FEMALE GENITAL SCHISTOSOMIASIS (FGS) AS A RISK FACTOR FOR SQUAMOUS CELL ATYPIA IN AN EPIDEMIOLOGICAL LONGITUDINAL COHORT OF YOUNG WOMEN IN KWAZULU-NATAL

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Submitted in partial fulfilment of the academic requirements for the degree of Doctor of Philosophy in the School of Nursing and Public Health Medicine, University of KwaZulu-Natal.

Purpose of the dissertation:

The research described in this PhD thesis was conducted in the Discipline of Public Health Medicine in the School of Nursing and Public Health University of KwaZulu-Natal, from January 2011 to December 2015, under the supervision of Professor Myra Taylor and Dr Eyrun F Kjetland.

These studies represent original work by the author and have not been submitted in any form for any degree or diploma to any other tertiary institution. Where use has been made of the work of others such work has been duly acknowledged in the text.

Format of dissertation:

This thesis will be presented as a thesis of manuscripts, which include published and prepared journal articles that have emanated from the research in this field.

Biomedical Research Ethics Committee
approval date and reference number
23rd June 2015, BF057/11

DECLARATION

I.....***Pavitra Pillay***.....declare
that

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DEDICATION

This study is dedicated to all who are at risk for the following preventable diseases: schistosomiasis, cervical cancer and HIV.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND DEFINITIONS

ASCUS	Atypical squamous cells of undetermined significance, this refers to cellular that appear to be atypical but fall short of a definitive diagnosis of squamous intraepithelial lesion.
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CIN	Cervical intraepithelial neoplasia, this refers to abnormal or disordered cellular growth that is still contained within the epithelial layers.
CIN1	This is a grade of intraepithelial neoplasia that is confined to the upper third of the squamous epithelium of the cervix.
CIN2	This is a grade of intraepithelial neoplasia that is confined to the two thirds of the squamous epithelium of the cervix.
CIN3	This is a grade of intraepithelial neoplasia that is confined to the full thickness of the squamous epithelium of the cervix.
Colposcopy	Visualisation of the cervix and vagina using a magnifying instrument with an attached light source called a colposcope.
Cytology	This is the study of cells in health and disease, and includes the detection of microorganisms and other entities that can be detected in cytological material using a microscope.
DNA	Deoxyribose nucleic acid
ELISA	Enzyme-linked immunosorbent assays
FGS	Female Genital Schistosomiasis, this entity refers to *Schistosoma* ova within the genital tract of women

FUS	Female Urogenital Schistosomiasis, this entity that refers to *Schistosoma* ova within the urinary and genital tracts of women.
HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus, is a sexually transmitted virus whose carcinogenic strains are implicated in cervical cancer.
HSIL	High grade squamous intraepithelial lesion
LAMP	Loop-mediated isothermal polymerase chain
LBC	Liquid based cytology, refers to the collection and processing of a cervical sample to ensure that cells are concentrated and excess mucus and background material is removed to enhance visualisation.
MDA	Mass drug administration, refers to the administration of anti-schistosomal drugs at population or community level.
MGS	Male genital schistosomiasis this entity refers to *Schistosoma* ova within the genital tract of males.
µl	Microliter
µm	Micrometer
mL	Millilitre
LSIL	Low grade squamous intraepithelial lesion
NTDs	Neglected tropical diseases
p16	Protein 16

Pap	Papanicolaou smear, this refers to the collection of exfoliated cells from the cervix, which are stained and viewed cytologically to detect cancer.
PCR	Polymerase chain reaction
%	Percentage
POC	Point of care
PZQ	Praziquantel, this is the anti-schistosomal drug.
rpm	Revs per minute
SCA	Squamous cell atypia, this refers to any category of cervical squamous atypia detected cytologically, and refers collectively to ASCUS, SIL and invasive cancer.
Schistosomiasis	This is the disease caused by infection with *Schistosoma* species.
S. haematobium	*Schistosoma haematobium*, this is a water-borne parasite that is found in the urinary and genital tracts.
SIL	Squamous intraepithelial lesion, this refers to abnormal cellular growth that is still confined to the squamous epithelium and this phase precedes invasion through the basement membrane into the stroma.
S. mansoni	*Schistosoma mansoni*, is a water borne parasite that is found mainly in the intestinal tract.

TBS	The Bethesda System, this is a cytologic based system of reporting that has uniform diagnostic terminology.
TZ	Transformation zone, this is an area on the cervix where squamous epithelium from the ectocervix meets with columnar epithelium of the endocervix.
UGS	Urogenital Schistosomiasis, this entity refers to the presence of *Schistosoma* ova in the urinary and genital tracts of both men and women.
VIA	Visual inspection with acetic acid
WHO	World Health Organization

ABSTRACT

Female Genital Schistosomiasis (FGS) is a clinical manifestation linked to the parasite *Schistosoma haematobium*. Studies have shown that parasite ova can lodge in genital tract tissue and cause increased contact bleeding and mucosal lesions called sandy patches. It is also hypothesised that FGS is associated with human immunodeficiency virus (HIV) acquisition and possible squamous cell atypia (SCA) of the cervix. The aim of this study was to investigate whether there is a link between FGS and cervical SCA using non-invasive diagnostic tools and to also investigate the effect of mass treatment among participants.

This study was nested in a large school based clinical FGS study among girls aged 10-12 (n=708) and young women aged 16-23 from two regions in the north (n=833) and south coast (n=394) of rural KwaZulu-Natal between 2009 and 2014. Non-invasive diagnostic tests used included Pap smear cytology and Liquid-based cytology (LBC), (only among young women) and *Schistosoma* PCR in urine (in girls and young women) and vaginal lavage (among young women only). Urine microscopy was also conducted. HPV genotyping was done in a subsample of young women from the north coast. Ethical considerations were adhered to.

Among the girls, the overall mean prevalence of *S. haematobium* in urine samples collected on three consecutive days was 31.8% and the *Schistosoma* PCR prevalence was 25.0%. Microscopy and PCR results were significantly associated with a history of gynaecologic symptoms among the girls. Highly focal distribution of urogenital schistosomiasis was found in the 18 primary schools based on PCR, (median= 27.0%, IQR=12.0-38.7%).

Among the young women, the prevalence of *S. haematobium* ova detected in cytology was 8/394 (2.0%) from the south coast and 12/833 (1.4%) from the north coast. *Schistosoma*-specific DNA was found in 38/394 (9.6%) of vaginal lavages and in 91/394 (23.1%) of the urine from the south coast. There was a significant association between *S. haematobium* eggs in Pap smears and the presence of *Schistosoma* DNA in lavage (p<0.001). The prevalence of SCA in the south coast was 107/394 (27.1%) and this was not significantly associated with schistosomiasis. The *Schistosoma* PCR urine and vaginal lavage results from the south coast were included in a comparison of two other countries in Africa (Tanzania and Madagascar) each with distinct *S. haematobium* and *S. mansoni* transmission patterns. In the north coast however, there was a significant association between SCA and genital and urinary *Schistosoma* ova (p=0.005). Additionally 19/833 (2.2%) of the young women had high grade squamous intraepithelial lesion

(HSIL). Possible risk factors for schistosomiasis and SCA were river water contact, age at sexual debut and having at least one child. LBC samples had an improved diagnostic yield compared to Pap smears and also enabled HPV genotyping to be done in a subsample of 10 cases with HSIL. HPV genotyping revealed at least one or more carcinogenic types of HPV.

The outcome of anti-schistosomal treatment on SCA was a challenge to analyze since mass drug administration (MDA) did not occur within the study population as planned. Despite this, the questionnaire data on reported anti-schistosomal treatment were described in relation to schistosomiasis and SCA. The findings of this study reveal that FGS does exist among girls and young women in *Schistosoma* endemic areas. Among the young women, *Schistosoma* PCR in vaginal lavage was an improved method to diagnose FGS rather than conventional cytology. There was a statistically significant association between ova in Pap smears and the other diagnostic methods used. LBC sampling should be used in FGS populations to detect schistosomiasis and SCA, since a single sample can be used for cytology, HPV genotyping as well as possibly *Schistosoma* PCR testing. The high prevalence of HSIL detected in this young population at risk for HIV and schistosomiasis raises concern that the screening programme commencing at 30 years of age in our study area might be starting too late. The preliminary results for the outcome of treatment require further investigation.

1.0 CHAPTER ONE

1.1 Introduction

Female Genital Schistosomiasis (FGS) refers to the presence and effects of *Schistosoma* ova within the female genital tract. Ova from the parasite Schistosoma haematobium lodge in genital tissues and can cause lesions which result in increased susceptibility to contact bleeding and alterations to the mucosa, leading to FGS (Kjetland *et al.* 2008; Norseth *et al.* 2014). This study focuses on the association between FGS and squamous cell atypia (SCA). SCA encompasses a spectrum of atypical changes to cervical cells including pre-cancerous or dysplastic changes as well as invasive cervical cancer diagnosed cytologically. An important carcinogenic agent in almost all cervical cancers is Human papillomavirus (HPV) (Schiffman *et al.* 2007). There is interest in FGS due to alterations in the mucosa caused by *Schistosoma* ova. Studies have shown that there is a possible link to SCA of the cervix (Petry *et al.* 2003; Kjetland *et al.* 2009a) and also to HIV acquisition (Mbabazi *et al.* 2011). Furthermore it has also been suggested that anti-schistosomal treatment with Praziquantel could have a positive effect on reducing genital lesions (Richter *et al.* 1996; Downs *et al.* 2013).

The aim of this study was to investigate whether there is a link between FGS and SCA. This thesis presents the findings of the prevalence and risk factors for FGS and cervical atypia that may lead to cancer. The preliminary findings on the effects of anti-schistosomal treatment on cervical atypia are also discussed. The analyses which form the basis of the five included manuscripts were conducted in three sub-samples, one among girls and two sub-samples among the young women over the five-year study period from two schistosomiasis endemic districts in KwaZulu-Natal, South Africa.

My link to this research is explained through a brief synopsis of my professional and academic background. My post-graduate qualification is a Master of Public Health degree. This has been the source of my motivation and grounding for wanting to investigate the public health aspects of this research. My undergraduate qualification is laboratory based, and since I was a student technologist, I have always had an interest in cytologically diagnosable parasites. As a qualified Cytotechnologist, I have a further interest in investigating cytology as a diagnostic tool for genital schistosomiasis and cervical atypia. I strongly believe that the girls and women of South Africa face much hardship and often bear the scourge of ill health due to preventable diseases like schistosomiasis, cervical cancer and HIV. I hope that through this research I am able to provide new knowledge and contribute to improving the plight of

girls and women in South Africa and globally to reduce their suffering from these preventable diseases. An area of new knowledge and technology that was brought into this research was the inclusion of *Schistosoma* PCR in cervico-vaginal lavage sampling and HPV DNA genotyping among young women from *Schistosoma* endemic regions.

1.2 Aims and Objectives

Aims To determine if FGS is associated with current Human papillomavirus (HPV) and squamous cell atypia (SCA) in young women.

To determine if early treatment for schistosomiasis reverses the cytologic indicators of gynaecological damage (cervical atypia) caused by *S. haematobium* ova and if this treatment has an impact on HPV and squamous cell atypia.

Objectives

The First Objective was to determine the prevalence of SCA and other cytopathology of the cervix in the presence of FGS, in young women aged 16-23 of rural KwaZulu-Natal. **(Paper 2, 4 and 5)**.

The Second Objective was to determine the epidemiological risk factors for SCA of the cervix with special reference to FGS. **(Papers 2 and 4)**.

The Third Objective was to develop early diagnostic tools for FGS. In girls aged 10-12 years and among young women aged 16-23 years. **(Papers 1, 2, 3 and 4)**.

The Fourth Objective was to explore the effect of mass-treatment with Praziquantel on early stages of genital disease in the young women. **(Paper 5-Short communication)**.

The Fifth Objective was to make recommendations for health policy and health services interventions that would reduce the prevalence and incidence of SCA and FGS. **(Papers 1, 2, 3, 4 and 5)**.

Hypothesis

We hypothesize that genital schistosomiasis in young women is associated with squamous cell atypia of the cervix and HPV infection.

1.3 List of Manuscripts

Paper 1

Real-time PCR detection of *Schistosoma*-DNA in small volume urine samples reflects focal distribution of urogenital schistosomiasis in primary school girls of KwaZulu-Natal, South Africa.

Pavitra Pillay, Myra Taylor, Siphosenkosi G. Zulu, Svein. G. Gundersen, Jaco J. Verweij, Pytsje Hoekstra, Eric A.T. Brienen, Elisabeth Kleppa, Eyrun F. Kjetland, and Lisette van Lieshout.
Status: Published in American Journal Tropical Medicine and Hygiene, 2014. 90 (3) 546-552.

Paper 2

Cervical Cytology as a diagnostic tool for Female Genital Schistosomiasis. Correlation to cervical atypia and *Schistosoma* PCR.

Pavitra Pillay, Lisette van Lieshout, Myra Taylor, Motshedisi Sebitloane, Siphosenkosi G. Zulu, Elisabeth Kleppa, Borghild Roald and Eyrun F. Kjetland
Status: Provisionally accepted for publication in CytoJournal 21st November 2015.

Paper 3

Detection of *Schistosoma* DNA by real-time PCR in vaginal lavages and urine: a comparison between five female African study populations originating from three countries with distinct *S. haematobium* and *S. mansoni* transmission patterns.

Pavitra Pillay, Jennifer Downs, Eric AT Brienen, Myra Taylor, Eyrun F Kjetland and Lisette Van Lieshout. (2 additional co-authors from Madagascar and Tanzania)
In manuscript anticipated submission American Journal of Tropical Medicine and Hygiene

Paper 4

Liquid-based cytology as a tool for a more precise diagnosis and risk-assessment of cervical squamous cell atypia among young women from *Schistosoma* and HIV endemic populations in South Africa.

Pavitra Pillay, Myra Taylor, Hashini N Galappaththi-Arachchige, Siphosenkosi G Zulu, Irene K Christiansen, Ole-Herman Ambur, Borghild Roald and Eyrun F Kjetland
In manuscript.

Paper 5 -Short Communication

Preliminary findings of the effect of prior treatment with Praziquantel on squamous atypia are described in young women at risk for female genital schistosomiasis.

Pillay Pavitra, Myra Taylor, Hashini N Galappaththi-Arachchige, Borghild Roald, Lisette Van Lieshout, Eyrun F Kjetland.

In manuscript.

1.4 **Summary of Chapters**

Chapter One: Provides the setting of the study in relation to the health and social context, as well as where the researcher fits into this context. The aims and objectives as well as a list of manuscripts are provided.

Chapter Two: Review of Literature: This chapter includes current literature on FGS. It covers the epidemiology, burden, history and developments of FGS research, the risk factors for and the development of cervical cancer. Possible diagnostic tools for FGS are also discussed. HPV genotyping in Liquid based cytology (LBC) samples is also discussed. Alternative diagnostic tools and new technology are also introduced. In addition, the issue of treatment for schistosomiasis is discussed as well as the public health issues pertaining to FGS. In addition, the gaps in the literature are highlighted.

Chapter Three: Materials and Methods: This chapter focuses on the implementation of the study and includes the protocol that was followed in order for the study to be conducted. It also includes background information on the study area, population and the ethical considerations. This research undertook a study of females living in communities from areas in which access to clean water and adequate sanitation remains a problem. Information on the two main cohorts that is included in this study, namely the primary-school girls in which microscopy and urine *Schistosoma* PCR results are compared with self-reported genital symptoms. This has been detailed in Paper 1. The cohort of young women in whom Pap smear cytology, microscopy and *Schistosoma* PCR in urine and vaginal lavage was conducted is discussed in detail in Papers 2, 3 and 4. The effect of treatment has been discussed in the Short Communication (Paper 5).

Chapters Four, Five, Six, Seven and Eight: In each of these chapters, one of the five manuscripts is presented. This is preceded by a synopsis of the manuscript indicating how it relates to the aims and objectives of the study.

Chapter 9: Unpublished Results

The cytology results which were not included in any of the manuscripts are presented in this chapter.

Chapter 10: Synthesis, Conclusions and Recommendations

Key findings are discussed in this chapter. The main purpose of this chapter is to reveal the current status of FGS and how it was evaluated in terms of the objectives of this study. The findings are critiqued in the light of similar studies and the strengths and weaknesses of the data are presented.

Chapter 11: Conclusions and Recommendations

This chapter also draws conclusions and recommendations from the manuscripts and recommendations are made. Areas for improvement for further studies are also highlighted.

Appendices

The appendices contain the letters of permission and ethical approval, as well as the questionnaire and supporting documents used in the data collection.

2.0 CHAPTER TWO

2.1 Introduction

In this chapter, current literature on FGS and the research questions in this study are discussed. The discussion includes the epidemiology and burden of schistosomiasis, the history and developments of FGS research, cervical cancer and the link to FGS. The risk factors are presented. Diagnostic tools for urogenital schistosomiasis are also reviewed, including urine microscopy, *Schistosoma* PCR in urine and vaginal lavage samples and cytology. Alternative diagnostic techniques that are available but were not used in this study are also discussed.

2.2 Epidemiology of schistosomiasis

Schistosomiasis is one of the 17 neglected tropical diseases (NTDs) that has been prioritised by the World Health Organisation (WHO) (World Health Organisation 2015). These diseases include a variety of entities that affect the majority of the population who comprise the world's poorest communities and collectively affect more than 1.4 billion people (World Health Organisation 2015). Schistosomiasis, affects approximately 261 million people world-wide, 46% of whom are children aged (5-14) years (World Health Organisation 2015). Africa has the highest distribution of schistosomiasis (Figure 1.1.) (World Health Organisation 2015)

The genus *Schistosoma* is made up of several species, those that most commonly infect humans are *S. haematobium*, *S. mansoni* and *S. japonicum*. The *S .haematobium* and *S. mansoni* parasites are mainly found in Africa and the Middle East, *S. mansoni* in South America, while *S. japonicum* is found in Asia (Colley *et al.* 2014). Schistosomiasis is known to cause several complications in the human host, depending on the parasite species involved, ranging from portal hypertension which can lead to death via bleeding in the gastrointestinal tract, to increased risks of developing urinary bladder cancer (Vennervald and Polman 2009). *S. mansoni* and *S. japonicum* are associated with intestinal schistosomiasis and *S. haematobium* is associated with urinary and genital schistosomiasis (DeMay.R 1996; King 2009)

Schistosomiasis is a neglected tropical disease that remains a global burden despite the efforts to eliminate this disease in the 1980's using the anti-schistosomal drug Praziquantel. Infection peaks within the first two decades of life, thereafter the prevalence and intensity decline. However in endemic areas, schistosomiasis can cause lasting pathology and morbidity.

Children are predisposed to infection due to factors such as poor hygiene related to excretion and their tendency to play in infested water (Wright 1986).

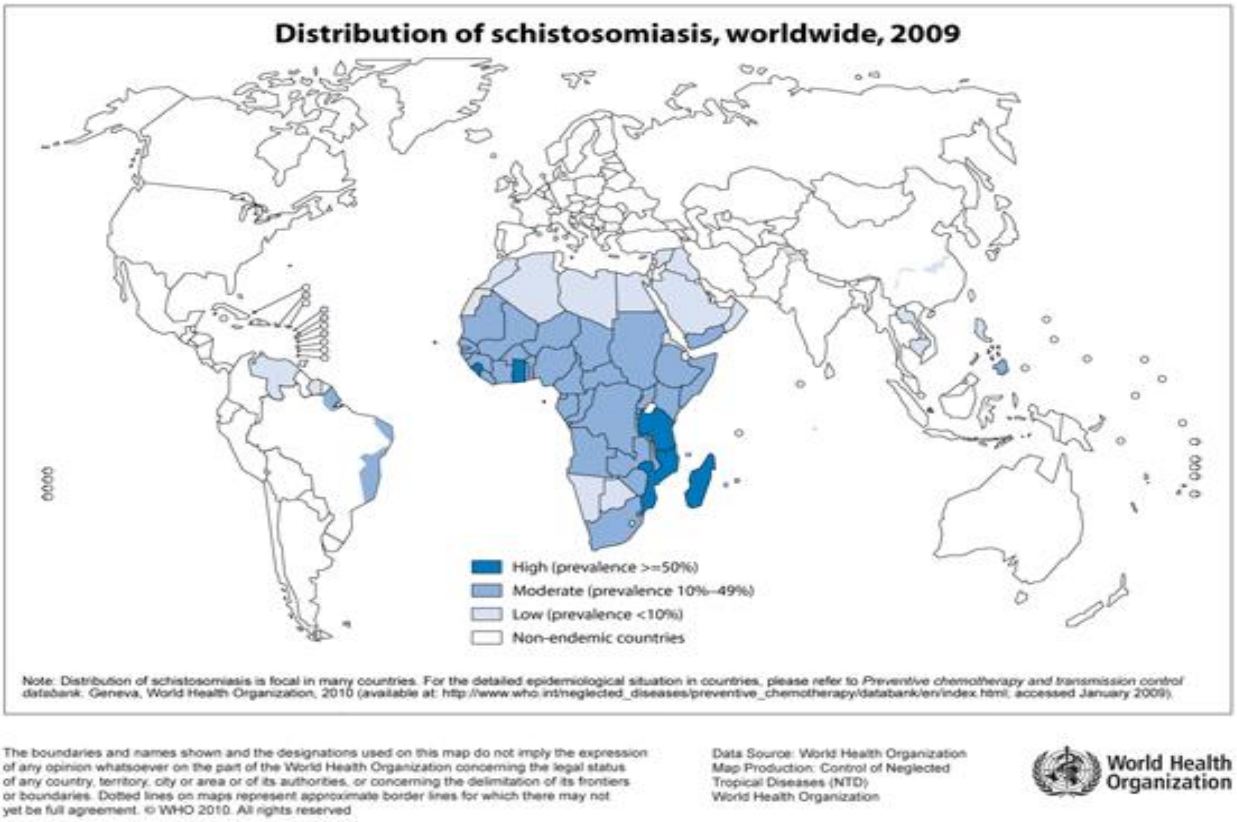


Figure1.1. Global prevalence of schistosomiasis (World Health Organisation 2015)

2.3 Life Cycle and Pathology

Schistosomiasis, or "Bilharzia" as this disease is commonly known was named after Dr Theodor Bilharz who elucidated the life cycle. Schistosomiasis is acquired through parasite infected water-contact mainly among the poor, who rely on rivers for their livelihood and water source for washing and cooking (Colley *et al* 2014). Each species of schistosomiasis has its own specific snail host; the *Bulinus* snail is the intermediate host for *S. haematobium* (Figure1.2.). This snail is found in vegetation surrounding water sources where it sheds thousands of infective cercariae, which can penetrate the skin of the human host. Once within the human host cercariae can take up to six or seven weeks to develop into adult worms that favour living in the venules of the host (Colley *et al* 2014). The adult worms copulate and produce thousands of fertilised ova excreted by the humans through urine and faeces, and because of the lack of sanitation, into fresh water such as rivers or dams. Here, these ova hatch to release miracidia which infect the snail host and the life cycle continues (Colley *et al.* 2014).

Adult worms can live for an average of 3 to 5 years but some may live for up to 30 years within the human host. The male and female worm pair remain as a "monogamous couple" together for their lifetime, with the female producing eggs and the male fertilizing them (Gryseels *et al.* 2006; Colley *et al.* 2014). Schistosomiasis can cause various phases of infection; initially the host has an immune response to antigenic secretions from the miracidia which clinically is presented as a rash at the site of penetration from cercariae. It is also known as "swimmers itch". A condition called Katayama fever due to parasite related hypersensitivity could ensue (Gryseels *et al.* 2006). This usually resolves within a few weeks and is not very common in endemic populations. In high risk populations, chronic pathology can occur in the genito- urinary tracts of both males and females because of *S. haematobium,* which is due to the eggs that are trapped in the mucosa (Leutscher *et al.* 2000; Gryseels *et al.* 2006; Stecher *et al.* 2015) It is not the adult worms that cause much damage to the host, but rather the viable and dead ova (Wright 1986).

Schistosomiasis has a complicated life cycle and while treatment efforts were made to kill the parasites using the anti-schistosomal drug Praziquantel, there was little control over re- infection nor the irreversible damage that this disease had caused within the human host. Eliminating the definitive snail host has also been a challenge for eco-systems, which compound this problem (King 2009).

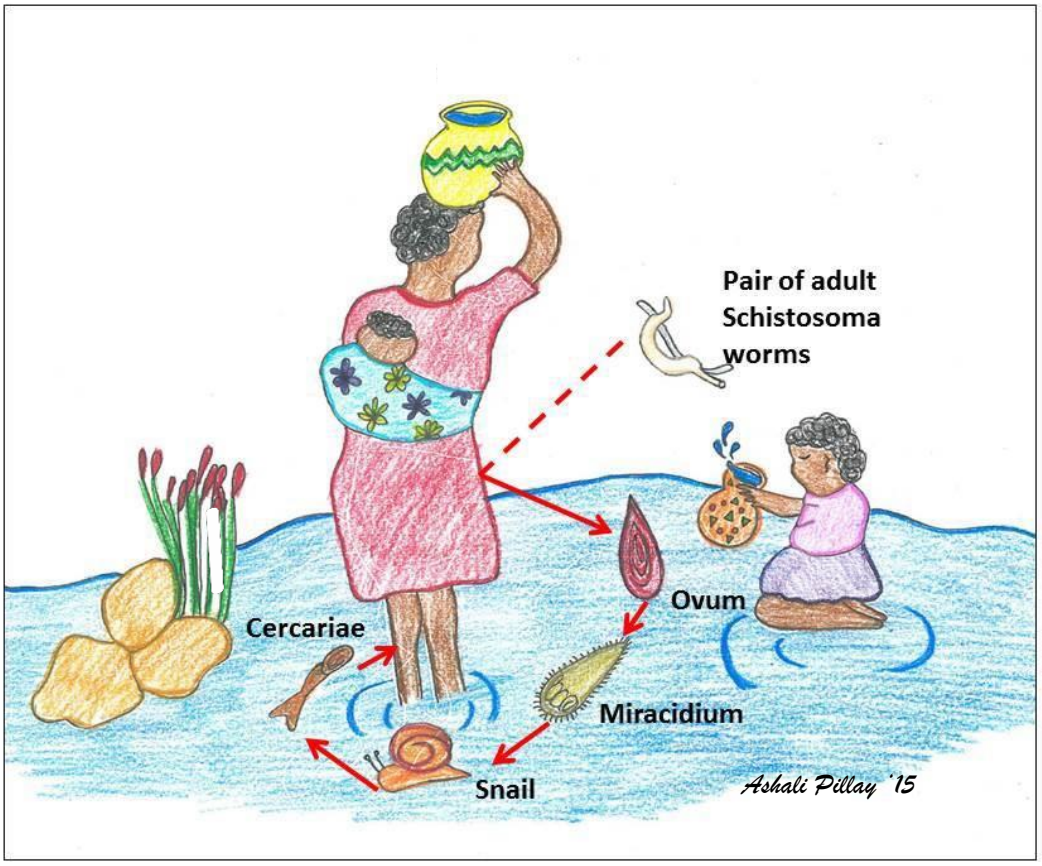


Figure1.2 Life cycle of *S. haematobium* adapted from (Gryseels *et al* 2006; and Colley *et al* 2014).

