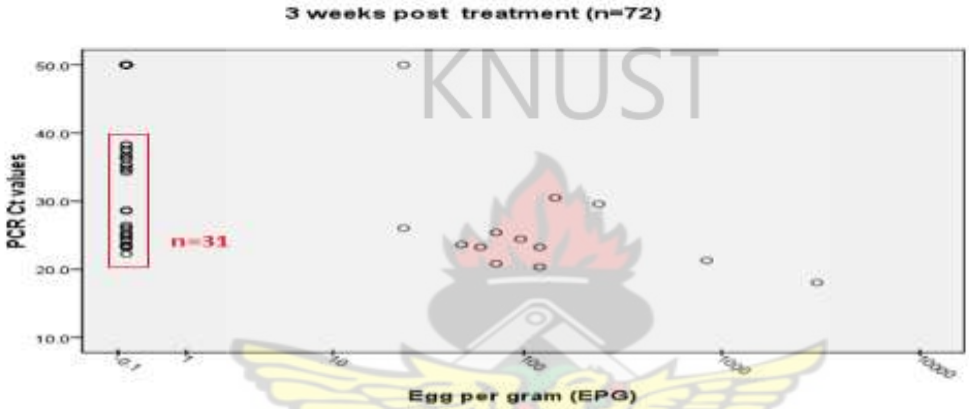


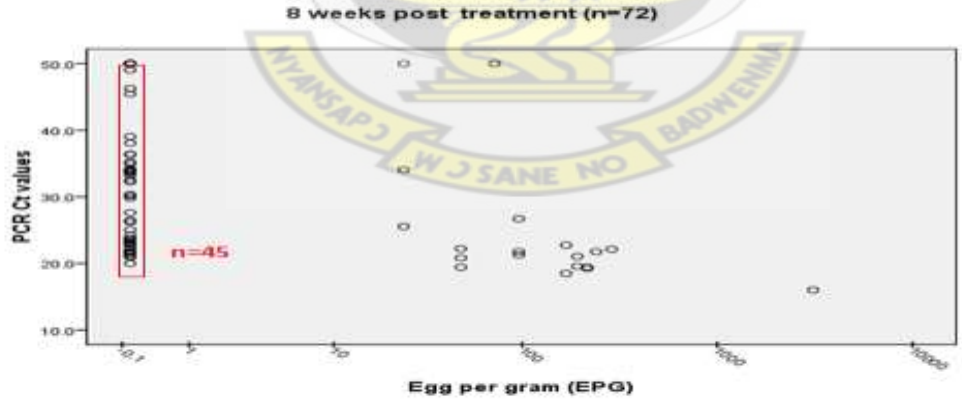
Figure 5: Scatter plots displaying urine egg count against RT-PCR Ct values at all time points



Spearman correlation coefficient= 0.604 (p= 0.000)



Spearman correlation coefficient= 0.312 (p= 0.008)



Spearman correlation coefficient= 0.058 (p= 0.621)

Figure 6: Scatter plots displaying egg per gram stool against RT-PCR Ct values at all time points

CHAPTER 5

DISCUSSION

Current estimates of the prevalence of schistosomiasis depend on the use of well-established, but imperfect, diagnostic tests where specimens are processed by routine parasitological methods and parasitological cure is judged by inspection of a cohort of children before and after PQZ treatment (Stothard *et al.*, 2013). Appropriate diagnosis of schistosomiasis is becoming increasingly important for several reasons; for example, clinical diagnosis might lose its value because of lack of specificity, and mass treatment might only remain cost effective through the use of appropriate diagnostic tools to only target further drug treatment to those groups of people actually infected (Koukounari *et al.*, 2009). With technological advancement and more efficient throughput, molecular diagnostics employing RT-PCR can potentially improve both the accuracy and precision of epidemiological studies of schistosome sp (Ten Hove *et al.*, 2008). This study which compared the detection of Schistosoma infections in urine and stool samples using microscopy and RT-PCR amplification was done at three time points; 1). Pre-treatment 2). 3 weeks post-treatment and 3). 8 weeks post-treatment, to assess the implied cure rates at each period following treatment with PQZ.