2.4 *Schistosoma* in the urogenital tracts

Genital schistosomiasis has been studied in the African population for several years as seen in Table 1.1. (Page 14). Early studies by Gelfand *et al*. and others reported sites where evidence of schistosomal infections was found other than the urinary tract. In men the sites were the prostate and testes and in women the sites included the uterus, fallopian tubes, ovaries, cervix and vagina. These studies dating back decades shed light on the organ preference and pathological changes due to *S. haematobium* and *S. mansoni* (Gelfand 1967). Studies focussing on the debate around the presence of schistosomiasis in the genital tract with or without involvement of the urinary tract have revealed that urinary and genital schistosomiasis can co-exist and also that genital schistosomiasis can occur in the absence of urinary schistosomiasis (Poggensee *et al.* 1998; Kjetland *et al.* 2005; Randrianasolo *et al.* 2015). Up to 75% of the women who excrete *S. haematobium* eggs in the urine may have schistosome eggs in the uterine, cervix, vagina or vulva (Renaud *et al.* 1989; Kjetland *et al.* 2005). In a study that was conducted among 547 women aged between 20-49 years to determine the morphologic effects

of schistosomiasis on the lower genital tract, it was found that 58% of women with urinary schistosomiasis had genital schistosomiasis (Kjetland *et al.* 2005).

In 2009, at a Schistosomiasis Working Group Meeting, consensus was reached that since the *Schistosoma* parasite is responsible for the development of lesions in the urinary and genital tracts of both males and females, this condition was renamed from urinary schistosomiasis to urogenital schistosomiasis (WHO 2009).

Urinary Tract: Pathological changes to the urinary bladder are usually associated with the intensity of infection, in humans with high egg loads, eggs are excreted via the urine. Over half the eggs produced by the parasite remain trapped in the host's tissues and never leave the body (King 2009). The eggs secrete enzymes that trigger an inflammatory response; the extent of the inflammation is dependent on the immune system of the host (Gryseels *et al.* 2006). This disruption to the tissues caused by ova is characterised initially by neutrophils and macrophages, then lymphocytes and finally eosinophils which result in a granulomatous reaction. Red blood cells are also a common finding. Giant epithelioid cells and plasma cells may also be found around the granuloma (Wright 1986). The ova are able to trigger an inflammatory reaction that can progress to erosion of the mucosa. Erosion is manifested clinically by painful micturition and haematuria among males and females. This common clinical symptom when seen among male children was termed "male menstruation" (Wright 1986). On cystoscopic examination, the bladder appears granular, which is due to inflammation and urothelial hyperplasia around the ova. While some eggs are degraded within the granulomas, others resist and become calcified. Within affected organs, fibrosis and scarring can be found as a result of trapped ova. The cellular reaction may progress from hyperplasia to metaplasia and then in some cases to dysplasia and bladder cancer (Herrera and Ostrosky-Wegman 2001; Vennervald and Polman 2009). In the present study, diagnostic tools are used to determine the presence of schistosomiasis in the urinary tract as well as in genital samples to further explore the existence of infection within the urinary and genital tracts.

Genital Tract: While there is evidence that *S. haematobium* is the causative agent for FGS, little is known about the effect of *S. mansoni*. The two *Schistosoma p*arasites, *S. haematobium* and *S. mansoni* are prevalent in Africa and in the Middle East, whereas *S. mansoni* can also be found in South America (Colley *et al.* 2014). In some regions in Africa, there is overlap between the geographical areas in which both species are endemic (Meurs *et al.* 2012). The *S. haematobium* worm is known to prefer residence in the pelvic venous plexus therefore the ova

tend to become lodged in urogenital tissues, while *S. mansoni* prefers the portal venous system and is usually found in the intestinal tract. It has been suggested that while *S. mansoni* ova are usually lodged in the liver or intestines, this parasite can also be found in the genital tract (Feldmeier *et al.* 1998). In the following studies conducted in Tanzania and Brazil, *S. mansoni* was found with FGS (Poggensee *et al.* 2001a; Cavalcanti *et al.* 2011).

Community based studies have linked *S. haematobium* as the more frequent parasitic agent in FGS (Kjetland *et al.* 2005). FGS is a neglected entity and has recently only gained interest in Africa through community based studies (Leutscher *et al.* 1997; Kjetland *et al.* 2005; Downs *et al.* 2011) despite being studied for many years prior to this (Table 1.1), (page 14). In other studies the genital changes as seen on macroscopic examination of the cervix, via histopathology and cytology have been reported over the last five decades (Berry 1976; Wright, Chipangwi and Hutt 1982; Poggensee *et al.* 2001b; Kjetland *et al.* 2005). The genital tract is affected when eggs are excreted into the venous system where they become trapped in surrounding organs, it is suggested that there is an overflow of eggs into the genital tract from other pelvic organs. The exact extent of damage that can occur within the organs is related to the host's response to the deposited ova. Ova have been found throughout the female genital tract: in the ovaries; fallopian tubes; uterus and placenta; cervix and vagina (Poggensee *et al.* 2001b; Kjetland, Leutscher and Ndhlovu 2012).

The genital manifestations may mimic cancer-looking lesions and different sexually transmitted diseases, such as ulcers, genital warts, polyps, and also cause mucosal immune activation and blood vessel friability (El-Zeneiny, Badawy and Iskander 1968; Edington, Nwabuebo and Junaid 1975; Leutscher *et al.* 1998; Poggensee *et al.* 2001b). Genital biopsies from thirty three women from Malawi were used in the study to examine the histopathologic and clinical changes seen in FGS. In this study it was found that sandy patches and vaginal tumours were mostly associated with FGS (Helling-Giese *et al.* 1996). In a recent expert committee meeting, a general consensus of clinical findings from several large community based FGS studies was in summary, the colposcopic findings of sandy patches, homogenous sandy patches, abnormal mucosal blood vessels and more recently rubbery papules in the cervico-vaginal region (Kjetland *et al.* 2005; Norseth *et al.* 2014; Randrianasolo *et al.* 2015). Classification of the clinical findings are also of importance since FGS is an under diagnosed entity which needs to be made known to health care workers (Norseth *et al.* 2014)

In histological sections of the cervix, ova have been described as few or numerous, and are usually seen in the connective tissue below the epithelium of the squamo-columnar junction.

It has been reported that ova could gain access from the stroma into the epithelial lining by either of the following mechanisms: hyperaemia within the egg containing venules resulting in eggs "piercing" into the epithelial lining with their spines, or through areas of ulceration within the epithelium (Berry 1966). The response to ova within cervical biopsies have been described as follows: in some biopsies no reaction to the ova was detected, while in others, varying degrees of changes similar to those in the bladder, including inflammation, granulation and ulceration were noted (Berry 1966; Poggensee *et al.* 2001b). In granulomatous inflammation, giant multinucleated histiocytes, epithelioid cells, lymphocytes, plasma cells and eosinophils may be seen. The ova can be detected in varying stages of maturity containing immature or mature miracidia or as calcified or dead ova. Empty shells may also be seen (Berry 1966).

In cytology Pap smears varying stages of inflammation may be seen depending on the stage, from acute (with mainly neutrophils), to chronic (mainly lymphocytes and histiocytes) and granulomatous inflammation (Wikeley 1989). Granulomatous inflammation is characterized by the presence of aggregates of macrophages and small lymphocytes. Macrophages become large with pale nuclei and abundant foamy cytoplasm and epithelioid cells have narrowed elongated/oval nuclei with soft frayed cytoplasm outlining the nuclei. Giant multinucleated histiocytes are also seen (Berry 1966). Freshly passed ova are oval with a chitinous shell. *S. haematobium* has a terminal spine whilst *S. mansoni* has a lateral one. Ova of *S. haematobium* range from 80-170 µm long and from 30-70 µm broad whilst those of *S. mansoni* are marginally larger and *S. mathee* are longer and narrower (Berry 1976). Inside the ovum an embryo miracidium may be seen in an under-developed or well-developed stage and emergent miracidia are not uncommon. In cytological samples of the female genital tract the following presentations of *Schistosoma* species as seen in histological sections have been described, including empty shells which are refractile, folded and crumpled with no visible internal structure; degenerative ova which have a variable appearance dependent upon the length of time they have been dead (Wikeley 1989) Partially blackened and completely black and opaque forms may also be seen. Immature ova have a granular appearance inside the refractile, chitinous shell. Mature ova may be seen with a visible miracidium. Emergent free-lying miracidia are oval, round and could also appear in elongated shapes with the exterior surface of miracidia being finely ciliated (Berry 1976).

In the present study, cytology is used as a diagnostic tool in young women who are at risk for schistosomiasis, since cytology has the advantage over cervical biopsies because it is a technique that causes minimal disruption to the epithelium. In addition to the detection of

parasites, it can be used for the detection of other entities like inflammation and cervical atypia. In the present study, the presence of *Schistosoma* ova in Pap smears or *Schistosoma* DNA detected in vaginal lavage samples, has been used as a proxy for FGS because demonstration of *Schistosoma*-egg derived pathology is not feasible in the study population.

2.5 Female genital schistosomiasis and cervical cancer

There has been a controversial relationship between genital schistosomiasis and squamous cell atypia of the cervix which has been studied over decades. Studies either supporting or rejecting this association may be seen in Table 1.1. (Page 14). It has been hypothesised that *Schistosoma haematobium* may play a role in the development of cervical cancer probably indirectly by increasing the susceptibility to HPV infection (Kjetland *et al.* 2009a). Others however question the aetiologic role of schistosomiasis in the development of cervical cancer (Moubayed *et al.* 1994). The causal relationship between schistosomiasis and cervical cancer is complex and hence may be difficult to determine since both entities occur in the genital tract and females may be asymptomatic for several years. In a study analysing the relationship between parasites and cancer, it was found that helminths can cause genetic instability and affect cellular communication mechanisms, through inflammation and alteration to the immune modulators which could promote tumour formation (Herrera and Ostrosky-Wegman 2001).

Similarly, several histopathological reports indicate that there may be inflammatory reactions around dead ova, which continue to pose a clinical problem (Kjetland *et al.* 2008). In a study conducted in Malawi it was found that several lesions including epithelial hyperplasia, basal cell activity and leukoplakia were frequently seen in genital *Schistosoma* infection. It has been suggested that these changes may induce chronic irritation and possible premalignant conditions (Helling-Giese *et al.* 1996). In the present study the association of FGS and squamous atypia has been investigated.

Table 1.1 Summary of literature on Female Genital Schistosomiasis in relation to symptoms and distribution, squamous cell atypia, HIV, and treatment

Focus of studies: Distribution of FGS and Clinical Symptoms				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Randrianasolo *et al.* 2015)	To compare Schistosoma PCR, histopathology and gynaecological findings among women with different intensities of urinary schistosomiasis	118 women aged 15-35 years	This study adds an additional clinical finding called "rubbery papule" to the previously classified sandy patches. It also confirms that live and dead ova can cause pathology in the mucosa. It confirms that women with *Schistosoma* negative urines may have *Schistosoma* positive genital samples.	Madagascar
(Hegertun *et al.* 2013)	An investigation of genital symptoms in young girls with urinary schistosomiasis	1057 school girls aged 10-12 years	Genital symptoms were significantly associated with urinary schistosomiasis and water contact	South Africa
(Yirenya-Tawiah *et al.* 2011)	To determine the prevalence of FGS in Ghana	420 women, aged 15-49	Prevalence of FGS was 10.6% and the women infected had associated reproductive health symptoms	Ghana
(Kjetland *et al.* 2005)	To investigate the FGS induced pathology in the lower genital tract	527 Women aged 20-49	Genital homogenous sandy grainy patches found in 46% of women with schistosomiasis also mucosal bleeding and abnormal blood vessels were found	Zimbabwe
(Poggensee *et al*. 2000)	To investigate the occurrence of and morbidity caused by genital schistosomiasis	134 women	Morbidity caused to the genital mucosa in women infected with schistosomiais could increase the risk of acquiring HIV	Tanzania
(Helling-Giese *et al.* 1996)	To examine the histopathologic and clinical changes seen in FGS	33 women	Sandy patches and vaginal tumours were mostly associated with FGS	Malawi
(Gelfand *et al.* 1971)	Histopathological analyses of Schistosoma infections from the uterus, and its adnexa and vagina.	64 Autopsies from African women aged 17-70	Most infections were due to *S. haematobium*. Heaviest egg loads were found in the bladder, similar egg loads were found in the cervix, fallopian tubes and ovaries	South Africa and Zimbabwe

Focus of studies: Female Genital Schistosomiasis Association with Cancer				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Toller *et al.* 2015)	Case report CIN3 and schistosomiasis eggs	44 year old HIV positive woman	Diagnostic features described. FGS must be included in the differential diagnosis in routine gynaecological investigations to prevent misdiagnosis	Angola
2013(Van Bogaert 2014)	To investigate urogenital schistosomiasis and HIV and cervical cancer	227 cervical biopsies, 50 were from HIV infected women and177 were non- HIV infected	No association of cervical schistosomiasis and HIV and also no HIV-schistosome co- infection with cervical cancer or its precursors	South Africa
(Kjetland *et al.* 2009a)	To investigate the impact of HPV and schistosomiasis in a 5 year follow up study	37 women	Schistosomiasis was associated with high grade intraepithelial lesions but not with persistent high risk HPV. Further studies warranted.	Zimbabwe
. (Moubayed *et al.* 1994)	To investigate carcinoma of the cervix and schistosomiasis	Retrospective study- 4520 histopathology cervical cancer cases	76 (1.7%) of the cases had schistosomiasis, schistosomiasis was not associated with cervical atypia	Tanzania
(Szela *et al.* 1993)	To investigate the possible association of FGS with cervical dysplasia and cervical cancer	2 groups of 24 women each- one *Schistosoma* endemic area and the other from a non-endemic region	46% schistosoma prevalence in the endemic group but no association with cervical dysplasia or cancer. No association from the non-endemic group. HPV was most likely the cause of cancer of the cervix rather than schistosomiasis	Ghana
(El Tabbakh and Hamza 1989)	To investigate the relationship bet cervical cancer and schistosomiasis	Case report well differentiated adenocarcinoma and schistosomiasis	Schistosomiasis and squamous carcinoma of the cervix is controversial- schistosomiasis associated with precancerous epithelial changes like basal cell hyperplasia, leukoplakia Regarding the *Schistosoma* squamous carcinoma relationship: association does not prove causality- since it is very complex to prove causality	Egypt

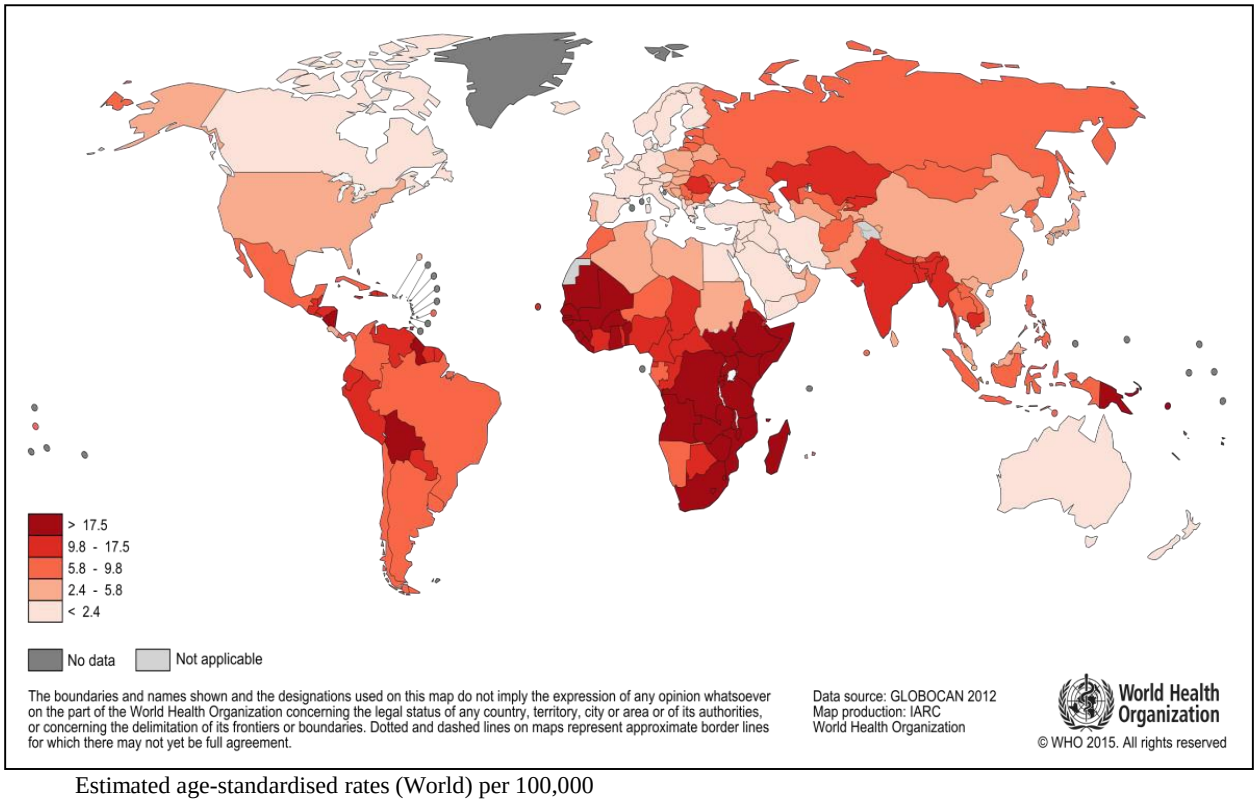
Focus of studies: Female Genital Schistosomiasis Association with Cancer				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Wright, Chipangwi and Hutt 1982)	To investigate the histopathology of FGS	176 histopathological sections	60% had genital schistosomiasis, no conclusive evidence that this was linked to cervical cancer	Malawi
(Youssef, Fayad and Shafeek 1970)	To investigate the association of FGS with genital atypia	121 cases of cervical schistosomiasis	Possible association of schistosomiasis with premalignant conditions- epithelial and basal cell hyperplasia and other changes. One case of CIS and 16 cases of invasive squamous carcinoma associated with schistosomiasis	Egypt
(Berry 1966)	To describe the cytopathology and histopathology of genital schistosomiasis	139 cytology smears 140 histological sections from the genital tract	Genital schistosomiasis was confirmed and should be considered in the diagnosis of populations at risk. FGS was not associated with cancer of the cervix, but further investigation necessary. Cervical smears can be of value in the diagnosis if properly collected.	South Africa
Focus of studies: Clinical Manifestations of Urinary Schistosomiasis				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Swai *et al.* 2006)	To review retrospective histopathological samples in order to determine the occurrence and features of FGS.	423 organ specimens in which schistosomiasis was diagnosed were reviewed in conjunction with age, clinical and other symptoms	172 (40.7%) had urinary schistosomiasis Genital schistosomiasis diagnosed in 176 cases, from peritoneum, uterus, ovary, cervix, vulva, labia, cervix, fallopian tubes, women aged between 5-61 years. Also evidence of cancer or precancerous lesions in 20% of the cervical biopsies with schistosomiasis	Tanzania
(Leutscher *et al*. 1998)	To assess morbidity due to schistosomiasis among women of reproductive age	176 women aged between 15-49	Schistosomiasis causes symptoms in both urinary and genital tracts and needs further investigation- small sample size and many confounders	Madagascar
(Poggensee *et al*. 1998)	To investigate genital schistosomiasis without egg excretion in urine	533 women, aged between 15-45 years	Urinary *Schistosoma* prevalence was 40%, Genital schistosomiasis was 32%. Co-existence of genital and urinary schistosomiasis was 62%. Genital schistosomiasis without egg detection in urine was 23%. FGS link to HIV possibly increased	Tanzania

Focus of studies: FGS and its Association to HIV				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Kleppa *et al.* 2014)	To investigate the relationship between FGS and HIV using HIV target cell density and HIV expression of the HIV CCR5 co receptor in blood and cervical mucus at baseline and post anti-schistosomal treatment.	14 young women attending high school	$CD14^+$ cells were elevated blood and $CD4^+$ cells expressing the CCR5 HIV co-receptor were increased in FGS+ women. Post anti-schistosomal treatment, only the $CD14^+$ levels decreased. FGS may increase HIV acquisition and alter HIV target cells as well as due to genital damage. Anti-schistosomal treatment can modify this.	South Africa
(Downs *et al.* 2011)	To determine female urogenital schistosomiasis (FUS) among women of reproductive age	457 women aged 18-50 years	Prevalence of FUS was 5% and HIV was 5.9% overall, but found in 17% of women with FUS-conclusion- FUS may increase acquisition of HIV	Tanzania
(Kjetland *et al.* 2006)	To determine the association between FGS and HIV	547 women aged between 20-49	Women with genital schistosomiasis had almost a 3-fold risk of HIV	Zimbabwe
Focus of studies: FGS and the Effect of Anti-schistosomal Treatment				
AUTHOR,YEAR	**AIMS OF STUDY**	**POPULATION**	**RESULTS**	**COUNTRY**
(Downs *et al.* 2013)	To explore the response of a single dose of praziquantel in women with schistosomiasis	33 women aged 18-50	6 months after a single dose of PZQ (8) 24% had persistent schistosomiasis and (11) 33% had genital abnormalities attributed to schistosomiasis. Treatment with PZQ more frequently than 6 month intervals and in younger women may help reduce genital pathology, and risk of HIV acquisition.	Tanzania
(Kjetland *et al.* 2006)	To study the effect of treatment with praziquantel	527 women aged 20-49 years	Results were analyzed at baseline and within 3-12 months post treatment. Genital lesions and bleeding did not reduce after treatment.	Zimbabwe
(Richter *et al.* 1996)	To investigate the reversibility of lower genital tract abnormalities in women with Schistosoma infections after treatment with praziquantel	21 women at 2-9 weeks after PZQ treatment	Although the sample size was small, and the follow-up time was short, there was partial resolution of genital pathology- more research is required.	Malawi

2.6 Epidemiology of Cervical Cancer

Cervical cancer is a serious public health concern and was ranked the second most common cancer among women after breast cancer in developing countries and third most common in developed countries (Arbyn and Dillner 2007). The highest prevalence is among developing countries from South and Central America, sub-Saharan Africa, and South and South East Asia as (Anorlu 2008; Ferlay *et al.* 2010). Cervical cancer in the developing world was estimated to account for approximately 79% of cancers that occurred in the world in 2002. About 80% of these cases are in developing countries where screening programmes are not well established or minimally effective (Sankaranarayanan, Budukh and Rajkumar 2001). In 2008, it was reported that more than 85% of the deaths due to cervical cancer occurred in developing countries (Jemal *et al.* 2011).

Estimated Cervical Cancer Mortality Worldwide in 2012



Estimated age-standardised rates (World) per 100,000

Figure1.3. Global cervical cancer mortality rates. (WHO 2015)

Cervical cancer is one of the few cancers that pass through a stage where it is confined to the epithelium before it invades through the basement membrane and into the stroma (Schiffman *et al.* 2007). The uterine cervix is lined on the outer region or ectocervix by non-keratinized stratified squamous epithelium and the endocervix is lined by a single layer of columnar cells.

The point at which the two epithelia meet is known as the squamo-columnar junction or transformation zone (TZ) (Figure 1.4). The TZ is found within the endocervical canal before puberty, at the onset of menstruation or at the first pregnancy, this zone "rolls" out into the ectocervix. This is the site where squamous metaplasia occurs in most women (Singer 1975). Squamous metaplasia is a benign proliferative reaction in which the fragile columnar epithelium becomes replaced by a more resilient squamous-like epithelium. During menopause, the TZ once again reverts into the ectocervix. The TZ is the most common site where cells become infected by viral infections like HPV and also where cancers develop (Schiffman *et al.* 2007).

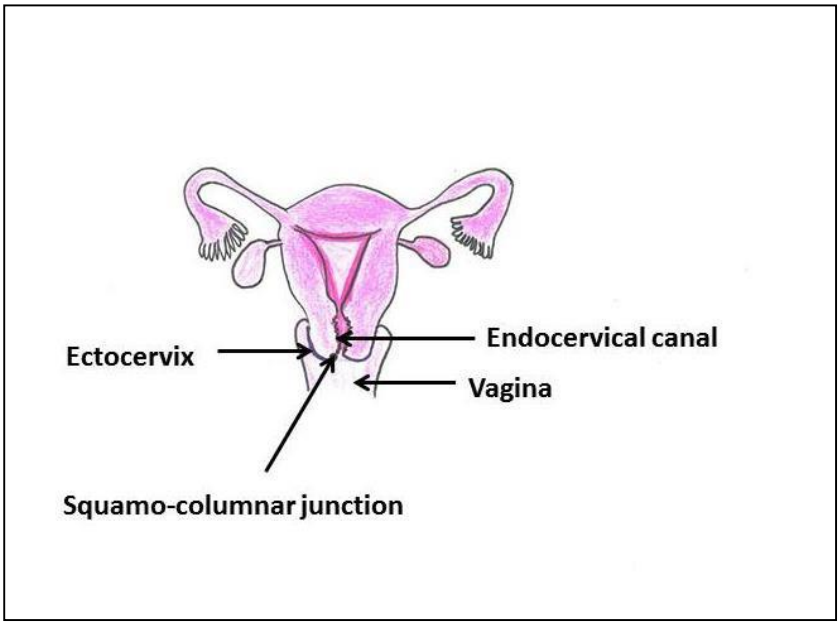


Figure1.4 Anatomy of the Female Genital Tract adapted from (Wheater P 1985)

The cervical lesions in the pre-invasive stage were initially described as "incipient cancer", "dysplasia" or "carcinoma in situ". Later these lesions were designated "cervical intraepithelial lesion" (CIN) and most recently they have been re-classified according to the Bethesda System of reporting as squamous intraepithelial lesions (SIL) (Solomon *et al.* 2002). The primary function of diagnostic terminology is to communicate to the physician the interpretation of a cervical smear in descriptive terms that will have clear universal implications for appropriate patient management. The Bethesda System (TBS) of reporting was adopted in 1991(Solomon *et al.* 2002). TBS was designed to be flexible so that it could evolve in response to changing needs in cervical cancer screening. TBS is divided into three main categories as depicted in Table 1.2. Since HPV is so strongly associated with dysplasia and often cannot be distinguished from CIN1/mild dysplasia it has been put in the same category under low-grade squamous intraepithelial lesion (LSIL). Early detection and treatment in the pre-invasive stage can in most cases lead to total eradication of this disease (Sankaranarayanan, Budukh and Rajkumar 2001).

The evolution of invasive squamous cell cancer involves a number of stages with increasing intraepithelial abnormality which steadily progresses from mild to moderate to severe to carcinoma in situ (CIS) and then to micro-invasive carcinoma (Vooijs 1991). Although it is not cytologically possible to predict the malignant potential of a pre-malignant lesion, evidence suggests that mild dysplasias are more prone to regress spontaneously. Conversely, severe dysplasia and carcinoma-in-situ dysplasia also known as high grade squamous intraepithelial lesion (HSIL) is more likely to persist and progress to invasive cancer. This can progress over a period of up to ten years (Vooijs 1991). Another important predictor of progression is the strain or genotype of HPV that is found with the intraepithelial lesion, there are some strains that are highly carcinogenic and associated with invasive cancer (Schiffman *et al.* 2007).

Table1.2 Terminology for cervical atypia (Solomon and Nayar 2004)

Bethesda System	Cervical Intraepithelial Neoplasia (CIN)	Dysplasia Terminology
Atypical Squamous Cells of Unknown Significance (ASCUS)	Cellular Atypia	Unspecified cellular changes
Low grade squamous intraepithelial lesions (LSIL)	HPV CIN1	HPV Mild Dysplasia
High grade squamous intraepithelial lesion (HSIL)	CIN2 CIN3 (including carcinoma in situ /CIS)	Moderate Dysplasia Severe Dysplasia/CIS

In South Africa a small proportion of women have been opportunistically over-screened in an uncoordinated and unscientific manner, while the vast majority of high risk and especially rural women have had no access to cervical screening (Anorlu 2008). Many women only present in the advanced stage of the disease, when the cancer is often untreatable (Anorlu 2008). Thus the lack of or poor implementation of screening has been a contributory factor in the development of cervical cancer. In 2000, the South African Department of Health launched the National Guideline on Cervical Screening which offers women three free Pap smears in their lifetime commencing at the age of 30. There are 10 year intervals between each smear, if women are found negative (Health 2000)

2.7 Risk Factors for cervical cancer

Sexually transmitted HPV infection of the cervix has been reported as the risk factor implicated in 99.7% of invasive cervical cancers in both developed and developing countries (Walboomers

et al. 1999). More specifically, the HPV types 16, 18, 31, 39, 45, 52 and 58 have been reported as "carcinogenic to humans" (Walboomers *et al.* 1999). The list of HPV types is updated by the International Agency for Research in Cancer (IARC) as those types with "sufficient" evidence for carcinogenicity and those with "limited" evidence for being carcinogenic (IARC, 2007). In addition, the following risk factors were identified from the results of the re-analysis of twelve invasive cervical cancer studies (Berrington and Green 2007). Early onset of sexual activity, a greater number of lifetime sexual partners, parity, younger age at full term pregnancy, use of oral contraceptives, immunosuppression and smoking were the most common risk factors (Berrington and Green 2007). In a South African survey among 20-25 year old women it was found that the average age at which they first had sex was 16.9 years (Fonn *et al.* 2002).

It is known, that women are generally infected with HPV in their teens, 20s, or 30s and cervical cancer can take up to 20 years after the initial HPV infection to develop (Schiffman *et al.* 2007). Studies also show that at least 96% of low grade lesions of the cervix in young women tend to regress within 36 months of acquisition (Szarewski and Sasieni 2004; Schiffman *et al.* 2007). Cervical screening amongst young women is therefore a topic of much debate in light of the theory that lesions tend to clear. There is concern about over diagnosis and over-treatment among young women (Szarewski and Sasieni 2004). The incidence of cervical cancer has been noted to increase in women between the ages of 35 to 40 years and reaches a maximum in women in their fifties and sixties (Denny, Quinn and Sankaranarayanan 2006). A Zimbabwean study, found the high-risk HPV prevalence to be 36% in young women aged 15-24 years (Baay *et al.* 2004). In developing countries like South Africa where young women are at risk for both HIV and schistosomiasis, and cervical cancer is the second common cancer, raising awareness of the effects of HPV among young women at risk for these diseases is important.

2.8 Implications for cervical cancer and HIV

Recent studies have shown that women with HIV have a higher risk of progressing faster to invasive cervical cancer (Ferlay *et al.* 2010; Firnhaber *et al.* 2012). In a cross-sectional study that was conducted in KwaZulu-Natal among 466 rural women to establish the relationship between HIV infection and cervical dysplasia, where the mean age among the women was 24.3 (range 15 to 55 years), it was found that there was a significant association with HIV and abnormal Pap smear findings (Karim *et al.* 2007). Of importance in this study was the age distribution of the subgroup of women with HSIL in which 5/6 cases occurred under the age of 30 and almost all these cases were from women co-infected with HIV (Karim *et al.* 2007). In a cross-sectional study conducted in Cape Town to determine the risk of pre-cervical cancer and cancer among HIV positive women, it was found that there was a 66,3% prevalence of abnormal Pap smears in women 18 years and older (Moodley *et al.* 2006). In a recent

exploratory study investigating screening South African women under the age of 30, since the current screening programme does not cater for this age group who have high grade lesions that could progress and also are at risk for HPV and HIV, it was suggested that policy makers should reassess the risk factors as well as the psychosocial impact of cervical cancer to ensure that health infrastructure supporting the prevention and management is provided for women at the onset of sexual activity (Learmonth and Learmonth 2014).

2.9 Association of schistosomiasis with HIV

This association has been investigated in several studies, as seen in Table1 (page 14). In a study among the same Zimbabwean population to determine the association between FGS and HIV, it was found that women with genital schistosomiasis had almost a 3-fold risk of having HIV (Kjetland *et al.* 2006). In a study investigating the immune cells in relation to HIV and schistosomiasis, it has been found that HIV target cells which are $CD4^+$ T lymphocytes found in genital mucosa from *Schistosoma* induced lesions may contribute to HIV transmission (Jourdan *et al.* 2011). It has been recently suggested that increased vascularity which makes the mucosa more prone to lesions within in the vagina and uterine cervix, caused by *S. haematobium* may also increase the risk of HIV (Jourdan *et al.* 2011). It has been shown that there is a significant overlap in the prevalence of HIV and schistosomiasis in Sub-Saharan Africa, supporting the hypothesis that schistosomiasis infection may be linked to increased risk for HIV (Mbabazi *et al.* 2011).

2.10 Anti-schistosomal Treatment

Praziquantel was developed in the 1970's and was found to be safe and easily administered via school or community based mass treatment campaigns (Gönnert and Andrews 1977; Ming-Gang 2005). The current WHO resolution is to eradicate schistosomiasis in multiple countries by 2020 and globally by 2025 (World Health Organisation 2015).The WHO mass treatment strategy includes all risk groups with school age children being a primary target (World Health Organisation 2015). Mass treatment for schistosomiasis is therefore done through schools in endemic areas and has already been implemented in many African countries (Gundersen *et al.* 1990; Fenwick and Webster 2006). In South Africa mass treatment was implemented in schools in KwaZulu Natal in 2001, however this programme was not sustained (Appleton and Kvalsvig 2006). While it was reported that there was a reduction in Schistosomiasis from this programme, it is also known that in populations at risk, there is the possibility of re-infection (Randjelovic *et al.* 2015). In South Africa a huge challenge has been the high cost of anti-schistosomal drugs (Berge *et al.* 2011). South Africa is one of the five remaining countries in Africa where mapping has to occur in accordance with the WHO resolution.

The mass treatment is known to be effective in killing the *Schistosoma* parasites, however this has not completely eliminated this disease because of various implementation, monitoring and re-infection challenges in high risk communities (King 2009). The WHO mass treatment strategy includes all risk groups with school age children being a primary target (World Health Organisation 2006).

Diagnostic tools for schistosomiasis detection are available, and for mass drug administration (MDA) it has been suggested that there are distinct stages in which infection is monitored, the first stage being the mapping and surveillance in which the prevalence of infection is determined, thereafter diagnostic tools are required for monitoring where the effect of treatment interventions can be measured and finally to monitor re-infection after treatment interventions have ceased (Utzinger *et al.* 2015).

While mass treatment is effective in killing adult worms responsible for laying eggs, it is not known what effect it has on FGS lesions. One study showed that treatment given to women with FGS lesions in adulthood appeared to leave the lesions unchanged (Kjetland *et al.* 2006). Similarly, several histopathological reports indicate that there may be inflammatory reactions also around dead ova, which are presumably PCR negative, yet continuing to pose a clinical problem (Kjetland *et al.* 2009b). The effect of treatment on FGS however requires further exploration.

2.11 Diagnosing FGS

In diagnosing genital schistosomiasis, visual inspection can be used whereby the cervix is magnified using a colposcope and the surface is examined for grainy sandy patches and other characteristic signs of egg-induced inflammation (Kjetland, Leutscher and Ndhlovu 2012). This procedure also has limitations since it is highly observer-dependent, requires extensive training and expensive equipment and cannot be used among children, since intra-vaginal inspections are normally not performed before the onset of sexual activity. Since FGS needs to be investigated among girls and young women due to their susceptibility to schistosomiasis, it is important to seek alternative and indirect mechanisms of investigating this disease.

Alternate diagnostic mechanisms to visual inspection include the identification of parasite eggs in urine, stool, or biopsy specimens, *Schistosoma* PCR analysis and using immunological disease markers on blood or other samples. For the specific diagnosis of FGS histological examination of a cervical biopsy was considered to be the gold standard (Poggensee *et al.* 2001b). In young populations who are risk for HIV, this procedure, searching for *Schistosoma*

eggs in biopsy tissue, is invasive and therefore not applicable. Urine microscopy may be used to diagnose schistosomiasis, however urine is an unspecific diagnostic tool for genital schistosomiasis, since it has been found that women may present with Schistosoma ova in their genital tract with or without ova in the urinary tract (Randrianasolo *et al.* 2015). Molecular techniques such as PCR in stool and urine have also been established (Verweij *et al.* 2007); (Aryeetey *et al.* 2013). In a Zimbabwean FGS study, *Schistosoma* PCR was done on cervico-vaginal lavage samples, however this aspect of diagnostics requires further investigation (Kjetland *et al.* 2009b).

In attempts to gain more insight into FGS, Pap smears may also be used to detect *Schistosoma* ova, although routine investigations from previous studies thereof, have shown mainly low sensitivity with the exception of a study conducted in Madagascar among known positives (Feldmeier, Helling-Giese and Poggensee 2001; Randrianasolo *et al.* 2015).

Immunologic tests for the *Schistosoma* antigen are also available, two antigen tests that have been in use for schistosomiasis are the circulating anodic antigen (CAA) and the circulating cathodic antigen (CCA) (Van Lieshout, Polderman and Deelder 2000). *Schistosoma* antigens are secreted into the host's circulatory system and are detectable within 3 to 5 weeks after infection (Van Dam *et al.* 1996). One of the methods of detection is thorough using the antibody sandwich enzyme-linked immunosorbent assays (ELISA) method (Van Lieshout, Polderman and Deelder 2000). In a study comparing direct parasitological methods and immunologic methods (using serum CAA and urine-CAA as well as antibodies) it was found that the results were comparable in populations with moderate to high intensity of infection (Van Lieshout 2000). A challenge with immunological markers is that these could not be used to distinguish between past and present infections (Obeng *et al.* 2008). A rapid field applicable point of care (POC) assay using CCA in urine samples was developed to overcome some of the disadvantages of the ELISA, however this tool is unable to distinguish between *Schistosoma* species (Obeng *et al.* 2008). These immunologic tests could possibly be useful for FGS if these are done on samples from the genital tract, like cervico-vaginal lavage. Antibody tests have proven to be highly sensitive in individuals who might be infected but originate from non-endemic areas such as travellers but are not for people who live in endemic areas, since antibodies can be detected in light infections and in those who have had a short exposure but are of little value in areas with low transmission due to their lack of specificity (Van Lieshout, Polderman and Deelder 2000; Gryseels *et al.* 2006; Van Lieshout and Verweij 2010). Indirect markers include the identification of clinical findings (for example, haematuria), radiographic examinations and questionnaires can be used to assess prevalence (Van Lieshout and Verweij 2010).

In populations at risk for schistosomiasis it is important to be able to identify those infected, ensure that they are treated accordingly and the measures are taken to reduce re-infection (Van Lieshout and Verweij 2010). A suitable diagnostic marker should not only provide information about the presence of a pathogen at the individual level but should also be able to provide additional information that will assist in the management of disease, such as intensity of infection or whether the infection is past or present. Schistosomiasis is a disease that can be viewed from two main perspectives, firstly from a health care perspective where infected individuals need treatment as well as from a public health perspective where diagnostic information provided can assist in managing populations at risk (Van Lieshout and Verweij 2010; Colley *et al.* 2014; Utzinger *et al.* 2015).

In the present study, the diagnostic tests used to determine *Schistosoma* prevalence at the population level included urine microscopy and *Schistosoma* DNA assays in urine and cervico-vaginal lavage and cervical cytology for detecting schistosomiasis in the urinary and genital tracts to map populations risk and to clarify trends and associations between FGS entities. These diagnostic tests will be discussed below in more detail.

2.12 Urine and stool microscopy

Traditionally, parasitology using urine microscopy and Kato-Katz for stool microscopy has been known to provide close to 100% specificity, whereas there is variation using the other methods (Gryseels *et al.* 2006). Sensitivity and specificity of the tests vary and depend on a range of factors such as the parasite species, host factors and epidemiologic characteristics of the area (Gryseels *et al.* 2006). The main goal in selecting a diagnostic method for schistosomiasis is to ensure that the test not only enables the parasite to be diagnosed, but is also helpful in providing additional information on whether the infection is past or present, the intensity of the infection and treatment (Van Lieshout and Verweij 2010). In endemic areas logistics of high tech laboratory analyses, and the lack of skilled personnel, as well as cost factors, need to be considered when selecting the appropriate laboratory test. Tests need to be reliable and reproducible (Van Lieshout and Verweij 2010).

The sensitivity of urine microscopy is optimal when using samples that are collected at times during maximum egg excretion (usually between 10am-12am) since it has been found that variation in egg excretion can occur among individuals, the exception being among those who are highly infected with a median egg output of at least 100 eggs/10 ml (Doehring, Feldmeier and Daffalla 1983; Doehring *et al.* 1985b). This study confirmed that peak egg excretion was

around midday and that excretion could be enhanced at any time of day with physical exercise and fluid intake prior to micturition (Doehring *et al.* 1985a). However individuals with light infections with low egg counts require repeated sampling and careful examination to enable high sensitivity and specificity. Light infections are often missed and underestimated, but should be viewed with more seriousness, since it has been found that the host's response to eggs and not the number of worm pairs determines pathology (van der Werf *et al.* 2003). It has been found that quantifying eggs is important in monitoring treatment programmes for schistosomiasis (Kosinski *et al.* 2011).

Urine microscopy is a relatively cheap and easy diagnostic test, but requires repeated sampling and careful examination to enable high sensitivity and specificity especially in light infections (Obeng *et al.* 2008). A 10mL sample of urine is usually examined microscopically and eggs are counted manually. Intensity of infection is expressed as the number of eggs detected per 10mL of urine; 50eggs is the threshold distinguishing between light and heavy intensities (Utzinger *et al.* 2015). A positive diagnosis is made if one intact ovum is seen (Thomassen Morgas *et al.* 2010). With urine microscopy, possible confounders include low egg counts, contaminants that can mimic eggs; the time the sample was collected (i.e. if the sample was collected when there was a low egg excretion) and also there may be variation in results due to human error (Braun-Munzinger and Southgate 1992). Urine samples must also be processed within 24 to 48 hours, and microscopic examination of a large volume of samples can be rather time consuming and tedious. An additional source of error in diagnosis is that some individuals can excrete variable numbers of eggs from day to day, so it is usually better to examine multiple samples (Van Lieshout and Verweij 2010). Using microscopy to detect schistosomiasis is used in many developing countries; however it is limited to monitoring the effect of treatment in cases where egg intensities are low and variable (Cavalcanti *et al.* 2013).

2.13 Real-time polymerase chain reaction (PCR) in Urine and Cervico-vaginal lavage

The use of PCR to detect schistosomiasis began with the amplification of DNA sequences in the detection of *S. mansoni* (Rabello, Pontes and Dias-Neto 2002). This approach was highly sensitive for the detection of the parasite DNA in stool samples. PCR for detecting the *Schistosoma* genus was compared to other diagnostic techniques and has been proven to overcome some of the drawbacks of the other detection techniques, especially in terms of sensitivity and specificity (Pontes, Dias-Neto and Rabello 2002). While the gold standard for the identification of parasites is via microscopy, PCR was found to be a more sensitive diagnostic test. DNA isolation and amplification was previously known to be a time consuming and laborious process, however in recent times, these processes have been simplified even with

the advent of real-time PCR. While conventional PCR has the advantage of being able to identify even minute quantities of parasite DNA, however it is unable to quantify the DNA (Gordon *et al.* 2011).

Multiplex PCR assays are able to detect multiple species in an individual host, using multiple primer pairs (Verweij *et al.* 2007). An advantage of real-time PCR over conventional PCR is the fact that the infection can be quantified. Multiplex real-time PCR assays have been established for specific and sensitive detection of a broad range of parasites in clinical samples, including *S. haematobium* (Verweij *et al.* 2007; Obeng *et al.* 2008; Ten Hove *et al.* 2008). In a study comparing real-time PCR, urine microscopy and other techniques for the detection of schistosomiasis it was found that previous limitations with PCR in detecting parasitic infections such as low sensitivity, and problems with contamination have been reduced (Obeng *et al.* 2008). *Schistosoma* PCR has also shown good specificity, sensitivity and reproducibility of results, especially in cases where no eggs were detected by microscopy in a study conducted among travellers (Cnops *et al.* 2012). Real-time PCR assays rely on samples collected and stored at optimal temperatures as well as require well defined laboratory facilities.

PCR as a diagnostic technique does not have the same limitations of microscopy. While PCR has limitations of cost as well as being a specialized technique that requires specific equipment and processes that are not field applicable, it has the advantage of enabling a large number of samples to be processed in a short period of time and has a reduced risk of contamination and human error. This application might prove to be effective for use in settings where epidemiological mapping of schistosomiasis is required. Quantification of *Schistosoma* DNA in urine samples using real-time PCR may be a specific and sensitive alternative to microscopy to determine the urogenital distribution in preadolescent girls, despite the issues of field applicability in developing countries. We have explored this as part of this PhD and further details are provided in Paper 1.

An earlier study has shown that genital schistosomiasis can occur among women while being negative in the urinary tract and that co-infection in both the urinary and genital tracts can occur (Poggensee *et al.* 1998). While urine and stool samples have been established assays for the detection of *Schistosoma* DNA using real-time PCR, a previous genital schistosomiasis study conducted among rural Zimbabwean women, investigated the use of cervico-vaginal lavage samples for *Schistosoma* DNA (Kjetland *et al.* 2009b). The results of this study revealed that positive *Schistosoma* ova detected in cervico-vaginal lavage PCR correlated with the clinical findings of bleeding and sandy patches. It was also found that younger women had increased schistosomal DNA compared to older women (Kjetland *et al.* 2009b). Since genital

schistosomiasis is more challenging to diagnose, it has been proposed that in resource poor settings, where rural gynaecological facilities and infrastructure are limited, this could be a complementary tool to gynaecologic examinations, especially for research purposes. The sample size in this study was small and further investigations are required to validate these assumptions (Kjetland *et al.* 2009b). PCR techniques used then have since evolved and improved. The PCR detection of schistosomiasis using cervico-vaginal washings or self-administered tampons or swabs would thus be beneficial in diagnosing FGS. Cervical washings for DNA detection are less traumatic to the cervical mucosa, and could be beneficial in early identification of disease. In this study, the use of PCR in the detecting of schistosomiasis is investigated in urine as well as vaginal lavage samples. The use of Schistosoma DNA in urine and cervico-vaginal lavage samples has been explored in Paper 2.

2.14 Cytology

The Pap smear was developed in the 1930's and initiated by the father of Cytology, Dr George N Papanicolaou (Papanicolaou 1942; Papanicolaou and Traut 1997). The first screening tool was the vaginal smear which was introduced approximately 50 years ago and supported by the American Cancer Society (Koss 1993). Pap smear programmes, also known as cytologic screening programmes have achieved remarkable results in reducing cervical cancer incidence and mortality in some developed countries. It is reported that the incidence of cervical cancer and the associated mortality have both decreased substantially in Nordic countries after the introduction of organized screening programmes using the Pap test (Hakama and Louhivuori 1987).

A Pap smear is a cytological sample of the cervix, designed to detect abnormal cervical cells. This involves scraping cells from the squamo-columnar junction (this exact location is most representative of the cervix) and then fixing them onto a glass slide. The slides are then sent to a cytology laboratory stained and evaluated by a cytologist. Pap smears are widely used and they are valuable in detecting pre-invasive cancer of the cervix. Smears obtained with the original "Ayre" spatula are often easier to screen (Ayre 1947). A wooden spatula is preferable to a plastic spatula, because of its mildly rough surface that can collect more material. The collection device may play an important role in the sample adequacy. The shape, surface, texture and material of the device may determine how much of the scraped material is deposited on to the glass slide and is available for screening and analysis. Several methods of obtaining cytologic material from the uterine cervix are available (Koss 1993).

Liquid-based cytology (LBC) is a newer technique which entails collecting the samples with a broom-like device that is placed into a vial containing a buffered fixative solution (Hutchinson *et al.* 1999). The principle of the LBC technique is that cells are processed and stained using an automated system, concentrating the cells onto a microscopic slide in a monolayer with the removal of excess mucous, blood and inflammatory cells. For both conventional and LBC samples the Papanicolaou staining technique is used (Papanicolaou 1942). This is a polychromatic stain that has gained worldwide acceptance for cytologic samples. It is designed to display many variations of cellular morphology showing degrees of cellular maturity and metabolic activity (DeMay 1996). In the present study, both conventional and LBC were used to investigate FGS and SCA.

2.15 Alternative cervical cancer screening tools to cytology

While the Pap smear is known to be an effective tool for the prevention, detection and management of cervical cancer it is not the most accurate test due to sampling errors and diagnostic errors (DeMay 1997; DeMay 1996) For a Pap smear to be a valuable tool in the diagnosis of cervical cancer, the following steps need to be followed: the smear needs to be properly collected so that it is well preserved and adequate (having the correct representation of cells from the cervix). Thereafter, the smear must be properly prepared or stained for cytological assessment and finally it must be correctly diagnosed by the cytologist (DeMay 1997). Three common diagnostic problems are that results from smears are issued without them being fully representative, or result are issued from an unsatisfactory smear and the issuing of "false negative" results (DeMay 1997). Cancer could be missed on a Pap smear if there are only few cells present or compared to the biopsy which is the gold standard, lesions may not be within reach of the sampling device used (Koss 1993). Cytology as a discipline requires concentration since screening many slides can lead to fatigue and human error (Koss 1993). Another common problem is the fact that there is a shortage of cytologists, and the capacity to provide results within a short period of time is limited in developing countries. Furthermore, it is a huge challenge in developing countries to get a woman to have a Pap smear, and expecting her to return for results after some time is also problematic. Not being able to interpret the cells correctly is another source of error. There are however, some alternatives to conventional cytology in addition to LBC cytology, which are available, and these include options such as visual examination and HPV testing and vaccination.

HPV DNA tests and genotyping: HPV originates from the papilloma virus family, there are several HPV types which have been identified in humans (Bibbo and Wilbur 2008). Some of these cause no symptoms in humans while others are sexually transmitted and potentially carcinogenic. Viral particles enter the body via small abrasions on the skin or mucous membranes. Viral replication occurs mainly in the nucleus, but the effects are seen in both the nucleus and cytoplasm. On the vulva, HPV causes multiple, warty, cauliflower-like (*verrucous*) lesions (Bibbo and Wilbur 2008). Histologically, HPV causes proliferation and thickening of the squamous epithelium. In the genital tract the following types of lesions have been identified: subclinical papillomavirus infection: flat warts, exophytic warts/ condyloma acuminatum, endophytic warts (Bibbo and Wilbur 2008).

With the discovery that HPV is central to the development of cervical cancer, scientists have been developing molecular diagnostic tests for detecting HPV (Walboomers *et al.* 1999). There is potential for HPV testing in screening programmes, both as an adjunct to cytological screening and in primary screening (Schiffman *et al.* 2007). The proposed uses of HPV testing in cervical cancer prevention programmes include the identification of HPV in areas where Pap smear findings show ASCUS (cells that are atypical but not definitely dysplastic) and to test for high-risk types of HPV so that such women may be identified, monitored and treated accordingly. In addition, testing may be used as a means of surveillance for women after treatment for high grade lesions (Schiffman *et al.* 2007).