Results from this study showed a high prevalence of infection (*S. haematobium* and / or *S. mansoni*) in the study area with no significant difference (p= 0.05) between microscopy (93%) and RT-PCR (97.2%) detection in urine and/ stool samples tested before treatment. This high prevalence of infection at baseline by both detection methods was characterised by a classical increase of both prevalence and intensity of infection with increasing age, which peaked in children aged between 10 and 13 years, which is in agreement with results reported by Saathoff *et al.* (2004).

Both methods detected more infection in stool than urines of the participants; microscopy identified eggs in 60.1% (86) of urine samples as against 80.4% (115) stool samples whereas

RT-PCR amplified Schistosoma DNA in 76.2% (109) urines as against 91.6% (131) stools. Similar prevalence rate (65.3% and 82% for *S. haematobium* and *S. mansoni* respectively) were recorded by microscopy egg count in a preliminary survey by the National Service Personnel group of Noguchi Memorial Institute for Medical Research (NMIMR) 2011, in the same population. Danso-Appiah *et al.* (2004) also reported prevalence rates of 70% and 78% for *S. haematobium* and *S. mansoni* respectively in a pilot study in Kokoetsekope, a community close to the current study area also along the banks of the Weija Lake. These results imply that intestinal schistosomiasis is becoming wide spread and could dominate over urinogenital schistosomiasis which was previously known to be dominant (Wagatsuma *et al.*, 1999).

Although studies have shown both prevalence and intensity of infection to be higher among males (Toure *et al*., 2008; Tohon *et al.*, 2008; Kosinski *et al*., 2012) this study did not reveal any significant differences in infection between the sexes by both detection methods. This could be due to similar exposures to water contact activities as the Weija Lake remains the only source of drinking water supply to the communities along its banks, for domestic use and water transport.

Co-infection between *S. haematobium* and *S. mansoni* was observed in this population with quite a number of participants excreting *S. haematobium* eggs in the stool and a lot more with *S. mansoni* eggs in their urine samples. Such occurrence has been reported in an increasing number of foci across Africa with instances, from studies in Cameroon (Ratard *et al.*, 1991) and Senegal (Cunin *et al.*, 2003) which revealed that in areas of transmission overlap, *S. mansoni*-shaped eggs may be excreted in urine as opposed to the usual faecal route. This developing situation of mixing infection where both parasites eggs are found in the same location will require further investigation in the near future to ensure that it does not lead to uncontrollable pathology as well as virulence of the disease on the individuals. The RT-PCR

primers used in this study did not differentiate between species but amplified all *Schistosoma* sp. DNA present in the samples.

Results from this study generally showed more positive detection by RT-PCR than microscopy for the detection of *Schistosoma* sp. infection in both urine and stool samples, with discrepancies between the two methods proving to be very minimal and mostly limited to lightly-infected cases which support earlier findings by Obeng *et al.* (2008) and Aryeetey *et al.* (2013). The higher sensitivity of the RT-PCR method at all the three time points investigated is supported by the ability of PCR technique to exponentially amplify a minute amount of DNA to a larger volume that can be detected and even quantified, and is consistent with previously reported underestimation of the prevalence of infection by microscopy detection of eggs when compared to PCR detection (de Vlas and Gryseels, 1992). Thus, eggs will be missed or not be detected using microscopy during the acute phase of infection when worms have not yet produced large numbers of eggs, or during active and chronic infections with low egg loads (Nicolls *et al.*, 2008; Clerinx and Van Gompel, 2011) and in cases with low egg excretions (Mwinzi *et al.*, 2004).

The observed drastic reductions of infection intensity and prevalence following treatment with PQZ to 25% and 30.7% at three and eight weeks post-treatment respectively by microscopy detection of eggs in urine and/ stool samples with no significant differences between sexes and age groups is supported by a previous study by Feldmeier and Chitsulo (1999) who reported similar reduction rates in prevalence after 3 weeks post-treatment with PQZ. Although there was a reduction in prevalence by RT-PCR (67% and 69.3% at 3 and 8 weeks post-treatment respectively) it was not as drastic as that of microscopy detection. Similar results were obtained by Pontes *et al.* (2003) in a comparison between the two methods in which RT-PCR detected more cases of infection than microscopy detection after treatment.