HPV genotyping is an important tool in determining the oncogenic potential of the virus. Many strains of HPV, types 16, 18, 31, 33 and 45, are thought to have a high oncogenic potential (Clifford *et al.* 2006). The worldwide prevalence of HPV in cervical cancer is 99.7%, and the association of so-called high-risk (likely oncogenic) HPV with cervical cancer makes HPV the highest attributable factor that is specific for any major human cancer (Walboomers *et al.* 1999). A global review conducted to assess the age specific prevalence of HPV in females, found that the most high-risk oncogenic HPV are types 16 and 18 (Louie, De Sanjose and Mayaud 2009). HPV vaccines have been developed and are aimed at protecting against oncogenic strains of HPV with the stimulation of antibody production among humans. The introduction of HPV vaccines in developing countries where cervical cancer is a huge burden could be a possible solution, however HPV vaccination is only effective in prevention among women who have no present or past HPV infection. HPV vaccination would therefore be more effective in pre-adolescent girls (Cutts *et al.* 2007).

***Molecular Markers p16*:** Immuno- markers may be used in order to identify oncogenic strains of HPV that are likely to progress, thus preventing over treatment or misdiagnosis by using the Pap diagnosis only (Lesnikova *et al.* 2009). The diagnostic test for the immune-marker p16 can be conducted on LBC samples as can the HPV DNA testing. HPV has been established as the common causative agent in cervical cancer since it is able to alter gene or protein expression in the host cells. There are different strains of HPV, some have a high risk oncogenic potential which results in cervical cancer. Two proteins caused by high risk HPV infection are E6 and E7 (Saqi *et al.* 2002). E7 binds to the retinoblastoma gene product (RB) resulting in its functional inactivation. The E7 HPV oncogenes inhibit the effect of tumour suppressive proteins, protein P53 and RBp respectively thus increasing the risk of mutations. Since the p16 is under negative feedback control of functional RBp, overexpression of the p16 gene occurs in cells infected by high risk HPV (Cuschieri and Wentzensen 2008). Because the p16 protein is detectable by immunocytochemistry it is a surrogate marker for HPV since this protein is not expressed in normal tissue (Lesnikova *et al.* 2009). It is observed in the nucleus and the cytoplasm of cells. In young populations, such as the present study population such an assay may be of value in identifying those young women with high risk for cervical cancer.

Visual inspection with acetic acid (VIA): Visual examination instead of cytology as the initial screening technique has been implemented in areas where resources are particularly scarce (World Health Organization 2012). The method entails a primary health care worker carrying out a visual examination of the cervix, using a speculum cervix which is swabbed with acetic acid, abnormal areas react with the acid and turn white. Once identified, these areas can be treated with cryotherapy or biopsy. This approach is advantageous since it is a "see and treat" approach. One of the limitations to this approach is that it is not always accurate in identifying pre-cancerous conditions. It has been found that VIA is promising in low-resource settings since it is a relatively simple, "low-tech" approach that is minimally dependent on infrastructure for its adequate performance, provided that treatment services are in place. Due to low cost, easy applicability and immediate results, it has been suggested that VIA could be a useful screening test in developing countries compared to Pap smear screening (World Health Organization 2012). Over-treatment may result in additional health risks to women as well as overburdening the health care system. VIA is less effective for screening women in their fifties because of the tendency for the squamo-columnar junction to recede into the cervical canal, making observation more difficult. Despite these drawbacks it is recommended that VIA be considered as an option for identifying pre-cancerous lesions in many settings, either in conjunction with or as an alternative to other screening approaches.

2.16 Management of Premalignant Cytologic Findings

The form of patient management is based on a correct cytologic diagnosis. It is not usually necessary to biopsy lesions with minimal or moderate severity, since only a small proportion of these lesions will progress to a more marked abnormality, whereas the time it takes a lesion to progress provides ample time to detect a lesion at successive cytologic examinations (Lindeque 2005). Only after persistence of the lesion has been confirmed are follow-up procedures, including colposcopy warranted. A cytologic diagnosis of severe dysplasia or in situ carcinoma is usually followed by colposcopy and biopsy, which at confirmation of the process are followed by cautery, laser treatment, conization or hysterectomy (Lim 2002). In cases where HPV genotyping is available, this helps inform the course of treatment (Schiffman *et al.* 2007).

2.17 Summary

Many school going females have contact with contaminated water sources and are at risk of schistosomiasis and FGS. Young women in South Africa are also at risk of HIV and cervical cancer and the high prevalence of these infections place many women at risk of premature mortality.

In this study we investigate the usefulness of diagnostic tools such as Pap smears and LBC samples together with *Schistosoma* PCR in urine and vaginal lavage samples which are ethically acceptable among women in the diagnosis of FGS.

3.0 CHAPTER THREE

3.0. Materials and methods

3.1. Study setting and design

The study included a cross sectional design. The study had different components. The sample for this study was drawn from a larger sampling frame among young females (aged 10-12 years) in 18 primary and 81 high schools (aged 16-23 years) who took part in a clinical study on FGS to investigate if annual school-based chemotherapy could prevent the development of FGS (Ref BF029/07). In a sub-sample of the young women a cohort was followed up in a prospective study.

Table1.3 Study areas and groups

Paper	Study Site	Age (years)	Sampling year - analyses	Number of participants included in analysis
1	18 Primary schools Ugu District Cross sectional	10-12	2009-10 - Questionnaire Urine collected over 3 days: microscopy, PCR	708
2, 3	41 High schools Ugu District Cross sectional	16-23	2011 - Questionnaire Single urine, microscopy, PCR	394
4, 5 (Short Communication)	40 High schools Ilembe and Uthungulu Districts	16-23	2012-13 – Questionnaire Cervico-vaginal lavage PCR, Pap smears and or LBC samples	833

3.2 Inclusion and Exclusion Criteria:

a) Primary school girls were included in the study provided that assent was obtained from each participant as well as written permission from their parents or guardians. Participants who were absent from school on the day of sampling were excluded.

b) Young women were included in the study. Pregnant women and virgins were excluded as well as those young women who did not wish to participate in this study. Participants provided written consent.

3.3 General Characteristics and Demographics of the study populations

The study was conducted in the KwaZulu-Natal province which is situated on the eastern coastline of Southern Africa. This province is made up of eleven districts and is the epicentre of the HIV epidemic in South Africa (South African Municipal Demarcation Board 2012; Directorate Epidemiology 2013). Two areas Ugu and Ilembe/Uthungulu situated alongside the Indian Ocean south and north of the city of Durban were included in the study. These districts are traversed by many rivers. The map below indicates the study areas.

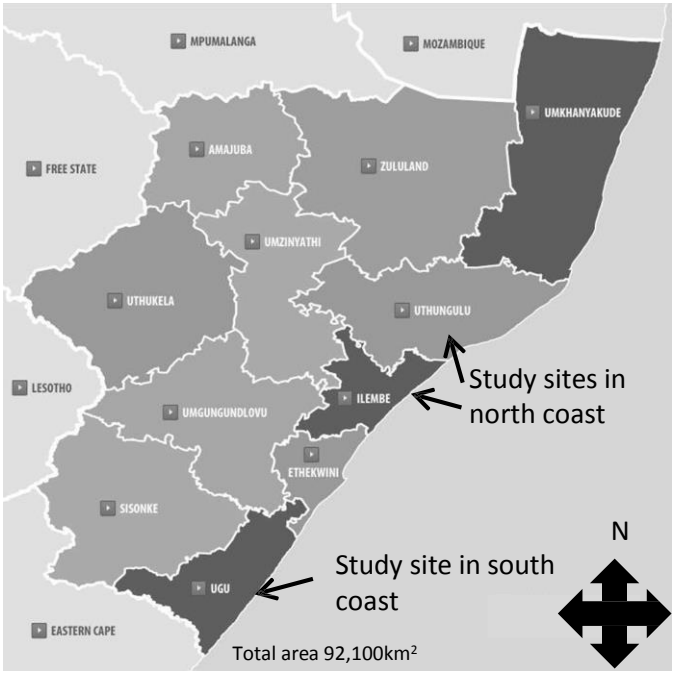


Figure1.5 Map of KwaZulu-Natal showing study areas adapted from (South African Municipal Demarcation Board 2012)

Study area

Within the province of KwaZulu Natal, Ugu District is situated along the coast south of Durban and has a population of 710.000 people, 51% are below the age of 20 years, and 55% are female. The area is endemic for both schistosomiasis and HIV. Ilembe and Uthungulu Districts are situated on the north coast of KwaZulu-Natal and this area of the study, hosts a population of approximately 570 000 people. Those who are under the age of 19 account for 49% of the population. Those who are 34 years or younger comprise 72% of this population. There are low levels of employment in these districts, with only about 29% of this population being actively employed (Manzini 2001). Many of the population from these districts reside in areas, where access to water sanitation and other infrastructure is poor. HIV and AIDS is a major health burden in this population, the national South African HIV prevalence among antenatal women was reported in 2013 at 29.5% of which KwaZulu-Natal had the highest prevalence of 37.4%.

Individual district prevalences for HIV among antenatal women were as follows: 38.3% in Ugu District, and 37.4% and 38.5% in Ilembe and Uthungulu districts respectively (Directorate Epidemiology 2013). These are some of the poorest districts in South Africa, similar to other schistosomiasis endemic areas in other African contexts. These are mostly rural communities where women do their laundry and children play in *Schistosoma* infected rivers, but also include some urban and semi-urban centres (Appleton and Kvalsvig 2006; Saathoff *et al.* 2004; Kvalsvig 1988). Schools were selected that were situated below an altitude of 400 metres.

3.4 Ethics and Permissions

The processes of obtaining ethical approval and permissions were the same for both study populations. The study was initially discussed with the relevant stakeholders including the Provincial and District representation, Department of Health, Department of Education, school staff, parents, and pupils. Their permission was obtained at this time. In the first meetings, the focus of the project, its importance, and benefits were explained to the stakeholders. Written informed consent was obtained and sought at this time **(Appendices 2, 3, 4, 5)**. For the young women logistical arrangements were made with school principals and other relevant staff, for suitable times for transportation of the participants to the study clinics for gynaecological examinations and interviews.

Ethical approval was obtained from the Biomedical Ethics Research Committee of the University of KwaZulu Natal for the main study entitled: "*Schistosomiasis in young women and girls in KwaZulu-Natal, manifestations, effect of treatment, association with HIV*" Ref BF029/07. Ethical approval was also received, Reference HRKM010-08. REK Øst-Norge and the Norwegian ethics committee, gave ethical clearance Ref 469-07066a1.2007.535, September 17th 2007. Permission was received from the Department of Health, Pietermaritzburg, KZN, February 3rd 2009. The Departments of Health and Education in Ugu, Ilembe and Uthungulu Districts, KwaZulu-Natal have also given permission. The present study protocol was submitted to the UKZN Biomedical Research Ethics Committee (BREC) and full approval was granted on the 19th of August 2011 and annual recertification approval has been received since then and to date, RefBF057/11.

3.5 Data collection

Teams of trained field assistants and school nurses visited the schools to give general information and recruit initially the primary school girls.

In the high school, a team of trained field assistants and school nurses visited the schools to give general information and recruit adolescent girls for laboratory investigations and treatment. After signed consent was received, participants were collected from school by research assistants and transported to the project clinic via clinic transport. Once all examinations were complete, the participants were transported back home.

Data storage: Each participant was allocated a unique number to be used as a code for all samples and data. The participants' names did not appear on any sample, container or interview sheet, only the code. Names and identity with codes were kept separately in a locked cupboard. Full confidentiality was maintained in all aspects of the research.

Interview Process.

Consenting participants underwent a private face-to-face semi-structured interview, conducted by research assistants in "isiZulu", the local language, including history of water contact, red urine and genital symptoms. The young women were asked about sexual history and number of children as these variables possibly could have bearing on the correlation to cervical atypia.

***Urine storage and microscopy*:** Collected urine samples for microscopy were stored in dark cooler boxes to ensure sample quality. Urine samples were processed in the field site laboratory by a laboratory technician. Urine samples for microscopy were preserved with 1ml of 2% tincture of merthiolate in 5% formalin solution (Thomassen Morgas *et al.* 2010). The samples were spun for 10 minutes at 4000 rates per minute (rpm) and the sediment examined microscopically, magnification with objective 10, by trained field workers. The samples underwent quality control by an independent microscopist on 10% of randomly chosen samples.

PCR analysis: For *Schistosoma* PCR analysis, an aliquot of 1 mL of each first day urine sample was transferred into cryotubes without any preservative. The aliquots were stored at 4 °C for a maximum of a week in the dark and thereafter stored at -80°C for several months, before being transported to the Netherlands in frozen conditions for DNA isolation and detection. For a sub-selection of 85 urines, 49 of them microscopy negative at all examinations, a second aliquot was PCR tested at a local laboratory in South Africa for quality control purposes.

High school cohort- Gynaecological Examination: The protocol used was adapted from a previous FGS study conducted in Zimbabwe (Kjetland *et al.* 2005). Gynaecologic examination was done by trained clinicians (Kleppa *et al.* 2014). It was commenced by photocolposcopic examination of the cervix using an autoclaved metal speculum after which cervico-vaginal

lavage samples were collected followed by cytology Pap smears or LBC samples in all the consenting participants. Acetic acid or iodine application for colposcopic examination was done last.

Collection of cervico-vaginal lavages Cervico-vaginal lavages were collected by spraying 10 ml saline on the vaginal wall and cervix twice, and then drawn back into the syringe. Thereafter, 1 mL of each sample was then dispensed into 6 labelled cryotubes. The samples (cervico-vaginal lavage and urine) for *Schistosoma* DNA detection were stored at 4 °C for a maximum of a week in the dark and thereafter stored at -80°C for several months, before being transported to the Netherlands in frozen conditions for DNA isolation and detection. The laboratory staff was blinded to urine microscopy and the cytology results.

3.6. Laboratory Analysis

3.6.1 *Schistosoma PCR analysis*

DNA analysis was conducted at the Leiden University Medical Center (LUMC) in the Netherlands, where colleagues from the Department of Parasitology have developed and perfected these assays. In order for samples to be transported abroad, permission was obtained from the Department of Health, South Africa, in addition to the initial ethical permission that was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee. The samples were transported to the Netherlands via courier at -80^0 Celsius, in dry ice.

The DNA isolation and the set-up of the PCR was performed with a custom-made automated liquid handling station (Hamilton®, Switzerland) (Figure 1.5). The protocols for urine and vaginal lavage were identical. In short, the DNA detection technique entails the following main steps: denaturation/isolation, annealing/hybridization and amplification.

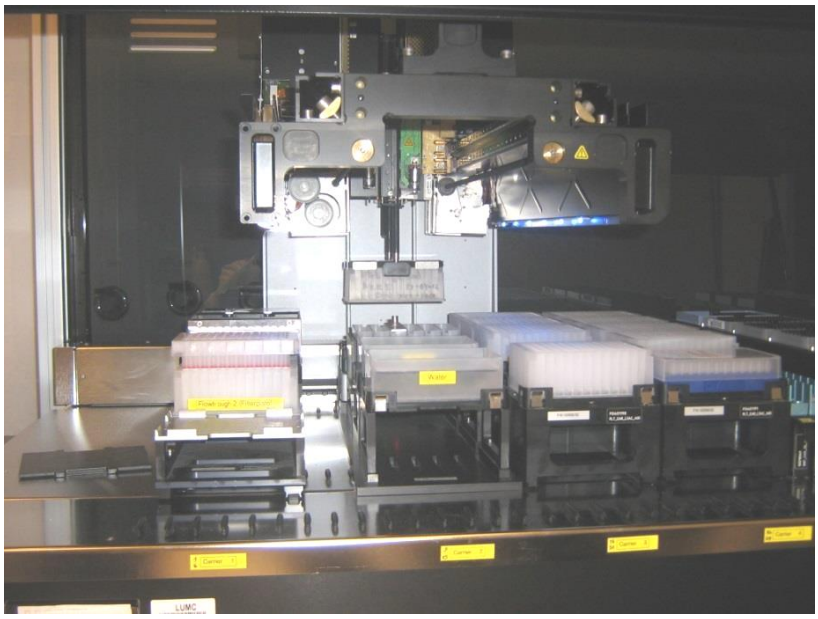


Figure1.6 Automated liquid handling station (Hamilton®, Switzerland) with block containing 96 samples being processed in each run.

DNA was isolated from a 200 µL subsample of each urine and vaginal lavage sample respectively using QIA Tissue Kit (QIAgen, Hilden, Germany) spin columns (Verweij *et al.* 2007; Obeng *et al.* 2008). The principle of DNA isolation using spin columns entails the separation of DNA molecules which bind to silica surfaces in the presence of chaotropic salts under certain pH conditions. Carbohydrates and proteins are not absorbed and are removed. Nucleic acids are eluted under low-salt conditions- this process helps free the DNA.

In the first step, the samples underwent the heating step after the addition proteinase K for 2 hours at 55^0C. The heating step facilitates the removal inhibitory factors and helps to break up eggs if present. The tissue lysis buffer containing the internal control which is the Phocin Herpes Virus (PhHV-1) was then added and the samples were incubated for 10 minutes at 70^0C (Niesters 2002). The purpose of the tissue lysis buffer is to break down the peptide bridges and destroys the cell walls to release DNA. It also facilitates the removal of interactions between DNA and histones.

For real-time PCR *Schistosoma*-specific primers Ssp48F and Ssp124R were used to amplify a 77-base paired fragment of the internal-transcribed-spacer-2 (ITS2). The double-labeled probe Ssp78T was used to detect amplification (Obeng *et al.* 2008). For the internal control, PhHV-1-specific primers PhHV-267S and PhHV-337AS and the specific double-labeled probe PhHV-1-305TQ were included in each reaction mixture. The process of primers binding to DNA is called hybridization/ annealing. Resultant bonds are only strong if the base pairs of the primer

and the DNA match. Polymerases then begin to attach to additional complementary nucleotides. Polymerases are enzymes that link individual nucleotides to form long DNA chains.

The amplification and detection of each DNA sample was achieved using the Bio-Rad CFX Manager (Figure 1.5). Amplification and detection commenced by using a 25ul reaction mixture containing PCR buffer (HotstarTaq mastermix QIAgen), 5mM MgCl2, 2,5ug buffer (HotstarTaq mastermix QIAgen), 5mM MgCl2, 2.5 µg bovine serum albumin (Roche Diagnostics Nederland BV, Almere, The Netherlands), 5 pmol of each *Schistosom*a-specific primers, 3.75 pmol of each PhHV-1-specific primers, 1.25 pmol of each of the *Schistosoma*-specific and PhHV-1-specific double labelled probes and 5µl of the DNA sample. In each amplification run, negative and positive controls were used. The thermocycler was set for 15 min at 95^0C, followed by 50 cycles, each of 15s at 95^0C, 30 seconds at 60^0C, and 30 seconds at 72^0C (Figure 1.6).

The PCR output consisted of a cycle-threshold (Ct) value. This value represents the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence and thereby indicating the presence of parasite-specific DNA in the sample that was tested. Based on microscopy, the infection was classified as high-intensity if the mean number of eggs of the three specimens was higher than 50 per 10 ml urine (Montresor *et al.* 1998).Based on PCR, the infection was classified as high-intensity ($Ct<30$), medium-intensity ($30\leq Ct<35$), low intensity ($35\leq Ct<50$) or negative ($Ct=50$) (Pillay *et al.*2014). These categories were chosen arbitrarily, based on previous experiences with protozoal infections where DNA loads with a Ct-value below 30 could generally be confirmed by microscopy and DNA loads with a Ct-value higher than 35 were always microscopy negative(Van Lieshout and Verweij 2010).

Ct-values of the internal controls were within the expected range for all samples. Fluorescence data from PCR assays are collected from PCR cycles that occur in the linear amplification portion of the reaction. In ideal conditions, the fluorescence accumulates in proportion to the amplicon. Negative samples have no DNA, therefore have no threshold value. Weakly positive sample imply that not much DNA was present, therefore this would take a longer number of cycles to reach the Ct-value. A sample with a high load of DNA will take less cycles to reach the threshold than for those samples with less DNA.

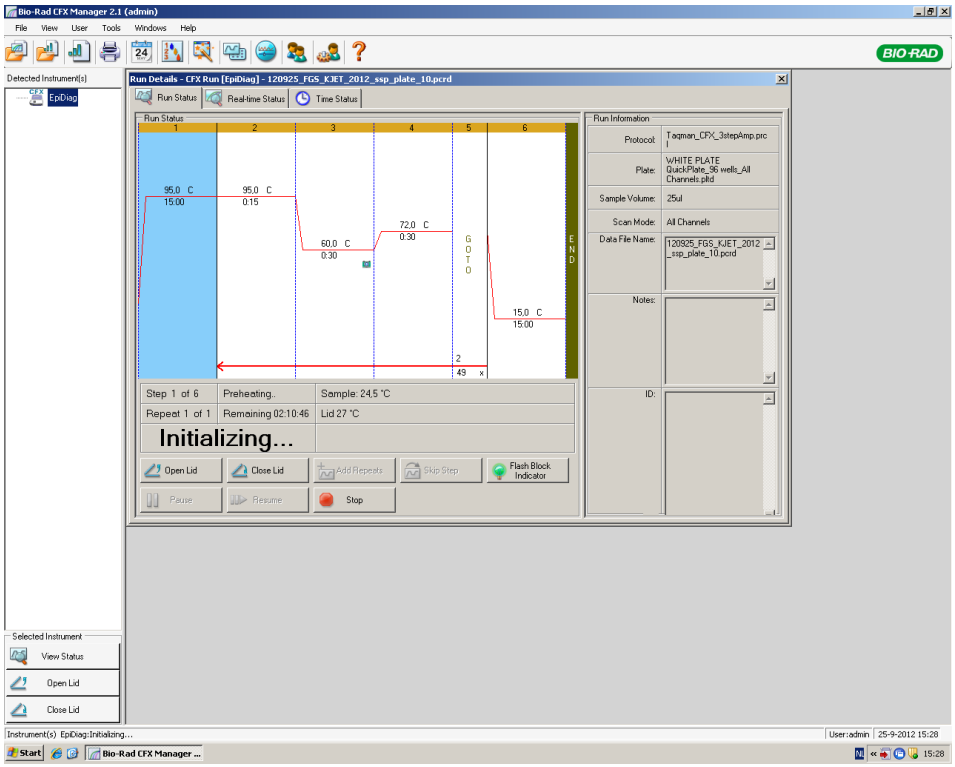


Figure 1.7 Display screen of the amplification cycle.

3.6.2 *Pap smears and liquid-based cytology samples*

The Pap smears or LBC samples were collected after the cervico-vaginal lavage samples were taken. Traditional Pap smears were collected by scraping a wooden spatula of the cervix and the cervical surface of the fornices. The cervical smears were then spray-fixed using a commercial cytological fixative for preservation. Traditional Pap smears were collected from all of the participants from the Ugu District and 519 of the participants from the Ilembe district.

Liquid-based cytology (LBC) is a newer technique, and entails collecting the samples with a broom-like device that is placed into a vial containing a buffered fixative solution. In this study the SurePath™ (Franklin Lakes, NJ, USA) LBC method was used. The principle of the processing of these samples is that on sample collection the cells are further processed and stained using an automated system concentrating the cells onto a microscopic slide in a monolayer with the removal of excess mucous, blood and inflammatory cells (Figure 1.8 A and B). LBC sampling was done for the remaining 327 participants from the Ilembe district.

All cytological smears were examined using an Olympus BX43F microscope with magnification of 10 and 40 times. The screening was done blinded to any of the other laboratory results or clinical findings other than the sample type. Smears were examined microscopically for all cytological entities that are expected to be found in cervical smears and reporting was

done in accordance to the Bethesda System of Reporting (Solomon and Nayar 2004). Smears were reported in a systematic manner, ensuring that all cytological entities were taken into account. The cytology report consisted of the following broad areas: a) smear adequacy; b) inflammation and benign conditions; c) infective agents; and d) squamous cell atypia.

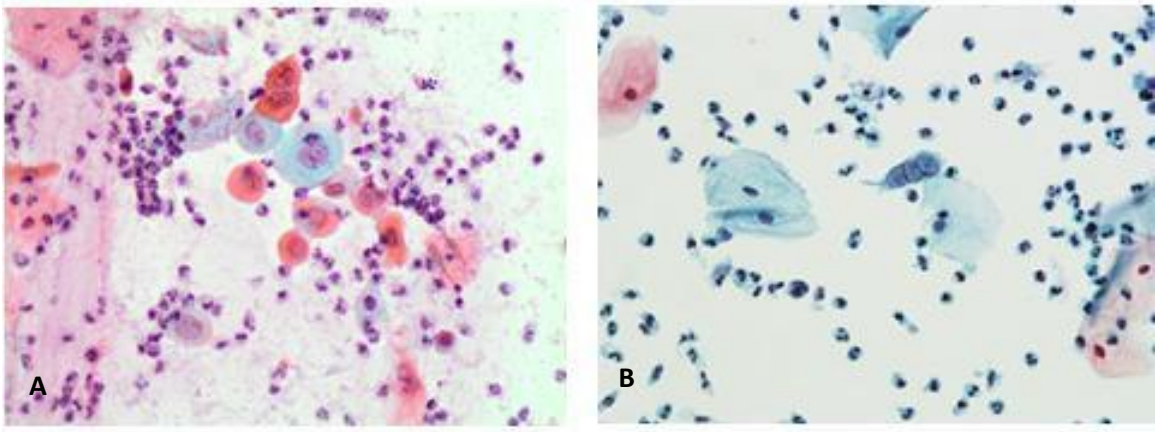


Figure 1.8 A) Pap 40x: showing cells which are not well preserved and there is excess background mucus and inflammatory cells.

B) LBC 40x: excess background material is removed and cells are well preserved and easily visualized.

Staining: For both conventional and LBC samples the Papanicolaou staining technique was used. It is designed to display many variations of cellular morphology showing degrees of cellular maturity and metabolic activity. Use of the Papanicolaou stain results in well stained nuclei, differential cytoplasmic counterstaining and cytoplasmic transparency (Figure 1.9). There are three main dyes used in this staining technique (DeMay 1996). The nuclear stain is Haematoxylin which stains the nucleus of cells blue. The first cytoplasmic counterstain is Orange G-6 (OG), which is a monochromatic stain. It stains keratin bright orange. Keratin is not a "normal" component of cells and is seen mainly in keratinizing squamous cell carcinoma. The other cytoplasmic stain is Eosin Azure-50 (EA). This is a polychrome stain and consists of eosin and light green. It stains the cytoplasm of metabolically active cells, intermediate squamous cells, parabasal cells, columnar cells, histiocytes and neutrophils. It also stains nucleoli. Superficial cells are stained pink or eosinophilic. Intermediate cells and parabasal cells are stained shades of blue or cyanophilic (DeMay.R 1996).

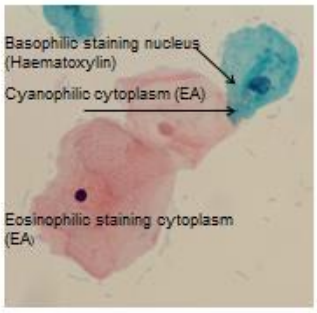


Figure1.9 Pap 40x: Showing different areas stained by the Papanicolaou stain

Cytology screening in South Africa is based on the Bethesda System of reporting (Solomon and Nayar 2004). The smears were examined microscopically for all cytological entities that are expected to be found in cervical smears and reporting was done in accordance to the Bethesda System of Reporting (Solomon and Nayar 2004). Smears were reported in a systematic manner, ensuring that all cytological entities were taken into account. Adequacy was reported according to the Bethesda System, and entities such as the adequate number of well- preserved epithelial cells and cellular composition and sampling of the transformation zone as well as any other indicators that may impact on the quality of the smear were considered. Smears which met these criteria were deemed "satisfactory for evaluation". Smears which did not meet all the criteria, but at the same time enabled a diagnosis were reported as "satisfactory for evaluation but limited by", followed by whichever criteria were lacking for example inadequate endocervical cells, inflammation etc. Smears were deemed "unsatisfactory for evaluation" in instances where either obscuring blood, excessive inflammation, thick areas, poor preservation, poor staining, air drying artefact or contaminants compromised. The background contents were also reported on, these included blood and inflammatory cells (neutrophils, eosinophils, lymphocytes and histiocytes). Benign changes such as repair and regeneration or hyperkeratosis or parakeratosis or squamous metaplasia are also reported. Infective agents which are cytologically detectable were also reported. Epithelial dysplasias

were graded according to the Bethesda System (Solomon and Nayar2004);(Figure1.10).

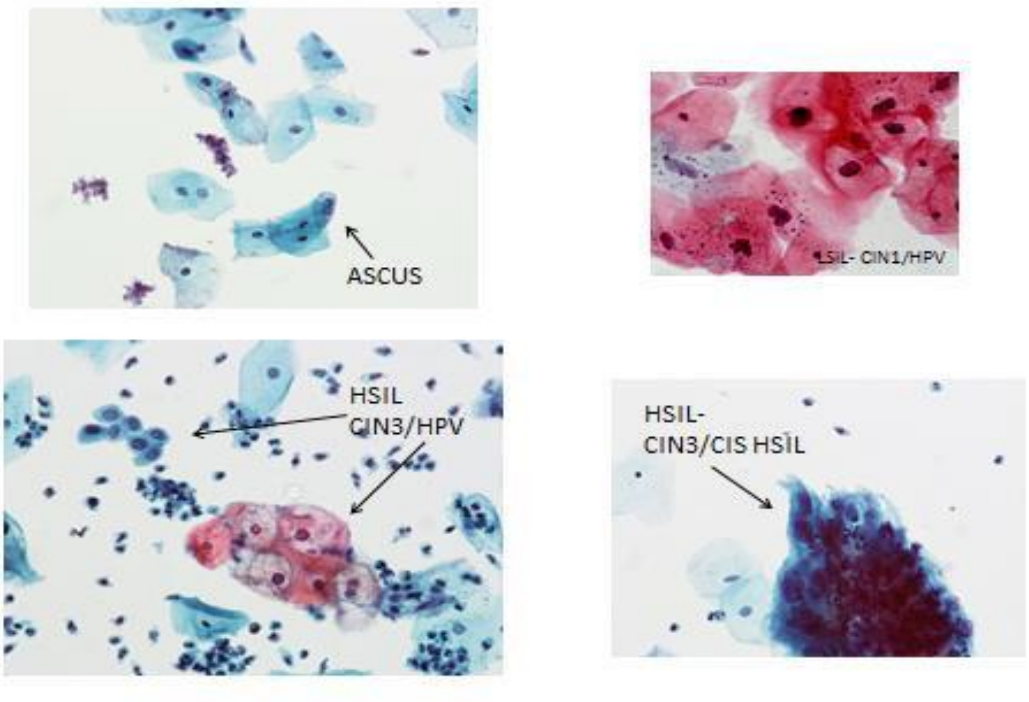


Figure 1.10 LBC 40x epithelial cell abnormalities as seen in cervical cytology samples.

ASCUS: This entity refers to cytopathologic changes that are not sufficiently clear-cut to permit a specific diagnosis. Cellular features include nuclear enlargement in squamous cells with mature, superficial/intermediate-type cytoplasm. Variation in nuclear size and shape, and binucleation, may be observed. The chromatin is slightly increased but still retains even distribution. Because the cellular changes in the ASCUS category may reflect as exuberant benign change or a potentially serious lesion, which cannot be unequivocally classified, they are interpreted as being of undetermined significance (Solomon and Nayar 2004).

Low Grade Squamous Intraepithelial Lesion (LSIL)/Mild Dysplasia/ (CINI): This category encompasses those changes associated with HPV including koilocytosis as well as mild dysplasia changes. Cells are seen lying singly or in sheets. The nuclear abnormalities occur within "mature" or superficial-type cells (Figure 1.11 A). There is also mild variation in nuclear size and shape. Nuclear hyperchromasia mild and chromatin is uniformly distributed. The chromatin may appear degenerated or smudged if associated with the cytopathic changes of HPV. In LSIL, cells rarely exhibit nucleoli. Cells usually have plentiful, clear, translucent cytoplasm with well-defined angular borders (Figure 1.11B). Cells resemble intermediate and superficial type squamous cells with a somewhat reduced cytoplasmic body and a slightly enlarged nucleus, occupying less than one third of the total area of the cell. Cells that demonstrate a well-defined, optically clear perinuclear cavity and a peripheral dense rim of

cytoplasm with the accompanying nuclear abnormalities are consistent with HPV (Solomon and Nayar 2004); (DeMay 1996).

Figure1.11 Pap 40 x A) Benign squamous epithelial cells with pyknotic nuclei. B) LSIL cell with slightly enlarged nucleus

High Grade Squamous Intraepithelial Lesion (HSIL): High grade lesions encompass moderate dysplasia/CIN II, severe dysplasia/CINIII, and carcinoma in situ/CIS. This category encompasses a spectrum of cytological changes that are more likely to persist or progress than low-grade lesions.

Moderate Dysplasia/ (CIN II): The size of abnormal cells is more variable. These abnormalities are usually confined to the "intermediate" squamous cells (Figure 1.12A). Most cells are round to oval, but occasionally spindle cells and elongated and bizarre shapes may be found. Cytoplasmic staining is usually cyanophilic, but some cells may show eosinophilia of the cytoplasm. Nuclei are enlarged and round to oval, sometimes elongated or irregularly shaped. Nuclear chromatin is evenly distributed and moderately hyperchromatic. Nucleoli are usually absent. The nucleocytoplasmic ratio is increased, both by nuclear enlargement and by reduction of the cytoplasmic volume. The nucleus generally occupies less than half of the total area of the cell (Abdel-Hadi and Talaat 2000; Solomon and Nayar 2004) (Figure 1.12B).

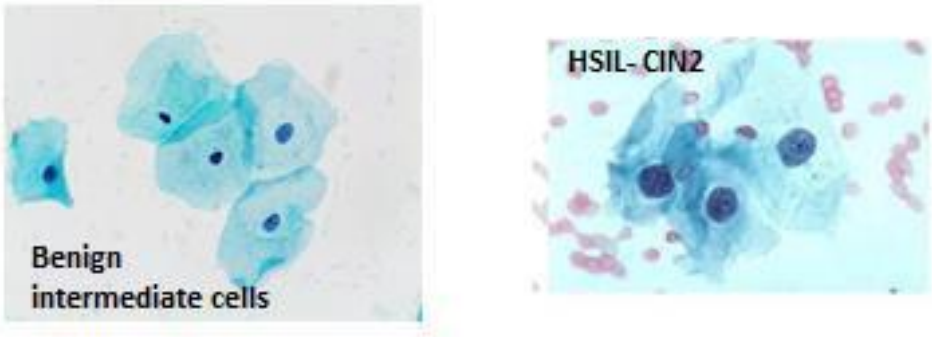


Figure1.12 Pap 40 x A) Benign intermediate squamous epithelial cells with nuclei have finely granular chromatin. B) HSIL- CIN2 Intermediate type cells with moderately enlarged nuclei and moderately hyperchromatic nuclei.

Severe Dysplasia/ Carcinoma in Situ (CIN III/CIS): An area in which discrepancies in the grading of lesions still occurs is the discrimination between severe dysplasia and carcinoma in situ. Cytoplasm was usually sparse, typically forming a small rim around the nucleus. Cells are mainly round or oval often irregular or elongated. Cells occur singly as well as in aggregates. In the most severe intraepithelial lesions aggregates have a syncytial arrangement with indistinct cell borders and irregularly arranged nuclei. The nucleus occupies usually at least two thirds of the total area of the cell. Nuclei have a hyperchromatic, irregularly distributed, coarsely granular chromatin (Figure 1.13A and B). In actively proliferating lesions eosinophilic- staining nucleoli may be observed, but more often these are obscured by the dense, hyperchromatic chromatin. Some severe dysplasias show an extreme irregularity in shape and size of the composing cells.

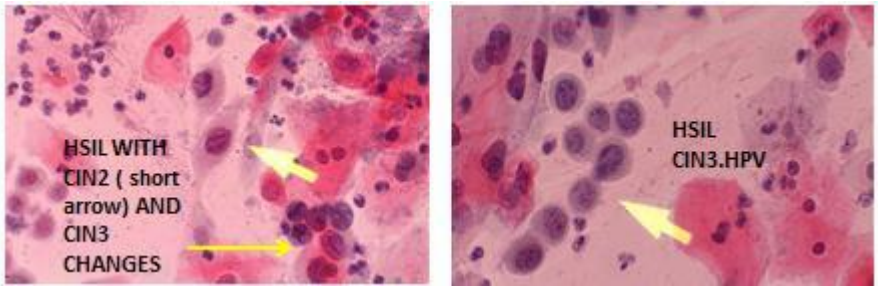


Figure 1.13 Pap 40 x A) HSIL- Cells from CIN2 and CIN3, B) HSIL- CIN3 Cells with marked hyperchromasia and a small volume of cytoplasm.

Cellular Morphology of schistosomiasis:

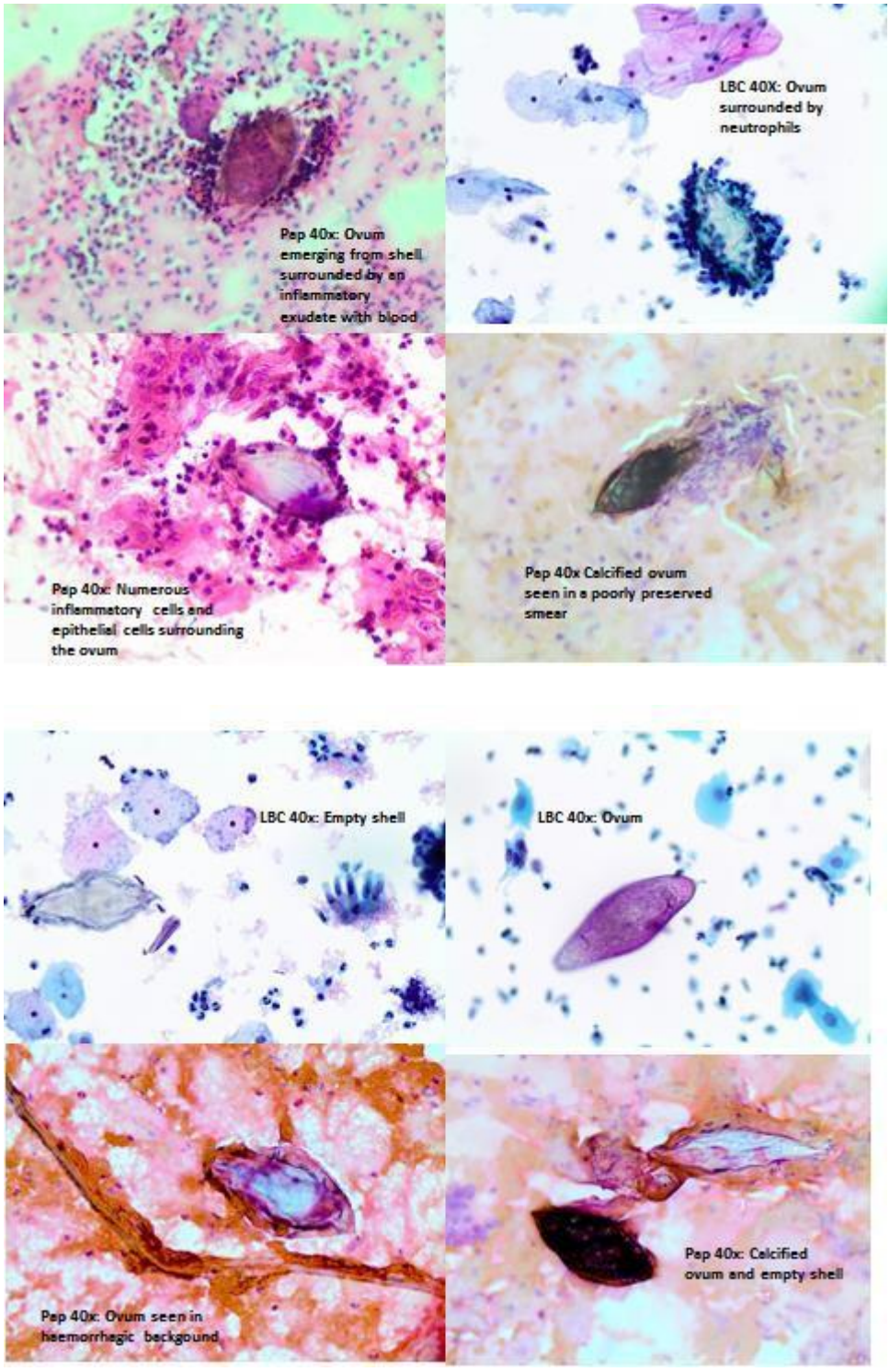


Figure 1.14 Pap and LBC 40x: Ova of *Schistosoma haematobium* were seen in varying presentations in the Pap smears and LBC samples as described in Paper 2, additional images are depicted.

3.1.7 Statistical analyses

Data were stored and grouped in Microsoft Access database and imported into IBM SPSS 20.0 (Chicago, Illinois, USA) and 23.0 (Armonk, NY, USA) for statistical analysis. Descriptive statistics were used in this exploratory study. Inferential statistics were done using the Fisher's Exact Test. The odds and risks ratios and the corresponding 95% confidence intervals were determined for 2 x 2 cross-tabulations. All *p*-values were two-tailed values and *p*-values less than 0.05 considered as statistically significant. Categorical variables were summarized by frequency and percentage and presented using bar charts. Where the data were not normally distributed, results were described as the total number, range, interquartile ranges (IQR) and median value of positive subjects. The McNemar statistical test was used to analyze concordance between the different diagnostic procedures. For the diagnostic tests the continuous variable output was given for the positive cases only using the Mann-Whitney rank-sum test.

3.1.8 Linking of objectives and papers

This study had five manuscripts, Papers 2 and 4 included the first three objectives. The papers are presented as follows: development of non-invasive diagnostic tools for FGS are included in Papers 1-4. Paper 1 describes the use of real-time PCR to diagnose schistosomiasis in girls 10-12 years of age and the sensitivity of the diagnostic tests were used to assess distribution of schistosomiasis in relatively large study populations by using small sample volumes.

Paper 2 builds on the use of PCR and focused on an older cohort. The value of Cervical Cytology as a diagnostic tool for FGS and comparison with real-time PCR for *Schistosoma* specific DNA in vaginal lavage and urine samples and urine microscopy for *S. haematobium* ova has been described. .

Paper 3 considers the generalisability of the use of real-time PCR for the detection of *Schistosoma* DNA in vaginal lavage and urine comparing populations from Tanzania, Madagascar and South Africa.

Paper 4 further investigates the value of traditional Pap smears comparing with LBC for FGS. This paper presents the prevalence of cervical squamous atypia and the association with urogenital schistosomiasis, possible risk factors for cervical atypia and schistosomiasis are identified.

Paper 5 considers whether treatment in young women with Praziquantel can reduce FGS and squamous cell atypia.

4.0 CHAPTER FOUR

4.1 Paper 1

Real-time PCR detection of *Schistosoma*-DNA in small volume urine samples reflects focal distribution of urogenital schistosomiasis in primary school girls of KwaZulu-Natal, South Africa.

Pavitra Pillay, Myra Taylor, Siphosenkosi G. Zulu, Svein. G. Gundersen, Jaco J. Verweij, Pytsje Hoekstra, Eric A.T. Brienen, Elisabeth Kleppa, Eyrun F. Kjetland and Lisette van Lieshout. 2014.

American Journal of Tropical Medicine and Hygiene 90: 546-552. (Published)

This paper was written to address the challenge to find non-invasive diagnostic tools since in children who are risk for genital schistosomiasis since gynaecologic examinations are not ethical (**Objective 2, 3**). The aim of this study was to investigate *Schistosoma* PCR in urine samples and compare results to microscopy and to investigate the association of genital symptoms using the laboratory and clinical data on a school level.

Urine samples from 708 primary school girls aged 10-12 years from 13 schools were analysed for *Schistosoma* ova microscopically using 2 subsamples of 10mL of urine collected over three consequtive days and using a single 200µl urine sample for *Schistosoma* DNA analysis. The interview data collected provided information on the living conditions, water contact and genital symptoms.

My role in this aspect of the research was to learn about the ITS-based real-time *Schistosoma* PCR assay, which was devised and developed at the Leiden University Medical Center (LUMC) and to process the urine samples with support and assistance from the staff at the LUMC. Performing the Schistosoma PCR laboratory work and analysing the data with assistance was new knowledgde gained. I was also first author in this publication with support and assistance from my supervisors and co-authors.

This was the first study to explore the association between *Schistosoma* PCR and female genital symptoms in children at school level. It was also found that schistosomiasis was focal and differed among schools. Such tools may be of value in disease mapping at population level for the detection and management of schistosomiasis.

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doi:10.4269/ajtmh.13-0406

Real-Time Polymerase Chain Reaction for Detection of Schistosoma DNA in Small-Volume Urine Samples Reflects Focal Distribution of Urogenital Schistosomiasis in Primary School Girls in KwaZulu Natal, South Africa

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Abstract. Schistosoma haematobium eggs and Schistosoma DNA levels were measured in urine samples from 708 girls recruited from 18 randomly sampled primary schools in South Africa. Microscopic analysis of two 10-mL urine subsamples collected on three consecutive days confirmed high day-to-day variation; 103 (14.5%) girls had positive results at all six examinations, and at least one positive sample was seen in 225 (31.8%) girls. Schistosoma-specific DNA, which was measured in a 200-mL urine subsample by using real-time polymerase chain reaction, was detected in 180 (25.4%) cases, and levels of DNA corresponded significantly with average urine egg excretion. In concordance with microscopic results, polymerase chain reaction results were significantly associated with history of gynecologic symptoms and confirmed highly focal distribution of urogenital schistosomiasis. Parasite-specific DNA detection has a sensitivity comparable to single urine microscopy and could be used as a standardized high-throughput procedure to assess distribution of urogenital schistosomiasis in relatively large study populations by using small sample volumes.

INTRODUCTION

Schistosomiasis is endemic to 76 countries and 85% of those infected live in rural areas of sub-Saharan Africa, where at least 100 million women and girls are at risk of having this helminth infection.[1] Those who are most vulnerable to infection are pre-school and primary school children, adolescent girls, and women of childbearing age. To reduce morbidity the World Health Organization recommends annual treatment of school-age children in areas of high endemicity.[2]

In sub-Saharan Africa, Schistosoma haematobium is the predominant species.[3] Adult worms lodge in the venules surrounding the pelvic organs, where they deposit their eggs. Some of the eggs are released into the urine whereas others get trapped in the bladder mucosa where they give rise to granulomatous inflammation causing hematuria and urinary symptoms. Up to 75% of the women who excrete S. haematobium eggs in the urine may also have Schistosoma eggs in the uterus, cervix, vagina, or vulva.[4] The genital manifestations may mimic cancer-like lesions and the different sexually transmitted diseases, such as ulcers, genital warts, polyps, and also cause mucosal immune activation and blood vessel friability.[5–7] Several studies have indicated that genital manifestation of schistosomiasis may make women susceptible to human immunodeficiency virus (HIV) infection and they may possibly also develop infertility.[8–10] Because S. haematobium affects the urinary and genital tracts, urinary schistosomiasis has been renamed urogenital schistosomiasis.[11]

The gold standard for diagnosing gynecologic schistosomiasis has been the demonstration of eggs in a crushed biopsy specimen.[6] However, this procedure is controversial because it is invasive and could make the cervical mucosa more susceptible to infections with other sexually transmitted infections, such as those with HIV or human papillomavirus.[4] The colposcope for investigating gynecologic morbidity has limitations because it is highly observer-dependent, requires extensive training and expensive equipment, and cannot be used among children because intra-vaginal inspections are normally not performed before the onset of sexual activity.

For urinary tract infection with S. haematobium, microscopic examination for eggs is considered the diagnostic gold standard.[12] However, the extent to which the presence and intensity of excreted eggs in urine is associated with the actual degree of genital morbidity is still under debate because studies have shown a substantial proportion of adult women with genital lesions to be urine microscopy negative.[4] In adults, gynecologic symptoms are often seen many years after reported water exposure.[4] Conversely, in school-age girls, one would expect infections to be more recently acquired, with adult worms actively producing eggs that are deposited at different tissues in the pelvic region and excreted into the urine in relatively large quantities.

The association between urine microscopy–detected S. haematobium infection and genital symptoms has been recently studied on a group of 10–12-year-old school girls in South Africa.[13] One third of the interviewed girls reported to have a history of genital symptoms, and multivariate regression analysis showed a significant association with the urine microscopy results. To avoid suboptimal diagnosis and not to miss light infections, intense microscopy was performed as generally recommended, which required repeated urine sample collection and examination.[12,13] Microscopy has the limitations of being an observer-dependent procedure, as well as laborious, when applied to large-scale population based surveys. For this purpose there is a clear need for more standardized and highly sensitive high-throughput diagnostic procedures.[14,15]

In recent years, several nucleic acid–based diagnostic tests have been established for specific and sensitive detection and quantification of a broad range of parasite DNA in clinical

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samples, including an internal transcribed spacer (ITS)–based Schistosoma-specific multiplex real-time polymerase chain reaction (PCR).[16,17] In this study, we further explored real-time PCR as a diagnostic tool for mapping school-based distribution of urogenital schistosomiasis by comparing urine Schistosoma DNA levels in the same cohort of school girls from South Africa with the findings of multi-sampling microscopy, as well as reported gynecologic symptoms.

MATERIALS AND METHODS

Study population. The study design is a school-based, cluster, randomized, cross-sectional study of girls 10–12 years of age in rural primary schools in KwaZulu-Natal, South Africa. Participants were recruited from 18 randomly selected schools, all situated in a coastal area of 5,866 km^2 within Ugu district, south of Durban.[13] The region is known to be endemic for S. haematobium and HIV.[18,19] Other common helminths are Ascaris lumbricoides and Trichuris trichiura but only occasional cases of infection with S. mansoni have been reported.[20] Recruitment of the study participants and their clinical symptoms have been described in detail elsewhere.[13] In brief, data and sample collection were performed during September 2009–November 2010. Before the study, information meetings were organized at each school and girls were invited to participate if their parents provided consent.

Interview. Research assistants invited consenting girls to face-to-face private interviews performed in the local language isiZulu. In brief, in this study we used four key variables: 1) living with both parents, 2) reported water contact, 3) history of red urine, and 4) history of genital symptoms because these variables potentially reflect social status, exposure to and clinical outcome of S. haematobium infection.

Each girl was questioned about her living conditions and relationship with her biological parents and about her own observations concerning red urine. High-risk water contact was defined as reported regular exposure to potentially infective water bodies covering at least 10% of the body surface or being in the risk water at least 60 minutes per exposure. History of genital symptoms consisted of reported symptoms of bloody discharge, malodorous discharge, genital itch or burning sensation in the genitals, and some additional symptoms, which were only occasionally mentioned. Further details about the interview are given elsewhere.[13]

Ethical considerations and permissions. This study project drew from a larger project that was approved by the Biomedical Research Ethics Administration, University of KwaZulu Natal (Ref BF029/07). The Departments of Health and Education (Ref HRKM010-08) of KwaZulu Natal also approved the study. Ethical permission was also granted from The Norwegian Ethics Committee, Regional Etisk Komité Øst-Norge (REK-Øst), (Ref IRB 0000 1870) and The European Group on Ethics in Science and New Technologies (Ref IRSES-2010:269245).

Parents or guardians provided written informed consent and consent was given by each girl. All girls were informed of the right to withdraw and to abstain from answering questions without negative consequences. To protect children from stigmatization, the disease was discussed in general terms as urinary schistosomiasis, known as isichenene in isiZulu. Treatment of schistosomiasis with praziquantel was offered to all participants, and all were informed about possible side effects. Support of a private psychologist was available to assist with cases that required psychological support. The project also had a referral system with local clinics and hospitals for those participants that required this system. Consent forms were returned and signed by 92% of the parents in the first 13 schools, but because of teacher strikes, the turnout was only 17% in the remaining five schools.[13]

Sample collection procedure and microscopy. A team of trained field research assistants and school nurses visited each school for general study information and urine collection between 10:00 AM and 2:00 PM on three consecutive days. Samples were transported to the laboratory on the same day in dark cooler boxes to ensure optimal processing. After arrival, two 10-mL urine samples (A and B) were preserved with 1 mL of 2% tincture of merthiolate in 5% formalin solution.[21] Within the same week, samples were centrifuged for 10 minutes at 4,000 rpm and microscopically investigated at a magnification of 10× for S. haematobium eggs. The egg counts were recorded for each 10 mL of urine separately. When more than 1, 000 eggs were seen, counting was stopped. Microscopy was performed blinded to previous results and by separate technicians. Quality control was performed by an independent senior microscopy expert on 10% of randomly chosen samples.