The high detection by the RT-PCR method use in this study also reflects in its utilization for the assessment of cure evaluation. Since RT-PCR detected more positive cases at all post-treatment surveys, it provided a lower estimation of the cure rate than the parasitological technique; 33% to 75% at 3 weeks and 30.7% to 69.3% at 8 weeks post treatment which supports previous reports by Pontes *et al.* (2003) whose results showed PCR detection to be higher than Kato Katz detection of *S .mansoni* in stool even after repeated sampling. The number of positive RT-PCR samples (urine and stool) drops 3 weeks after treatment, and that of stool samples increases later with egg counts eight weeks after treatment.

Based on microscopy alone, it seems as though mass treatment was effective. However, RT-PCR seems to suggest otherwise, as the drop in prevalence is not as dramatic and continues to show a large number of individuals having DNA in their urine, many even with lower C_t value representing higher initial DNA loads. These data clearly suggest that our RT-PCR is more sensitive in detecting schistosome infections status than microscopy that only reveals the presence or absence of parasite eggs both before and even after effective PQZ treatment, especially in diagnosis of individuals for the evaluation of the efficacy of chemotherapy where lightly infected cases are easily missed by microscopy (Wang *et al.*, 2011), calling into question the effectiveness of PQZ. This can be explained in part that the drug (PQZ) is active against adult worms alone and not juvenile stages (immature worms) which can be detected by PCR and not microscopy because they are not laying eggs yet. It is also known that poor cure rates are reported in communities with a heavy intensity of infections (Olliaro *et al.*, 2011), thus even if the drug is killing the vast majority of worms, there may still be residual worms and/or juvenile stages that survive initial treatment (Montressor *et al.*, 2011).

The data generally shows a good correlation for intensity of infection when comparing both microscopy and RT-PCR for the entire study population over all three time periods, as illustrated in Tables 18 and 19 for urine and stool, respectively. An especially good

correlation was observed for highly intensive infections in urine (egg counts of 50< eggs per 10ml urine), for which all samples tested positive by RT-PCR with a great majority of urine samples showing low C_t values (<30), indicating higher DNA loads. In the same egg class, only a few samples had relatively high C_t values (>35) while none were negative. RT-PCR in stool samples also achieved 100% sensitivity for all moderate (101- 400 EPG) and heavy (>400 EPG) intensity infections with a positive correlation between RT-PCR and microscopy. This suggests a good positive correlation between intensity of infection as measured by either microscopy or RT-PCR. The tables also shows that lower egg counts is somewhat evenly distributed over the three RT-PCR classes with simple majority of the samples showing C_t value less than 30 cycles. Result of this study therefore goes to support a number of other reports (Obeng *et al.*, 2008; Aryeetey *et al.*, 2013) which also indicated 100% detection of high intensity egg counts by RT-PCR amplification. However, 20 urine and 3 stool samples with light egg intensity (diagnosed by microscopy egg count) were missed and considered negative by the RT-PCR which can be explained as the presence of insufficient schistosome DNA in the isolated samples for positive detection (Obeng *et al.*, 2008) and also as a result of the possibility of variation in egg output and uneven distribution in the samples (Engels *et al.*, 1996).

CONCLUSION

Detection of parasite-specific DNA is becoming an important means of identifying and hopefully eliminating risk foci. There is no inference here that DNA detection is promoted to supersede other standard means of diagnosing schistosome infection. These methods are well

tried and serve a role in most circumstances; however, as the need for more sensitive tests arise, DNA detection adds another diagnostic tool which will expand the ability of the epidemiologist to collect data pertinent to any programme designed to eliminate schistosomiasis or warn clinicians of the potential problem of this debilitating parasitic infection.

The high detection by the RT-PCR explored in this study suggest its usefulness and value for the identification of schistosomiasis infections in low transmission settings and among individuals with low worm burden after treatment, which is important in the context of transmission control and disease surveillance.

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