Detection of *Schistosoma* DNA. Aliquots of 1 mL of each first-day urine sample, before the merthiolate-formalin was added, were transferred into cryotubes. The aliquots were stored at 4°C for maximum of one week in the dark and thereafter stored at −80°C for several months, before being transported to the Netherlands in frozen conditions for DNA isolation and detection. For a subselection of 85 urine samples, 49 of them that were microscopy negative at all examinations, a second aliquot was tested by PCR at a local laboratory in South Africa.

In the Netherlands, DNA isolation and the set-up of the PCR was performed at the Leiden University Medical Center with a custom-made automated liquid handling station (Hamilton, Bonaduz, Switzerland). DNA was isolated from a 200-μL subsample of each urine sample by using a proteinase K heating step and QI Amp spin columns (QIAGEN, Hilden, Germany) as described.[16] Phocin herpes virus 1 (PhHV-1) was added to the lysis buffer as an internal control.[22] For real-time PCR, Schistosoma-specific primers Ssp48F and Ssp124R were used to amplify a 77-basepair fragment of ITS2. The double-labeled probe Ssp78T was used to detect amplification.[16] For the internal control, PhHV-1-specific primers PhHV-267S and PhHV-337AS and the specific double-labeled probe PhHV-1-305TQ were included in each reaction mixture. The amplification and detection of each DNA sample, with use of the Bio-Rad (Herciules, CA) CFX Manager, was performed as described.[16]

Similar DNA isolation and PCR procedures were used at the laboratory in South Africa, with some minor modifications. All steps were done manually. The PhHV-1 internal DNA control was replaced by Lambda DNA (1 μL Lambda DNA; dilution of 10^{-6} per 400 μL of lysis buffer). Therefore Lambda-specific primers and the Lambda-specific CAL Fluor Orange 560–labeled probe Lambda-TMp were used in the reaction mixture. The amplification of each DNA sample was performed in a 25-μL reaction mixture containing PCR buffer (containing 10× Gold Buffer, dNTPs, and TaqGold), 5 mM $MgCl_2$, 2.5 μg bovine serum albumin (Roche Diagnostics

Nederland BV, Almere, The Netherlands), 1.5 pmol of each Schistosoma-specific primer, 1.5 pmol of each Lambda-specific primer, 1.25 pmol of each of the Schistosoma-specific and Lambda-specific probes, and 5 mL of the DNA sample. The thermocycler was set for 15 minutes at 95°C, followed by 50 cycles, each for 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Amplification, detection, and data analysis were performed with the Corbett Rotor Gene 3000 (QIAGEN).

In both laboratory settings, the PCR output consisted of a cycle threshold (C_t) value, which represented the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence and thereby indicating the presence of parasite-specific DNA in the sample that was tested. The C_t values of the internal controls (PhHV in the Netherlands, Lambda DNA in South Africa) were within the expected range for all samples and will not be further discussed within the results.

Data analysis and statistical testing. The PCR analysis was performed blinded from microscopy or other field data. The results of the real-time PCR analysis were stored and grouped in a Microsoft (Redmond, WA) Access database and imported into IBM (Chicago, IL) SPSS 20.0 for statistical analysis together with provided field data, including results of the interviews and urine microscopy examinations.

Based on microscopy, the infection was classified as high intensity if the mean number of eggs of the three specimens was > 50 per 10 mL of urine.[23] Based on PCR, the infection was classified as high intensity (C_t < 30), medium intensity (30 £ C_t < 35) or low intensity (35 £ C_t < 50).[15] These categories were chosen arbitrarily, based on previous experiences with protozoal and helminth infections where DNA loads with a C_t value < 30 could generally be microscopy confirmed and DNA loads with a C_t value > than 35 were always microscopy negative. For better visualization in the scatter plots, C_t values were also recalculated into arbitrary units (AU) of copy numbers of Schistosoma DNA. An arbitrary value of 1 AU was assigned to each positive sample with a C_t value ³ 35. Assuming 100% efficacy of the DNA multiplication process up to 35 cycles, a duplication of AU was calculated for each PCR cycle, meaning for each single C_t value reduction starting with a C_t value of 35.

Because the data were not normally distributed, results were described as the total number, range, interquartile ranges (IQRs), and median value of positive subjects. For the evaluation of S. haematobium infection intensity, concordance between sample egg counts and PCR output (AU) was statistically analyzed by using the non-parametric Spearman's rank order correlation coefficient (r) because both variables were skewed even after log transformation. The McNemar statistical test was used to analyze concordance between the different diagnostic procedures. Statistical significance was considered at P < 0.05.

RESULTS

General characteristics of the study population. A summary of the sample selection procedure is shown in Figure 1, and a general description of the study population is shown in Table 1. A total of 1,948 school girls were included for participation. However, some participants were excluded because unreturned consent forms, refusal to participate, absenteeism, and not being within the specified age range. Of the interviewed girls, 708 pupils provided urine samples for complete microscopy data and PCR, and 13 schools had more than 20 participants. Discrepancy analysis showed no relevant differences between the 708 girls tested and those excluded because of incomplete sampling. At the interview, 27.5% of the 708 girls reported not living with their parents, 60.4%

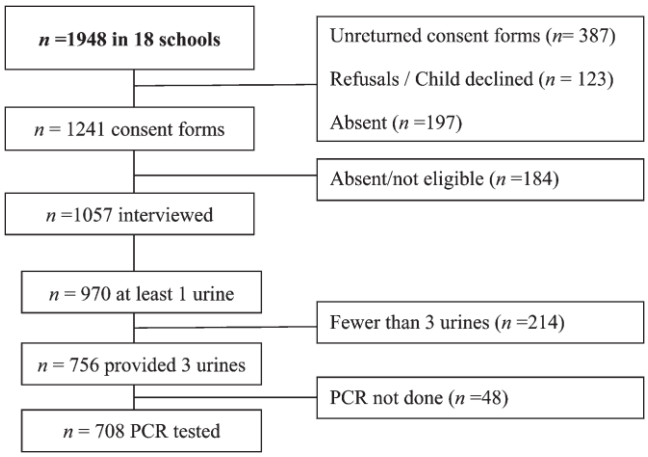

Figure 1. Flow chart of study participation and adherence by 708 school girls in KwaZulu Natal, South Africa. PCR = polymerase chain reaction.

Table 1
Epidemiological and parasitological characteristics of urine Schistosoma PCR tested school girls (n=708)

Characteristics	n	(%)	Range
Number	708		
Age (years)			
Median (range)	11		10–12
Number of schools	18		
Participants per school			
Median (range)	33		4–158
Reported during interview [1]			
Situation at home [2]:			
Living with both biological parents	163	(23.1)	
Living with one biological parent	348	(49.4)	
Possible exposure to infected water:			
Low risk water contact	180	(25.4)	
High risk water contact	248	(35.0)	
Red urine:			
Sometime before	50	(7.1)	
Last week	60	(8.5)	
Genital symptoms[3]:			
History of bloody discharge	52	(7.2)	
History of burning sensation in the genitals	101	(14.3)	
History of any symptoms	231	(33.1)	
Urine microscopy (S. haematobium eggs)			
60 mL examination	225	(31.8)	
Positive in all 6x 10 mL examinations	103	(14.5)	
Showing mean egg count > 50 eggs/10mL	60	(8.5)	
10 mL examination (day 1)			
S. haematobium egg positive cases detected	171	(24.2)	

1) Full details are presented in Hegertun et al.(2013) definitions describes at material and methods section.
2) In 3 cases no answer was given.
3) In 5 cases no answer was given.

reported possible exposure to infested water, 15.6% reported a history of red urine, and 33.1% reported any history of genital symptoms (Table 1). These characteristics were within the same range as the answers given by all 1,057 interviewed girls, being 27.3%, 62.6%, 17.8% and 35.0%, respectively.

Schistosoma microscopy. The day-to-day variation in counted S. haematobium eggs is shown in Figure 2A, which depicts egg counts of sample day 1A and sample day 3A. Intensity of infection between the two sample readings correlated significantly in those positive (n = 195; Spearman's $r = 0.32$, $P < 0.001$). At the same time, discrepancies were seen. Although 139 girls were microscopy positive for both samples, 32 girls were positive in urine sample 1A and negative in sample 3A. In 24 girls, the opposite result was seen. Less than 50 eggs/10 mL of urine were counted in 43 of the 56 girls; there was only one positive urine sample. Identical patterns were seen when microscopy data for other urine readings were compared.

The overall prevalence of S. haematobium based on the detection of eggs in urine samples collected on three consecutive days was 31.8%, with an average egg count of 0.17–624 eggs/10 mL (median = 20, IQR = 5–55). One hundred and three (45.8%) of the 225 microscopy-positive girls showed eggs in all six urine examinations, and average high intensity infections were seen in 60 (26.7%) infected girls. Based on readings from the three consecutive days, the prevalence of each day was 25.8–26.4%, and based on the individual 10-mL urine examinations, the prevalence was 22.5–24.2%.

Schistosoma PCR. The PCR analysis of first day urine samples showed Schistosoma DNA in 25.4% of the samples,

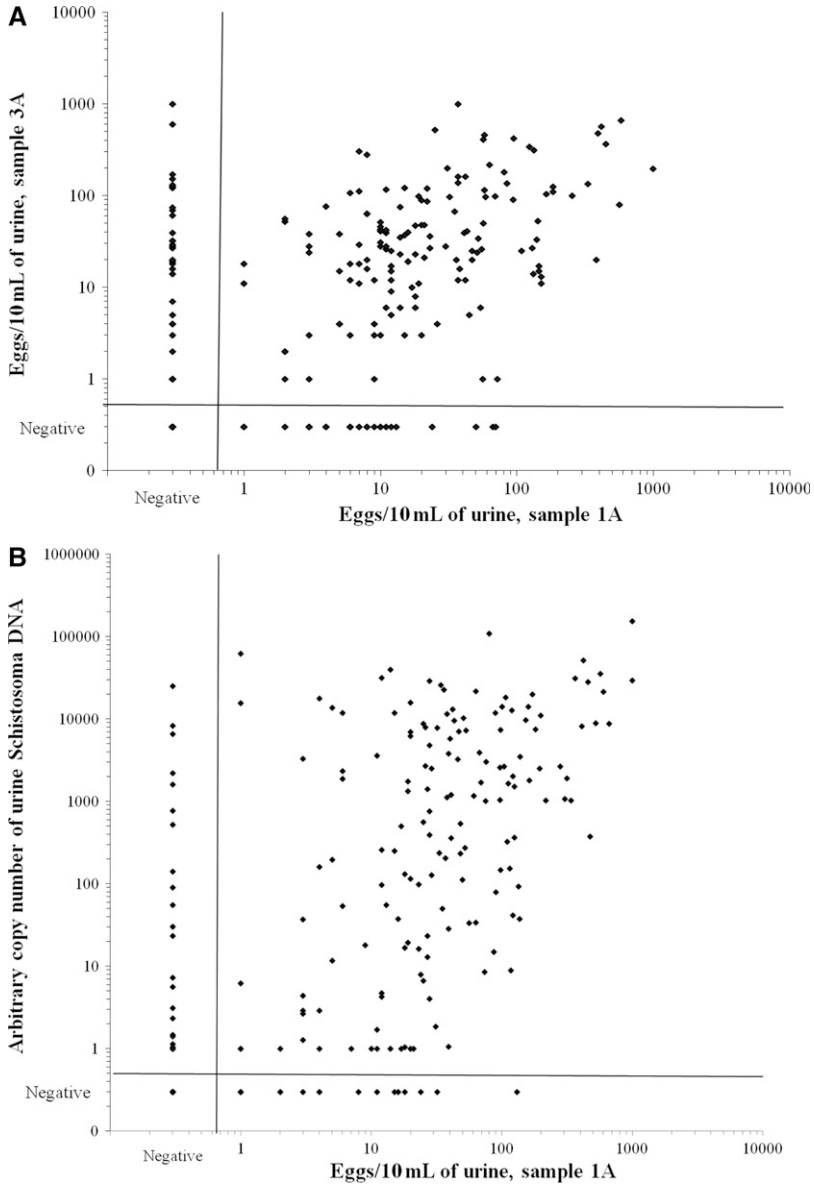

Figure 2. Scatter plots of 708 school girls 10–12 years of age in KwaZulu-Natal, South Africa. (A) Comparison of urine microscopy of the first 10-mL sample of day 1 (sample 1A) and the first 10-mL sample of day 3 (sample 3A). (B) Comparison of urine microscopy of the first 10-mL sample of day 1 (sample 1A) and urine Schistosoma DNA loads determined in the same day sample (200 mL) by using real-time polymerase chain reaction (PCR).

and 125 of these 180 samples had high DNA loads. The AU of DNA copies ranged from 1 to 154 × 10^3 (median = 448, IQR = 12–7× 10^3). The association between urine Schistosoma DNA load quantified in 200-mL samples and the number of eggs counted in a 10-mL sample of the same portion is shown in Figure 2B. A significant correlation was seen between the two procedures (n = 199; Spearman's $r = 0.51$, $P < 0.001$). Discrepancies were also observed. Nineteen samples were PCR negative and microscopy positive, all samples except one had egg counts < 50 eggs/10 mL. In addition, Schistosoma DNA was detected in 28 urine samples that were microscopy negative. In 14 of these samples, eggs were seen in at least one of the five other 10-mL urine samples by microscopy, and the remaining 14 had low DNA levels, which indicated that half of them had 1 AU and the maximum seen was 90 AU. The number of detected cases by urine PCR did not statistically differ from the number of detected cases by each of the 10-mL urine microscopy examinations, or each of the three consecutive day readings, but was significantly less compared with the 225 cases detected when all microscopy readings were combined. Also, the average egg counts correlated significantly with the measured urine AU of DNA copies (n = 239; Spearman's $r = 0.70$, $P < 0.001$).

A comparison between PCR outputs in the two PCR laboratories showed only minor discrepancies. Four of the 36 microscopy-positive urine samples were missed in South Africa; these samples showed positive results in the Netherlands. Conversely, 1 of the 49 microscopy-negative samples had a high DNA level in South Africa, which could not be confirmed when tested in the Netherlands.

School level analysis. To fully explore the diagnostic value of urine PCR to identify high-risk communities, findings were further analyzed at school level. Data for 13 schools with at least 20 participants per school, representing 666 school girls, were aggregated. The prevalence of S. haematobium per school based on urine PCR ranged from 3.4% to 45.9% (median = 27.0%, IQR = 12.0–37.8%) and showed a significant correlation with the prevalence range based on complete microscopy (n = 13; Spearman's $r = 0.95$, $P < 0.001$). The prevalence of detectable Schistosoma DNA showed a significant correlation with reported history of genital symptoms at school level (Figure 3) (n = 13; Spearman's $r = 0.74$, $P < 0.01$). Also, reported history of high-risk water contact and history of red urine showed a significant correlation with school-based Schistosoma prevalence determined by PCR or microscopy (n = 13; Spearman's $r = 0.62$–0.73, $P < 0.03$). A total of 58.6–83.8% of the girls reported living with at least one parent, and 13.9–34.5% reported living with both parents. No association was seen between household composition and prevalence of schistosomiasis determined by PCR or microscopy.

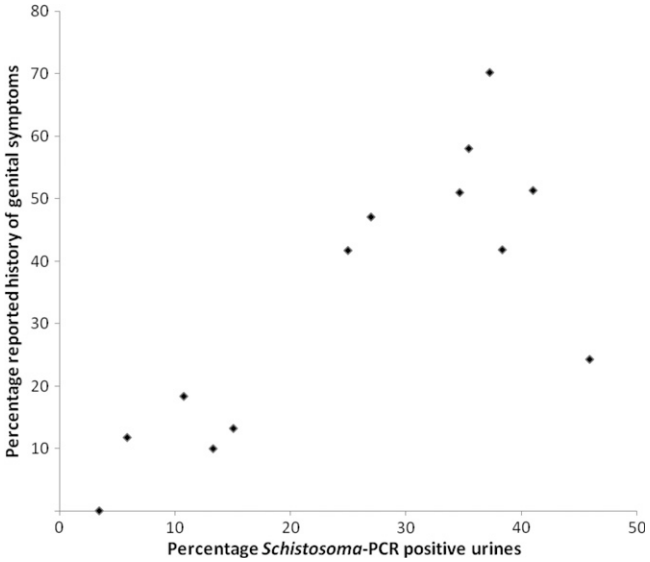

Figure 3. Scatter plot comparing school-based prevalence of Schistosoma DNA in urine samples and reported history of genital symptoms in school girls in KwaZulu Natal, South Africa. Schools (n = 13) with at least 20 participants were included, which included 666 school girls of 10–12 years of age. PCR = polymerase chain reaction.

DISCUSSION

In this study, we used a PCR with 708 urine samples collected from girls in 18 primary schools from KwaZulu-Natal, South Africa. Microscopy was performed six times on a 10-mL urine sample collected over three consecutive days. We found substantial dispersion of counted eggs in urine; < 15% of the girls showed positive results for all six 10-mL urine sample examinations. This high day-to-day variability in Schistosoma eggs shedding is generally acknowledged, but data have been published only sparsely.[24]

Interestingly, we found the sensitivity of the ITS-based PCR performed with a 200-mL subsample to be comparable to that of each of single 10-mL microscopy examinations. However, the sensitivity was lower when compared with repeated microscopy (complete 60-mL urine sample examination). DNA amplification was detected in 98.3% and 88.3% of the samples with > 50 eggs/10 mL of urine and £ 50 eggs/10 mL of urine, respectively, and in 28 (5.2%) of 537 urine samples in which eggs were not detected at the first 10-mL urine sample examination. In one considers that our population of school girls was truly infected if at least one of the six microscopy examination results was positive, or if Schistosoma DNA was detected in urine, this resulted in a negative predictive values for complete 60-mL microscopy, 10-mL microscopy, and urine PCR of 98%, 86%, and 88%, respectively.

In a previous study, the same ITS-based real-time PCR for specific detection of Schistosoma DNA showed excellent sensitivities for 730 urine samples collected from children in five primary schools from different communities in the Greater Accra region of Ghana.[17] Compared with our study, infection levels were low in the selected schools in Ghana; the overall prevalence was 7.8% and only 10 of 57 children had > 50 eggs/10 mL of urine. Schistosoma DNA was amplified in 100% and 85.2% of urine samples with > 50 eggs/10 mL of urine and £ 50 eggs/10 mL of urine, respectively. In addition, Schistosoma DNA was detected by PCR in 102 (15.2%) of 673 urine samples in which eggs were not detected by single 10-mL urine microscopy. These findings resulted in higher negative predictive values for real-time PCR (> 94.6%) than for microscopy (54.3–95.7%).[17]

Although Schistosoma DNA was quantified in both studies according to the same PCR procedure, microscopy showed better performance in South Africa. Results could not be explained by the higher overall S. haematobium prevalence

in samples in South Africa because concordance between PCR output and microscopy did not shift between the selected schools (e.g., the five schools with a microscopy prevalence < 20% showed a similar range in prevalence based on urine Schistosoma DNA detection, while the five schools tested in Ghana showed substantial higher infection levels based on PCR).[17]

A possible explanation for the comparatively poor PCR performance in the current study could be the procedure of sample collection (i.e., the time lag between receiving the samples and definitive storage in a frozen condition). Because of logistics, freezing of samples could only be performed a number of days, up to a week, after collection. When mixed with ethanol, fecal samples can be kept at room temperatures for weeks to months, even in tropical conditions, before transported to centralized laboratory facilities for DNA isolation.[25] For urine samples, optimal storage conditions without immediate need for a cold chain still need to be further explored.

Alternatively, the ITS-based PCR may not be the most optimal procedure for amplifying Schistosoma DNA in all geographic settings. Other procedures have been described and some of them seem to show promising diagnostic performance.[26,27] However, not all of these PCRs have been transformed into a multiplex real-time PCR format. Thus, they are substantially more laborious, still observer-dependent, and lack the option to include a proper internal control.[27] Also, some need relatively large serum volumes.[28] Thus, their feasibility for large-scale population based surveys still needs to be fully explored.

Human errors, including missed observations during microscopy and mislabeling and swapping of samples, can never be completely excluded. This problem is illustrated by the single sample showing > 50 eggs/10 mL of urine that showed negative results when tested by PCR. This person showed S. haematobium eggs at all six microscopy examinations, almost all with high egg counts. Thus, it is likely that aliquots were swapped during sampling for PCR analysis. The potential influence of human error is also illustrated by the performance of the real-time PCR at the separate laboratory facilities. Overall results were highly reproducible, but in case of discrepancy, the output produced by using an automated liquid handling station has been more in concordance with the microscopy data than the PCR output produced manually.

The use of an automated liquid handling station also enables processing of large sample numbers in a short period (i.e., we completed DNA isolation and detection, including automated data handling, for all 708 samples within 10 working days). The small sample volume required and the potential number of additional DNA targets that can be quantified, in particular when working with fecal samples, makes this approach highly attractive for large-scale population screening for the presence of multiple urinary and intestinal microorganisms.[17,29,30] In this study, we also introduced arbitrary units (AU) of copy numbers of Schistosoma DNA as PCR output, which provides a more realistic view on the existing high linear range of intensity of Schistosoma infection than C_t values. In addition, for more in depth comparison between different PCRs, further standardization, including converting DNA loads into egg counts, is needed.

In a previous study, the overall association between urine egg excretion and reported urinary and gynecologic symptoms was described. In particular, bloody discharge and a burning sensation in the genitals were found to be significantly correlated with repeated urine microscopy findings, even after controlling for confounders.[13] As expected, we found the same associations between PCR-diagnosed schistosomiasis and reported genital symptoms. Because of the young age of the girls included, we were not able to collect any gynecologic samples, which would be of interest for quantification of Schistosoma DNA levels.[31] However, for confirmation, urinary and vaginal samples should be collected from adult women.

Although schools were randomly selected from a relatively small rural area of KwaZulu-Natal that had comparable ecologic and socioeconomic circumstances, we found S. haematobium infection prevalence to range from < 5% up to approximately 60% between the different schools. Schistosoma prevalence and the prevalence of reported urinary and gynecologic symptoms varied greatly between included schools. Conversely, we found no association between household composition, reflecting social status in this particular region, and the prevalence of S. haematobium. Focal distribution of schistosomiasis has been described and besides behavioral components in relation to exposure to infected water bodies, many other factors may influence prevalence and intensity of infection, including micro-level environmental circumstances.[32] The consequences are obvious. Although local hot-spots require intensive and repeated mass treatment with appropriate anti-schistosomal drugs in combination with intense control measures and monitoring, other communities will be served sufficiently by targeted treatment or even passive case detection. Based on this knowledge, there is also a clear need for a rapid and field applicable diagnostic test to allocate these hot spots in resource-poor settings without the need of high technology laboratory equipment.[33,34]

In conclusion, our findings illustrate that the diagnostic potentials of urine Schistosoma real-time PCR is dependent on the quality and intensity of performed microscopy. Repeated microscopy may in certain settings be more sensitive but at the same time it is a highly laborious as well as observer dependent procedure. Although improved sensitivity is still anticipated, parasite-specific DNA detection already seems an attractive and efficient automated high-output system for large screening programs. Only small sample volumes are needed to identify communities at risk for development of genital morbidity caused by S. haematobium infection.

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5.0 CHAPTER FIVE

5.1 Paper 2

Cervical Cytology as a diagnostic tool for Female Genital Schistosomiasis.Correlation to cervical atypia and *Schistosoma* PCR.

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The purposes of writing this paper were to expand on developing early diagnostic tools among young women at risk for genital schistosomiasis, to increase the knowledge and awareness of FGS among health professionals in endemic areas, and explore the association between schistosomiasis and squamous cell atypia in this paper, since this has been under debate through several previous studies using different indicators. In this paper possible risk factors for both schistosomiasis and squamous atypia were described. Analysis of cervical cytology, urine microscopy and *Schistosoma* PCR in cervico-vaginal lavage and in urine samples were used to answer the research questions. The paper was also aimed at raising awareness of these entities and to provide laboratory and other health personnel with additional insight into the diagnosis and implications of FGS.

Consenting young women aged 16-23 from high schools underwent structured interviews and the following samples were collected Pap smears for cytological analysis including *Schistosoma haematobium* ova and cervical squamous cell atypia, vaginal lavage and urine samples for real-time PCR for *Schistosoma*-specific DNA, and urine microscopy for the presence of *S. haematobium* ova.

I was responsible for generating the Cytology results in the research project. Being a Cytotechnologist, I used my knowledge and expertise that I have gained over the years in the analysis and checking (of problematic) Pap smears. I was assisted by a Cytotechnician with the screening and another Cytotechnologist did the quality control. I facilitated the transportation of the urine and vaginal lavage samples to the Leiden University Medical Center (LUMC), where I did the ITS-based real-time *Schistosoma* PCR assay together with support and assistance from the staff at the LUMC. Performing the Schistosoma PCR on such a large sample of urines and vaginal lavage samples and comparing them to the cytology results was novel

research among a young population and has furthered our understanding of the early development of FGS symptoms in young women.

It was found that the *Schistosoma* PCR lavage analysis was six times more sensitive than Pap smears in detecting schistosomiasis. There was no significant association with genital schistosomiasis and squamous cell atypia but this could represent a Type 2 error because of the low prevalence of schistosomiasis. Cytology is a useful diagnostic technique that has provided insight on cervical cytological atypia detected amongst the sexually active young women who are also at risk for schistosomiasis and HIV since screening is not routinely done among young women, the cytology findings could contribute to the prevention of cervical cancer.

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Research Article

Cervical cytology as a diagnostic tool for female genital schistosomiasis: Correlation to cervical atypia and *Schistosoma* polymerase chain reaction

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Abstract

Background: Female genital schistosomiasis (FGS) is a tissue reaction to lodged ova of *Schistosoma haematobium* in the genital mucosa. Lesions can make the mucosa friable and prone to bleeding and discharge. Women with FGS may have an increased risk of HIV acquisition, and FGS may act as a cofactor in the development of cervical cancer. **Objectives:** To explore cytology as a method for diagnosing FGS and to discuss the diagnostic challenges in low- resource rural areas. The correlation between FGS and squamous cell atypia (SCA) is also explored and discussed. Cytology results are compared to *Schistosoma* polymerase chain reaction (PCR) in vaginal lavage and urine and in urine microscopy. **Materials and Methods:** In a clinical study, 394 women aged between 16 and 23 years from rural high schools in KwaZulu- Natal, South Africa, underwent structured interviews and the following laboratory tests: Cytology Papanicolaou (Pap) smears for *S. haematobium* ova and cervical SCA, real- time PCR for *Schistosoma*- specific DNA in vaginal lavage and urine samples, and urine microscopy for the presence of *S. haematobium* ova. **Results:** In Pap smears, *S. haematobium* ova were detected in 8/394 (2.0%). SCA was found in 107/394 (27.1%), seven of these had high- grade squamous intraepithelial lesion (HSIL). *Schistosoma* specific DNA was detected in 38/394 (9.6%) of vaginal lavages and in 91/394 (23.0%) of urines. Ova were found microscopically in 78/394 (19.7%) of urines. **Conclusion:** *Schistosoma* PCR on lavage was a better way to diagnose FGS compared to cytology. There was a significant association between *S. haematobium* ova in Pap smears and the other diagnostic methods. In low- resource *Schistosoma*- endemic areas, it is important that cytology screeners are aware of diagnostic challenges in the identification of schistosomiasis in addition to the cytological diagnosis of SCA. Importantly, in this study, three of eight urines were negative but showed *Schistosoma* ova in

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their Pap smear, and one of them was also negative for *Schistosoma* DNA in urine. In this study, SCA was not significantly associated with schistosomiasis. HSIL detected in this young population might need future consideration.

Key words: Cervical atypia, cytology, female genital schistosomiasis, gynecology, laboratory diagnostics, real- time polymerase chain reaction

INTRODUCTION

In terms of the public health impact, schistosomiasis (bilharzia) is globally second only to malaria among parasitic diseases. It affects approximately 262 million people worldwide.[1] The disease is most commonly found in sub-Saharan Africa.

Schistosoma haematobium infection is the most common schistosome infection in KwaZulu-Natal, South Africa,[2] with reported focal prevalences between 40% and 98%.[3] A recent study performed in coastal, Southern KwaZulu-Natal showed a prevalence of *S. haematobium* of 32% among girls aged 10–12 years.[4]

Schistosomal lesions can be seen in the urinary and gastrointestinal tracts. It is, however, well known that the ova also can be trapped in a variety of sites within the human definitive host including the brain, pancreas, kidneys, testes, the vagina, and uterine cervix.[5] This partly random distribution of schistosomal lesions makes it difficult to identify a diagnostic gold standard for a specific region where infections can occur. It has recently been suggested that schistosomal lesions in the vagina and cervix uteri, i.e., female genital schistosomiasis (FGS), may increase the risk of HIV acquisition.[6] FGS has also been suggested a possible cofactor in the development of precancerous lesions and cervical cancer.[7,8] FGS lesions are caused by the human host's response to living and dead parasite ova that are lodged or trapped in the submucosa of the uterine cervix and vagina. Visual inspection by colposcopy and targeted biopsies are considered a more accurate way of diagnosing FGS; however, they are expensive, and the latter is not feasible in populations at risk for HIV.[9,10]

Papanicolaou (Pap) smears are commonly used in low-resource areas by doctors and nurses for the diagnosis of cervical cancer. The aim of this study was to explore the possibilities and limits of diagnosing FGS using Pap smear cytology. The cytology results were compared to *Schistosoma* polymerase chain reaction (PCR) in vaginal lavage and urine, and in urine microscopy in a young, female population who resided in a rural area. We also wanted to explore a possible correlation between schistosomiasis and squamous cell atypia (SCA) of the cervix.

MATERIALS AND METHODS

The study area in coastal KwaZulu-Natal South of Durban is endemic for schistosomiasis. This area had a population of 710,000 people, 51% below the age of 20 years of which 55% are female. In these mostly rural communities, women do their laundry and children play in *Schistosoma* infested rivers. As part of a school-based, clinical study of FGS, females from 42 randomly selected high schools were included. Included in this cross-sectional study were 394 consenting young women aged between 16 and 23 years (median age 19 years); all were examined in the period 2010–2012. Pregnant women, virgins, and those who were not ready for gynecological examination were excluded.

The study was initially discussed with the relevant stakeholders including the KwaZulu-Natal Department of Health, Department of Education, school staff, parents, and pupils. A team of trained field assistants and school nurses visited the schools to give general information and recruit young women for laboratory investigations, treatment, and gynecological examinations. In the first meetings, the focus of the project, its importance, benefits, and possible negative consequences were explained to the stakeholders. Written informed consent was sought at this time. It was explained that the research samples would be investigated in batches and hence, an extended waiting period for results could occur. Participants were encouraged to visit local health care facilities should they experience any problems in the meantime. Logistical arrangements at suitable times to transport the young women to the clinic for their gynecological examinations and interviews were discussed with school principals and other relevant staff. According to practice in rural clinics, a syndromic protocol was used to diagnose and treat findings at the point of investigation. When laboratory results became available, patients were contacted and asked if they had been treated and helped with further management of the disease.

Permission was also obtained from the Biomedical Research Ethics Administration, University of KwaZulu-Natal (Ref BF029/07), the Departments of Health, and Education of KwaZulu-Natal (Ref HRKM010-08), the Norwegian Ethics Committee, Regional Committee for Medical and Health Research Ethics (REC), South Eastern Norway (Ref 469- 7066a1.2007.535), and the European

Group on Ethics in Science and New Technologies (Ref IRSES-2010:269245).

Consenting participants underwent a private face-to-face structured interview, conducted by research assistants in isiZulu, the local language, including their history of water contact, observed red urine and genital symptoms. In addition, the research assistants asked questions about their sexual history and parity as these variables could have had a bearing on the risk of cervical atypia.

During the gynecological examination, the surfaces of the vulva, vagina, and cervix were examined visually and colposcopically in accordance with the protocol from a previous FGS study.[11] Thereafter, cervicovaginal lavage samples were collected by spraying 10 ml saline on the vaginal wall and cervix 4 times, which was then drawn back into the syringe and divided into six sets of cryotubes and stored in a −80 freezer with CO_2 and generator backup systems. The Pap smears were then collected with a wooden spatula or a cytobrush. The Pap smears were preserved by using a commercial cytological spray fixative. Urine samples for PCR analysis were stored as described for vaginal lavage samples. The urine samples for microscopy were stored in dark cooler boxes to ensure sample quality.

For cytological analyses, the smears were Pap stained[12] and examined by a cytologist using an Olympus B×43 microscope and viewed at magnification with objectives 10 and 40. The smears were examined for all cytological entities in addition to *Schistosoma* ova and SCA. The diagnosis of *S. haematobium* was based on finding intact living or dead ova within oval chitinous shells with a terminal spine [Figure 1a].[13]

Epithelial dysplasias [Figure 1b] were graded according to the Bethesda system.[14] In short, the SCA was stratified into atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), a category that encompasses mild dysplasia/cervical intraepithelial neoplasia (CIN) 1 and human papillomavirus (HPV), and high-grade squamous intraepithelial lesion (HSIL). High-grade lesions encompass moderate dysplasia/CIN 2, severe dysplasia/CIN 3, and carcinoma *in situ*. To ensure quality control, 10% of all negative smears were re-screened.

Internal transcribed spacer (ITS)-based real-time PCR with quantification of *Schistosoma*-specific DNA in cervicovaginal lavages and urines were performed as described previously.[4,15,16,] Cycle threshold (Ct) values were used as the output of the PCR, reflecting the parasite-specific DNA load in the sample tested. Ct-values were arbitrarily categorized in the following infection intensity groups:

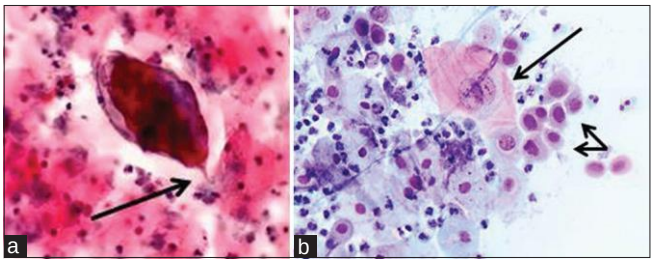

Figure 1: (a) Cervical smear, (Papanicolaou stain, ×40). *Schistosoma haematobium* ovum with an embryo miracidium. Note the diagnostic terminal spine (arrow). (b) Cervical smear, (Papanicolaou stain, ×40). Cytological changes consistent with high grade squamous intraepithelial lesion including human papillomavirus infection. Long arrow: Human papillomavirus cytopathic changes including nuclear and cellular enlargement, and koilocytosis. Double arrow: Cells with increased nuclear to cytoplasmic ratios, nuclear membrane irregularities and marked hyperchromasia consistent with high- grade squamous intraepithelial lesion. The background contains neutrophil granulocytes denoting inflammation

High-intensity (Ct <30), medium-intensity (30≤ Ct <35), low-intensity (35≤ Ct <50), or negative (Ct = 50).[15]

Urine microscopy samples were preserved with 1 ml of 2% tincture of merthiolate in 5% formalin solution.[4,15] The samples were spun for 10 min at 4000 rounds/min and the sediment examined microscopically by trained fieldworkers. The samples underwent quality control by an independent microscopist on 10% of randomly chosen samples, and any discrepancies were discussed with fellow field workers.

Data were analyzed using IBM SPSS version 23.0 (Armonk, NY, USA) Ct-values were not normally distributed and therefore described by median and range and compared using a nonparametric statistical method. Inferential statistics was done using the Fisher's exact test. The odds and risks ratios (OR and RR) and the corresponding 95% confidence intervals (CIs) were determined for 2 × 2 cross-tabulations. All *P* values were two-tailed and $P < 0.05$ considered significant.

RESULTS

The flowchart of the study population is presented in Figure 2. The population characteristics are presented in Table 1 and show that 376/394 (95%) of the participants had a prior risk of water contact.

In the Pap smears, *S. haematobium* ova were detected in 8/394 (2.0%). *Schistosoma*-specific DNA was found in 38/394 (9.6%) of vaginal lavages including the 8 Pap smears with *S. haematobium* eggs. Significantly lower *Schistosoma* DNA loads were seen in the lavages of Pap-smear negatives (median Ct-value 32.9, range 20.4–37.4). In 78/394 (19.8%), *Schistosoma* ova were detected microscopically in the urines while *Schistosoma* DNA was detected in 91/394 (23.1%) of

the urines [Table 2]. There was a significant association between *S. haematobium* eggs in Pap smears and the presence of *Schistosoma* DNA in lavage (OR: 12.87, $P < 0.001$; 95% CI 9.13–18.14), *Schistosoma* DNA in urine (OR: 25.1, $P < 0.001$; 95% CI 3.1–207.4) as well as eggs in urine determined by microscopy (OR: 7.1, $P = 0.009$; 95% CI 1.7–30.6).

Table 1: Participant characteristics

Characteristics	*n (%)*	*Standard deviation*
Study population	394	
Age (years)		
Mean	19	1.64
Reported in interview		
History of sexual activity		
Sexually active	392 (99.5)	
Mean age of sexual debut	16.4	1.30
No sexual partners within the last month	114 (28.9)	
One sexual partner within the last month	271 (68.8)	
Two sexual partners within the last month	6 (1.5)	
Three sexual partners within the last month	1 (0.3)	
Number of children alive		
One	163 (41.3)	
Two	12 (3.0)	
Three	1 (0.3)	
Types of risk water body contact		
None	18 (4.6)	
River	284 (72.1)	
Dam	38 (9.6)	
Standing water	7 (1.8)	
Several types	41 (10.4)	
Red urine		
Sometime earlier	83 (21.1)	
Last week	46 (11.7)	
History of bloody discharge/bleeding		
Sometime earlier	58 (14.7)	
Last week	56 (14.2)	
Postcoital bleeding	138 (35.0)	
Spotting	148 (37.6)	

Table 2: *Schistosoma* prevalence using cytology, vaginal lavage, and urine PCR or urine microscopy

Diagnostic test	*n (%)*	*Median*	*Range*
Pap smear cytology	8/394 (2.0)		
PCR[a] vaginal lavage	38/394 (9.6)	31.0 Ct	19.5- 37.4 Ct
PCR[a] urine	91/394 (23.1)	28.6 Ct	19.2- 40.2 Ct
Urine microscopy	71/394 (17.8)	13 ova/10 mL	1- 71 ova/10 mL

[a]*Schistosoma* PCR. Ct refers to cycle threshold output of the PCR. PCR: Polymerase chain reaction, Pap: Papanicolaou

Between 95% and 97% of the corresponding Pap smears in which *Schistosoma* ova were identified either in vaginal lavage or urines were deemed suboptimal [Table 3]. Smears were deemed suboptimal or "inadequate" due to technical interpretability or inadequate cellular composition or incorrect sampling. In poorly preserved and bloodstained smears, it was not often possible microscopically to identify the cellular characteristics of epithelial cells, *Schistosoma* ova; however, they were identifiable [Figure 3a and b].

Ova of *S. haematobium* typically range in size from 80 to 170 μm in length and are 30–70 μm wide and have a diagnostic terminal spine. Within the ova embryo, miracidia were seen, under-developed, or well-developed, or as emergent miracidia with a finely ciliated outline [Figure 4a]. Empty, refractile, folded, and crumpled shells with no visible internal structure were also seen and read as ova with signs of degeneration [Figure 4b]. Partially or completely blackened and opaque, nonviable forms were also seen.

The local inflammatory reaction in the cytological smears with *S. haematobium* ova varied from sparse to marked or chronic inflammatory changes. The heavily blood-stained smears might reflect the clinical finding of abnormal mucosal blood vessels seen in the FGS lesions prone to contact bleeding [Figure 3b]. All Pap smears where *Schistosoma* ova were identified, 8/8 (100%), included blood, as did 25/35 (71.4%) of the *Schistosoma* positive PCR vaginal lavage samples [Table 3].

On comparing the diagnostic methods used in this study, *Schistosoma* PCR lavage analysis was 6 times more sensitive than Pap smears in detecting schistosomiasis.

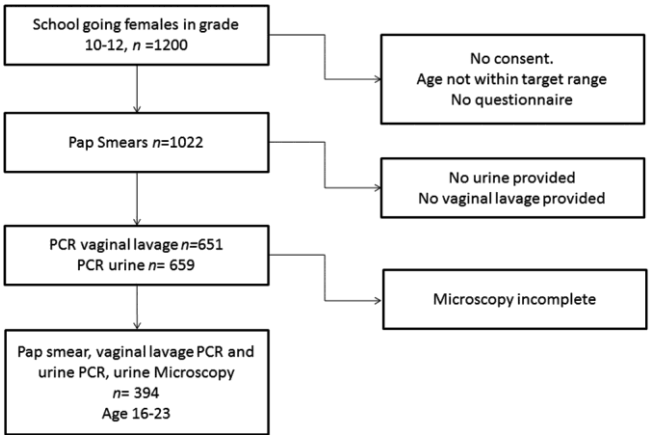

Figure 2: Flowchart of the study participation

Table 3: Summary of cytology results. For definitions of suboptimal Pap smears, ASCUS, LSIL, HISL, HSIL with possible invasion and SCA

Cytological entity	*n (%)*
Suitability of smears	
Smears containing blood	193/394 (49.0)
Blood- stained smears/*Schistosoma* Pap smear positive	8/8 (100)
Blood- stained smears/*Schistosoma* vaginal lavage PCR positive	25/35 (71.4)
Suboptimal Pap smears/urine microscopy *Schistosoma* positive	74/78 (95.0)
Suboptimal Pap smears/*Schistosoma* urine PCR positive	87/91 (96.0)
Suboptimal Pap smears/vaginal lavage PCR positive	37/38 (97.3)
SCA	107/394 (27.1)
ASCUS	33/394 (8.6)
LSIL	67/394 (17.0)
HSIL	6/394 (1.5)
HSIL possible invasion	1/394 (0.3)
Any SCA	107/394 (27.1)

SCA: Squamous cell atypia, ASCUS: Atypical squamous cells of undetermined significance, LSIL: Low- grade squamous intraepithelial lesion, HSIL: High- grade squamous intraepithelial lesion, PCR: Polymerase chain reaction, Pap: Papanicolaou

All *Schistosoma* positive Pap smears were also positive for *Schistosoma* PCR in vaginal lavage [Table 4]. There were, however, three cases with *Schistosoma* positive Pap smears and *Schistosoma* PCR lavage that had microscopy negative urine samples; one of them also negative for *Schistosoma* DNA in the urine. The distribution of SCA is shown in Table 3. HSIL was diagnosed in 6/394 (1.5%). One additional case was reported as HSIL with possible invasion. SCA was not significantly associated with any tests for schistosomiasis used in this study. The SCA was found across the whole age range examined [Figure 5].

DISCUSSION

With emerging knowledge of possible serious lifelong complications of FGS, there is an increasing demand for better diagnostic tools. It is important to find ways of diagnosing FGS that can be applied in rural areas with low resources. An increased awareness of the symptoms and signs of FGS is also needed among gynecologists and other health care workers that encounter women from *Schistosoma*-endemic areas.[10] FGS mucosal lesions can clinically mimic sexually transmitted infections and cervical neoplasias.[7-9,17]

The anti-schistosomal drug praziquantel is effective in killing adult worms responsible for laying eggs. The effect that the drug has on FGS lesions has not been fully explored. Treatment given to adults apparently did not change their FGS lesions.[18] Previous studies using histopathological reports indicate that the inflammatory reactions also around dead ova, presumably PCR negative, continue to pose a clinical problem.[19,20] This is in contrast to another study among adults with urinary schistosomiasis, showing that anti-schistosomal

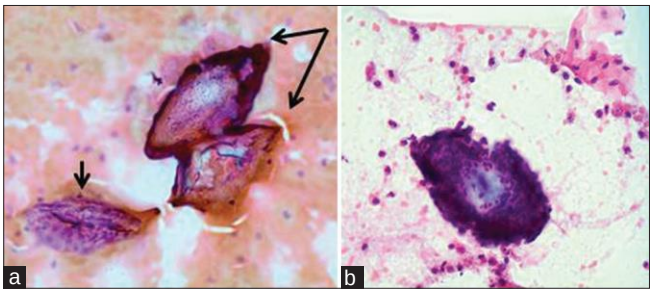

Figure 3: (a) Cervical smear, magnification (Papanicolaou stain, ×40). Poorly preserved smears with cellular detail obscured by blood and inflammatory cells. Small arrow: Non- diagnostic, poorly preserved epithelial cells. Double arrow: Two *Schistosoma haematobium* ova, identifiable by their refractile shells and characteristic terminal spines. (b) Cervical smear, magnification (Papanicolaou stain, ×40). *Schistosoma* ovum surrounded by many neutrophil granulocytes. Terminal spine detected on fine focus, however, not seen clearly in this image

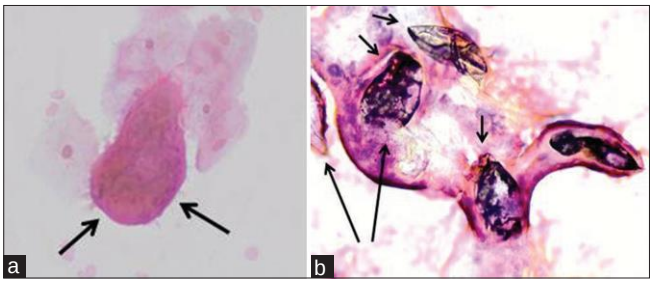

Figure 4: (a) Cervical smear, magnification (Papanicolaou stain, ×40). Arrows: emergent miracidium with a finely ciliated outline. (b) Cervical smear, magnification (Papanicolaou stain, ×40). Numerous calcified, dead ova (four calcified ova - short arrows and two non- calcified ova - long arrows) of *Schistosoma haematobium*

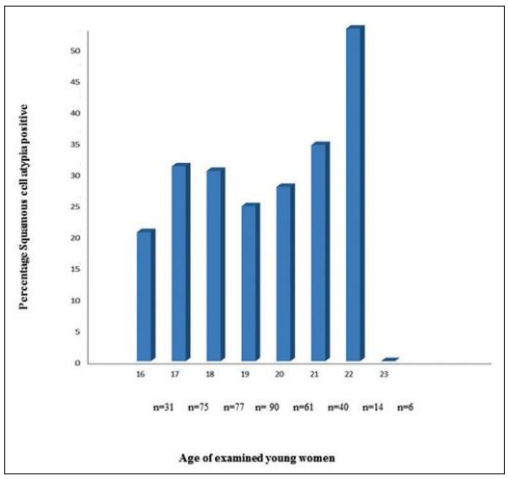

Figure 5: Percentage of squamous cell atypia positive in relation to age of the 394 examined young women. The number of examined women per year of age is indicated

Table 4: Eight cases positive for *Schistosomiasis* by cytology compared to *Schistosoma* PCR in vaginal lavage, *Schistosoma* PCR in urine and urine microscopy

Pap smear	*Vaginal lavage PCR (Ct)*	*Urine microscopy/ova per 10 mL*	*Urine PCR (Ct)*	*Participant's age*
Positive	Positive (19.5)	Positive (75 ova)	Positive (23.8)	16
Positive	Positive (28.3)	Positive (40 ova)	Positive (20.6)	18
Positive	Positive (26.6)	Positive (21 ova)	Positive (28.6)	23
Positive	Positive (23.0)	Positive (15 ova)	Positive (32.9)	17
Positive	Positive (21.9)	Positive (13 ova)	Positive (34.0)	19
Positive	Positive (25.4)	Negative	Positive (22.3)	18
Positive	Positive (21.9)	Negative	Positive (31.7)	17
Positive	Positive (27.2)	Negative	Negative (50.0)	19

Ct refers to cycle threshold output of the PCR. PCR: Polymerase chain reaction

treatment could contribute to the resolution of bladder lesions.[21] All the young women in the study were offered anti-schistosomal treatment. The effect of treatment on FGS will be followed and discussed in future publications.

A targeted biopsy is a method that has been used to diagnose FGS; however, this procedure is invasive and unethical in a young population that is also prone to HIV. Alternatively, diagnosis of FGS can be achieved directly by identification of parasite ova or parasite DNA in material from the vagina. In addition, the diagnosis can be made or suspected by visual inspection at a gynecologic examination through finding typical FGS lesions with the naked eye, by colposcopy, or by a camera with a magnification lens.[9] The latter has, however, never been tested. FGS should also be considered high on the list of differential diagnosis, once sexual abuse and sexually-transmitted infections have been precluded. The typical FGS lesions have been described and discussed extensively in recent publications and will be addressed in a forthcoming WHO FGS pocket Atlas with posters.[10] These mucosal lesions include two types of sandy patches, abnormal blood vessels, and rubbery papules.

In a recent study, primary school girls reported similar symptoms as adults with genital schistosomiasis There was also an association between genital symptoms and urinary schistosomiasis.[22] An unresolved question relates to diagnosing FGS in pre-pubertal young girls, who may have severe genital symptoms with bloody discharge, genital pain, and spotting. In our study, we had young women with negative urine tests, but positive tests from the genital tract [Table 3], in line with the seemingly random submucosal allocation of the ova-producing parasites to epithelial linings generally in the body, including the genital or urinary tracts. This is in line with a recent FGS study from Madagascar where women between 15 and 35 years with *Schistosoma* negative urines had *Schistosoma* DNA in genital samples as well as cases in our study.[23] The important notion of this is that testing only urine samples for schistosomiasis may be insufficient to diagnose FGS.

In our study, the diagnostic yield of *Schistosoma* infection in Pap smears was low which is consistent with previous findings from mainland Africa.[24,25] The number of positive results for schistosomiasis in cytology Pap smears in this study was small (8/394); hence, deductions need to be interpreted with caution. The Pap smear yield in our study might be low due to the sampling techniques as vaginal lavage samples were collected prior to the cervical smears. This might have removed *Schistosoma* ova. The lavage additionally possibly altered the cervical mucosal surface, resulting in suboptimal or unsatisfactory smears. Poor fixation could also contribute to the suboptimal smears. Furthermore, it might be that the ova load was low either proportional with a low parasite load or proportional with a different genotype that did not produce as many ova as found in, for example, Madagascan population.[23]

A crucial aspect of the cytology report is the issue of adequacy. According to the Bethesda system, adequacy is expressed in terms of an adequate number of well-preserved epithelial cells including cells from the transformation zone.[14] Smears which do not meet the criteria but at the same time, enabled a diagnosis are reported as "satisfactory for evaluation but limited by" followed by whichever criteria was lacking, for example, inadequate endocervical cells, inflammation, etc., Smears deemed "unsatisfactory for evaluation" may reveal obscuring blood; excessive inflammation; thick, multicellular areas; poor preservation; air drying artifact; or contaminants. In this study, the interpretation was compromised in approximately 75% of the smears.

Furthermore, in this study, all cytology Pap smear *S. haematobium* cases identified were also positive for vaginal lavage *Schistosoma* PCR. Some of the smears included nonviable ova. They are; however, significant as they indicate lesions where inflammation continues to develop.[9] Pitfalls exist, when contaminants (foreign

bodies) introduced into smears either during sampling, transportation or processing, with similar oval shapes as seen in Figure 6a are encountered. The depicted structure; however, lacks the diagnostic terminal spine, essential for the cytological diagnosis of *S. haematobium*. It is important to view such cases carefully since ova can be hidden among the contaminants and easily overlooked [Figure 6b]. *Schistosoma mansoni* can be differentiated from *S. haematobium* by its lateral spine.

The diagnostic yield in our study could probably have been higher with an alternative to conventional Pap smears by using liquid-based cytology (LBC) samples. In a population-based study among Costa Rican women who also have a high prevalence of cervical cancer, LBC was compared with conventional Pap smears. The samples processed via LBC were significantly more sensitive in detecting HSIL and other abnormalities.[26] Specimens from LBC testing could possibly also be tested for *Schistosoma* PCR and HPV subtypes.

In our study, the inclusion criteria required that all of the participants were or had been sexually active at some point in their lifetime to undergo the gynecological examination. The mean age of sexual debut was 16 and 163/394 (41.0%) had at least one child. SCA occurred across the age spectrum; the majority was ASCUS and LSIL 100/107 (94.3%). Seven cases of HSIL occurred between ages of 17 and 22 years, including one case of HSIL with possible invasion, which occurred at age 19. While studies have shown that at least low grade 96% of abnormalities in young women tend to regress within 36 months,[27] in contrast to this it has been shown that progression of squamous lesions occurs more rapidly in HIV-positive women.[28] It is not known if this will occur in individuals with schistosomiasis. In South Africa, cervical cancer is the second most prevalent cancer affecting women. A screening program does exist for women at the ages of 30, 40, and 50. Much of the screening is done in family planning clinics or antenatal clinics on symptomatic women.[29] Many women are unaware of the routine screening program.[29] As a consequence, women usually reach healthcare facilities when they have advanced or terminal cervical cancer, which could have been prevented by early diagnosis.

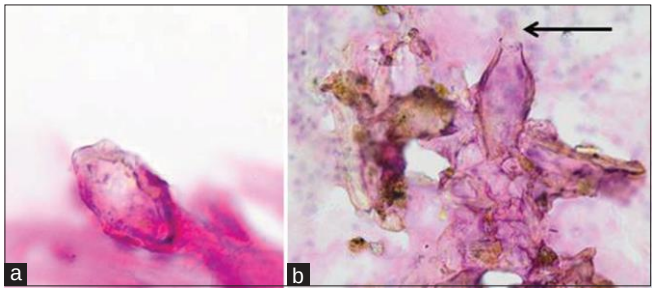

Figure 6: (a) Cervical smear, magnification (Papanicolaou stain, ×40). A contaminant easily misdiagnosed for *Schistosoma haematobium* ova. Note the lack of a diagnostic terminal spine. (b) Cervical smear, magnification (Papanicolaou stain, ×40). Arrow: *Schistosoma* ovum with a terminal spine found among contaminants

The long-term outcome of HSIL in a young population who are at risk for contracting HIV and schistosomiasis raises public health issues. The young age of women with HSIL makes it important for policy makers to consider increasing efforts to educate women on the dangers of cervical cancer and to screen young women in *Schistosoma*-endemic areas for SCA.

CONCLUSION

Pap smear cytology is a simple diagnostic test, that is, relatively cheap and noninvasive. It can be widely used for the detection of SCA and may also detect FGS. In our study, the "high tech" real-time PCR detects *Schistosoma* DNA, reflecting FGS, in cervical lavage almost 6 times more often than Pap smears. In this limited sample size, there was no significant association between cervical atypia and the tests which were positive for genital schistosomiasis. It is, however, important to note that SCA consistent with at least HPV infection was detected in 27% of this young population who are not conventionally screened for cervical cancer, some of them with advanced atypia. A shift in cytology sampling to LBC could open for a triage of cytology, HPV DNA testing, and *Schistosoma* PCR; this will be considered in future.

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COMPETING INTERESTS STATEMENT BY ALL AUTHORS

The author(s) declare that they have no competing interests.

AUTHORSHIP STATEMENT BY ALL AUTHORS

PP, LvL, MT, MS, SGZ, EK, BR, and EFK have all participated in this original research and the process of writing the manuscript. They all concur with its submission and subsequent revisions by the corresponding author, Pavitra Pillay. Each author acknowledges that this final version was read and approved.

ETHICS STATEMENT BY ALL AUTHORS

This study was conducted with approval from and permission from the Biomedical Research Ethics Administration, University of KwaZulu-Natal (Ref BF029/07), the Departments of Health, and Education of KwaZulu-Natal (Ref HRKM010-08), the Norwegian Ethics Committee, Regional Committee for Medical and Health Research Ethics, South Eastern Norway (Ref 469-07066a1.2007.535), and the European Group on Ethics in Science and New Technologies (Ref IRSES-2010:269245). Authors take responsibility to maintain relevant documentation in this respect.

LIST OF ABBREVIATIONS (In alphabetic order)

CIN - Cervical Intraepithelial Neoplasia
CIs - Confidence Intervals
Ct - Cycle Threshold
FGS - Female Genital Schistosomiasis
HPV - Human Papillomavirus
HSIL - High-Grade Squamous Intraepithelial Lesion
ITS - Internal Transcribed Spacer
LBC - Liquid-Based Cytology
LSIL - Low-Grade Squamous Intraepithelial Lesion
Pap - Papanicolaou
PCR - Polymerase Chain Reaction
SCA - Squamous Cell Atypia.

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EDITORIAL/PEER- REVIEW STATEMENT

To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was conducted under a double-blind model (authors are blinded for reviewers and vice versa) through automatic online system.

6.0 CHAPTER SIX

6.1 Paper 3

Detection of *Schistosoma* DNA by real-time PCR in vaginal lavage and urine: a comparison between five female African study populations originating from regions with distinct *S. haematobium* and *S. mansoni* transmission patterns.

Pillay Pavitra, Downs Jennifer, Brienen Eric AT, Taylor Myra , Kjetland Eyrun F and Van Lieshout Lisette.

In manuscript for submission to American Journal of Tropical Medicine and Hygiene

The purpose of this paper was to review diagnostic performance among comprehensive recent studies using microscopy, PCR urine and cervico-vaginal lavage and cytology (only comparing those which were *Schistosoma* positive) with the aim of showing the potential use of vaginal lavage as a diagnostic indicator for FGS in five regions in Africa each with distinct patterns of schistosomiasis prevalence. Since some of the regions were endemic for *S. mansoni,* the role of *S. mansoni* in FGS was investigated.

This explorative descriptive study compares schistosome real-time PCR analysis of 933 vaginal lavages of women from five different study populations among the following three countries in Africa; South Africa, Tanzania and Madagascar. In this paper the results of Schistosoma PCR in urine, stool, vaginal lavage, urine and stool microscopy and cytology are compared.

My role in this research was to participate in the analysis and writing of this manuscript using the PCR results in urine and cervico-vaginal lavage, urine microscopy and cytology results that were generated from my current research. Professor van Lieshout facilitated the writing of this collaborative manuscript since it was at her laboratory where the PCR analysis for the studies from Tanzania and Madagascar were conducted.

This study confirmed real-time PCR for the detection of *Schistosoma* DNA in gynecological samples to be a valuable diagnostic tool, in comparison to the classical Pap smears, to study the distribution of urogenital schistosomiasis at a population level. To elucidate the contribution of *S. mansoni* in the pathogenesis of FGS however there is a need for a species-specific ultra-sensitive diagnostic tool. This paper aims to contribute to the body of knowledge on cervico-vaginal lavage PCR in the diagnosis of FGS.

Paper 3

Detection of *Schistosoma* DNA by real-time PCR in vaginal lavages and urine: a comparison between five female African study populations originating from three countries with distinct *S. haematobium* and *S. mansoni* transmission patterns

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Key words: Female genital schistosomiasis, Laboratory Diagnostics. Laboratory Diagnostics, real-time polymerase chain reaction (PCR), Cervico-vaginal lavage,

List of abbreviations

DNA	deoxyribose nucleic acid
CVL	cervico-vaginal lavage
epg	eggs per gram
FGS	female genital schistosomiasis
HIV	Human Immunodeficiency Virus
PCR	polymerized chain reaction
US	urinary schistosomiasis

ABSTRACT

Background: Early life genital symptoms such as contact bleeding, homogenous sandy patches, grainy sandy patches and rubbery papules are important manifestations of *Schistosoma haematobium* infection in girls and these seem to increase HIV susceptibility when women with urogenital schistosomiasis become sexually active. Although lesions, seen during colposcopy, facilitate the diagnoses of female genital schistosomiasis (FGS), they are not pathognomonic. Additional detection of *Schistosoma* DNA in gynecological samples can contribute to the diagnosis, at least at a population level, but this procedure needs further investigation. This explorative descriptive study compares schistosome real-time PCR analysis of 933 vaginal lavages of women from five different study populations. **Methods:** *Schistosoma* DNA levels were determined by real-time PCR in Cervico Vaginal Lavages (CVL) of 422 women from Mwanza, Tanzania, of which 112 originate from a *S. haematobium* endemic region (site 1A) and 310 from a region where *S. mansoni* is endemic (site 1B). Findings were compared with data of CVL collected from 394 women from a *S. haematobium* region in South Africa (site 2) as well as of 79 pre-selected *S. haematobium* positive women and 39 urine microscopy negative women, from two regions with different transmission levels in Madagascar (respectively, site 3A and site 3B). While urine and stool microscopy and gynecological procedures varied between study protocols, standardized DNA isolation and *Schistosoma* real-time PCR was performed in a centralized laboratory. **Principle findings:** *Schistosoma* DNA in CVL samples generally reflected the distribution and intensity of urine PCR determined *S. haematobium* infection between the selected study groups, with the exception of 2.9% DNA positives in the *S. mansoni* endemic group of Tanzania (1A) who were negative in their urine samples. In groups with high *S. haematobium* infections levels, the PCR demonstrated a substantial number of *Schistosoma* DNA containing stool samples while being microscopy negative. Potentially this may be due to leakage of urine during defecation. **Conclusion:** This study confirmed real-time PCR for the detection of *Schistosoma* DNA in gynecological samples to be a valuable diagnostic tool, in comparison to the classical Pap smears and to study the distribution of urogenital schistosomiasis at population level. To elucidate the contribution of *S. mansoni* in the pathogenesis of FGS there is a need for a species-specific ultra-sensitive diagnostic tool.

INTRODUCTION

Female genital schistosomiasis (FGS) is a neglected disease of poverty for which millions of women and girls are at risk especially in developing countries (Kjetland, Leutscher and Ndhlovu 2012). The causative agent for FGS is *Schistosoma haematobium*, however little is known about the role of *S. mansoni* in FGS endemic areas (Kjetland, Leutscher and Ndhlovu 2012). FGS has been associated with an increased risk for HIV acquisition which is a consideration that is often overlooked in developing countries (Mbabazi *et al.* 2011)

One of the major challenges however, is to make a direct diagnosis of FGS, given that schistosomiasis occurs most frequently among young women and girls who may also be at risk for HIV. Taking a direct biopsy from the genital tract is not applicable for this age group. Diagnostic tests for schistosomiasis have been described using microscopy in urine and stool samples as well as with *Schistosoma* PCR (Obeng *et al.* 2008; Meurs *et al.* 2015; Utzinger *et al.* 2015). However, it is also suggested that urine analysis alone may not be the ideal indicator for FGS (Poggensee *et al.* 1998). The use of Pap smear cytology has also been shown to have a low sensitivity, despite the Pap smear being a direct sample from the cervical mucosa causing minimal epithelial disruption (Pillay 2015 submitted); (Poggensee and Feldmeier 2001);. Results from an earlier study investigating *Schistosoma* DNA in cervico-vaginal lavage (CVL) samples had limitations since the samples examined were frozen for several years and since the *Schistosoma* PCR technique has also evolved (Kjetland *et al.* 2009). In more recent studies, however *Schistosoma* DNA has been detected in CVL of women living in *S. haematobium* endemic areas and has shown clear correlation with genital symptoms (Randrianasolo *et al.* 2015); (Pillay 2015, submitted).

In this paper we review the diagnostic performance among comprehensive recent cross sectional FGS surveys using microscopy, *Schistosoma* PCR in urine and more specifically CVL samples from three different countries in Africa namely Tanzania, South Africa and Madagascar, each with distinct patterns of schistosomiasis. The main aim of these studies was

to determine the prevalence of FGS and contribute to knowledge about FGS diagnostics especially from regions with low transmission intensities. The possibility of FGS in an *S. mansoni* endemic area is also considered. Also given that *Schistosoma* DNA in CVL has been found to be an acceptable laboratory indicator of FGS; (Kjetland *et al*. 2009; Randrianasolo *et al*. 2015); (Pillay 2015, submitted), we further explore the presence of *Schistosoma* DNA in CVL, including samples collected in a *S mansoni* endemic region.

MATERIALS METHODS

Study Population, ethics, recruitment and sampling: *Schistosoma* DNA levels of 993 CVL were evaluated and compared to the *Schistosoma* DNA levels in urine and where available with *Schistosoma* DNA levels in feces. The flow chart enlisting the study sites is presented in Figure 1.

Site 1. The Tanzania study was nested within a larger cross sectional survey conducted during 2009-2010 at eight rural primary clinics in Mwanza, south of Lake Victoria. Two of the clinics were located in a region with predominantly *S. haematobium* transmission, while the six other clinics were located more to the North, closer to Lake Victoria, where mostly *S. mansoni* infections were seen. Ethical permission was received from the Bugando Medical Center, the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania and the Institutional Review Board of Weill-Cornell Medical College. An informed consent was part of the inclusion procedure and menstruating women were excluded from participation (Downs *et al.* 2011). Full details of the original study site, the ethical aspects and the followed procedures have been described elsewhere (Downs *et al.* 2011; Downs *et al.* 2012). In brief, out of the 550 eligible female visitors to a free cervical cancer screening program who were invited to participate in the study, 422 completed the required study procedures and could be tested for the presence of *Schistosoma* DNA in their lavage, urine and stool samples (Figure 1). This group consisted of 310 women from the *S. mansoni* predominant northern region, named site 1A, and 112 women from the *S. haematobium* predominant southern region, named site 1B. The median age of group of 1A was 30 years (range 13-60) and of 1B was 27 years (range 18-45). Samples were collected and examined as follows. During gynecological examination, CVL samples were taken by washing the surface of the cervix three times with normal saline and collecting 2 mL aliquots. In addition Pap smears were made using a plastic spatula and these were stained with 0.5% Trypan Blue and examined for the presence of *Schistosoma* eggs. Participants were requested to deliver a single urine and stool sample, which were examined for *Schistosoma* eggs by experienced microscopists, using respectively

a urine filtration procedure and a Kato-Katz smear examination as described in previous publications (Downs *et al.* 2011) An aliquot of approximately 1.0 -2.0 mL of each urine and stool sample was transferred to a cryotube on the day of collection and together with the CVL aliquots stored at -80^0 C. All samples were transported frozen to the Netherlands for *Schistosoma* DNA isolation and detection. PCR outcomes of a small subgroup of 33 *S. haematobium* infected women have been described before in order to specifically study the effects of Praziquantel treatment (Downs *et al.* 2013).

Site 2. For comparison *Schistosoma* PCR data were included from a cross sectional study performed in South Africa during 2010-2012 among young women living in rural KwaZulu-Natal, in a region in which the *S. haematobium* prevalence among girls aged 10-12 years was found to be 31.8%, based on microscopic examination of urines collected over three consecutive days (Hegertun *et al.* 2013; Pillay *et al.* 2014). Ethical approval was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal and the Norwegian ethics committee as well as the European Group on Ethics in Science and New Technologies. The Departments of Health and Education of KwaZulu-Natal gave permission. An informed consent was part of the inclusion procedure and pregnant women and virgins were excluded from participation. Full details of the study design, ethical aspects, sample collection and major outcomes have been described elsewhere (Holmen *et al.* 2015) (Pillay, 2015 submitted). In brief, 394 out of the 1200 eligible women, ranging in age from 16 to 23 years (median 19 years) met the inclusion criteria and completed all required procedures (Pillay, 2015 submitted). CVL samples were taken during gynecological examination by spraying 10 mL of saline on the vaginal wall and cervix twice, whereupon it was drawn back into a syringe and divided amongst 2 mL cryotubes. Pap smears were collected by using a wooden spatula and thereafter stained via the Papanicolaou technique and examined by a trained cytologist for the presence of *Schistosoma* eggs in addition to other cytological entities. Participants were requested to deliver a single urine sample, of which two times 10 mL were examined for *Schistosoma* eggs by experienced microscopists as previously described (Pillay *et al.* 2014) An aliquot of

approximately 1.0 mL of urine was transferred to a cryotube on the day of collection and together with the CVL aliquots stored at -80^0 C until transportation. In this study no stool samples were collected, as *S. mansoni* was found to be close to absent in the region after performing microscopy and real-time PCR analysis on approximately 600 stool samples of young school girls (unpublished data); (Pillay *et al.* 2014).

Site 3. Finally, *Schistosoma* PCR data were used from an FGS-study conducted in 2010 among rural farming communities across five villages in the district of Miandrivazo in the western part of Madagascar. Four of these villages were situated in a region known to be hyper-endemic for *S. haematobium*, with a prevalence >50% (site 3A), while the fifth village was situated in a region with low *S. haematobium* endemicity, showing a prevalence below 20% (site 3B) (Randrianasolo *et al.* 2015). Eligible women were stratified into different categories based on the location of their village and the outcome of a pilot survey on the prevalence of *S. haematobium* performed some months before actual sample collection took place. Within the hyper-endemic villages 79 women, age ranging from 15-33 years (median 20 years) were selected, based on the average urinary *S. haematobium* egg excretion at microscopic examination of three consecutive urines, being either highly infected, i.e. > 50 eggs per 10 mL of urine (n=40), or infected with a low worm burden, i.e. <20 eggs per 10 mL of urine (n=39). In total 38 urine microscopy negative women from the low *S. haematobium* transmission site 3B were included, with ages ranging from 15-35 (median 23 years). The Ethics Committee at the Ministry of Health in Madagascar granted ethical approval for this cohort. An informed consent was part of the inclusion procedure and pregnant women and virgins were excluded. Full details on the study design, ethical aspects, sample collection and major outcomes have been described elsewhere (Randrianasolo *et al.* 2015).

In brief, samples at site 3 were collected and examined as follows. CVL samples were taken during gynecological examination by spraying 10 mL of saline on the vaginal wall and cervix in total five times, whereupon it was drawn back into a syringe and divided amongst 2 mL cryotubes. Pap smears were made by using an endocervical brush and after staining with

Papanicolaou examined by a trained cytologist for the presence of *Schistosoma* eggs. Participants were requested to deliver a single urine and stool sample on the day of gynecological examination. Stool samples were examined for *Schistosoma* eggs by experienced microscopists, using a Kato-Katz smear examination (Randrianasolo *et al.* 2015). An aliquot of approximately 1.0 to 2.0 mL of each urine and stool sample was transferred to a cryotube. All collected samples were immediately stored at -80^0 C and transported frozen to the Netherlands for *Schistosoma* DNA isolation and detection.

DNA isolation and detection. DNA isolation and PCR reaction set-up steps were performed using a custom-made high throughput Hamilton robot platform (Hamilton Robotics GmbH, Germany) at the Leiden University Medical Centre in the Netherlands. Full details on the used DNA isolation procedures and set-up of the multiplex real-time PCR for semi-quantitative detection of the *Schistosoma* genus-specific internal-transcribed-spacer-2 (ITS2) target are given elsewhere (Obeng *et al.* 2008);(Downs *et al.* 2013);(Meurs *et al.* 2015). In brief, DNA was isolated using DNeasy 96 Blood & Tissue Kit spin columns and Phocin Herpes Virus-1 (PhHV-1) was added to the lysis buffer in each sample as an internal control. Negative and positive control samples for each parasite species were included in each PCR run. A CFX96 real-time detection system (Bio-Rad laboratories) was used for DNA amplification and detection. Cycle threshold (Ct) value results were analyzed using Bio-Rad CFX software (Manager V1.6.541.1028). These Ct-values represent the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, reflecting the parasite-specific DNA load in the tested sample. The amplification is considered to be hampered by inhibitory factors if the expected Ct value of 33 in the PhHV-specific PCR was increased by more than 3.3 cycles. DNA intensities of each species were arbitrarily classified as either high-intensity ($Ct<30$), medium-intensity ($30\leq Ct<35$), low intensity ($35\leq Ct<50$) or negative ($Ct=50$) (Pillay *et al.* 2014).

Data Analysis and statistical testing: PCR analysis was performed blinded from microscopy or other provided data. The results of the real-time PCR analysis were stored and grouped in a Microsoft Access database and imported into IBM SPSS 20.0 (Chicago, Illinois, USA) for statistical analysis. Descriptive statistics were used in this exploratory study. Categorical variables were summarized by frequency and percentage and presented using bar charts. Continuous variables for the diagnostic tests were summarized by median and interquartile range (IQR), (except when less than 10 total range), of all positive cases only using the Mann-Whitney rank-sum test. Proportions were compared using Fisher's exact test and McNemar statistical test. Two-sided hypotheses were assumed for all confidence intervals and *p*-values. Statistical significance was set at *p* value less than 0.05.

RESULTS

Figure 2 summarizes the prevalence and intensity of *Schistosoma* infection based on different diagnostic procedures performed on urine, stool and lavage.

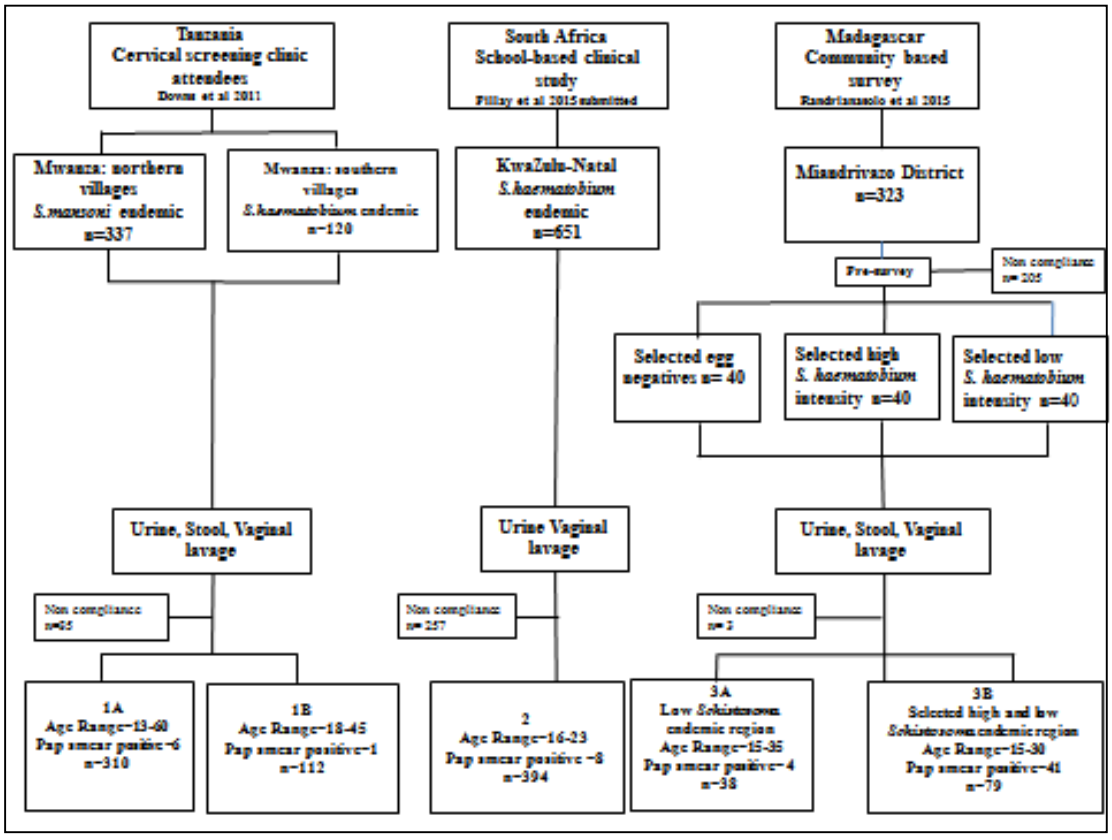

Figure 1. Participation flow and sampling among the 5 *Schistosoma* endemic regions. 1A and 1B represent northern (*S. mansoni* endemic) and southern (*S. haematobium* endemic) villages in Mwanza, Tanzania respectively, region 2 represents an *S. haematobium* endemic area in South Africa and Regions 3A and 3B represent low and high *S. haematobium* endemic regions of Madagascar. All 5 study areas had urine and vaginal lavage analysis and Pap smear analysis. Stool analysis was not included in the South African study region.

Tanzania urine examination. *S. haematobium* eggs in urine were demonstrated in 4 of the 310 examined women (1.3%) of the northern study area in Tanzania (site 1A) with counts ranging from 2-12 eggs/10mL. At the southern study area (site 1B) 13 of the 112 included women (11.6%) were positive in urine microscopy, with counts ranging from 1-54 eggs/10ml (median 8 eggs/10ml). Based on the urine PCR results, the detection rate was higher at both sites compared to microscopy, still showing *S. haematobium* to be more prevalent in the southern region (19.6%) compared to the northern region (8.1%). Accordingly, overall *Schistosoma* DNA loads in urine were significantly higher in the southern region (Mann-Whitney U=15.3, n=422, p=0.001).

Tanzania stool examination. *S. mansoni* eggs were seen at site 1A in 12.3% of the 310 Kato-smears examined, with intensity ranging from 12 to 917 epg (median 36 epg) while none of the 112 women from site 1B showed *Schistosoma* eggs in stool. PCR on these 112 stool samples showed DNA to be present in 10 (8.9%) of them, but with a median Ct of 34 the *Schistosoma* DNA load was generally low (Figure 2). Nine of these 10 women with *Schistosoma* DNA in stool had no detectable *Schistosoma* DNA in urine. Overall *Schistosoma* DNA loads in stool were significantly higher in the *S. mansoni* endemic northern region (Mann-Whitney U=21.8, n=422, p<0.001).

Tanzania lavage examination. PCR analysis of the lavages showed detectable *Schistosoma* DNA in 9/310 (2.9%) of the women from site 1A compared to 18/112 (16.1%) of the women from site 1B. Accordingly, overall *Schistosoma* DNA loads in lavage were significantly higher

in the *S. haematobium* endemic southern region (Mann-Whitney U = 15.0, n=422, $p<0.001$). (Figure 2)

Tanzania compared to 3 other sites. Either when analyzing microscopy egg counts or *Schistosoma* DNA loads in urine or lavage, the findings from the *S. haematobium* endemic site 1B were comparable to the finding of the South African site 2 (Mann-Whitney, n=506, $p>0.05$) (Figure 2).

On the other hand in all the *Schistosoma* positive DNA lavages from the *S. mansoni* endemic site, women had negative urine microscopy and were negative for urine *Schistosoma* DNA, only one woman showed *Schistosoma* eggs in stool microscopy. *Schistosoma* DNA in stool was found among 6 (66.6%) of these 9 women who were positive in their lavage samples.

Relation to cytology outcome. The relation between the outcome of the lavage PCR and histopathological examination differed per study site. In Tanzania 5 women of region 1A and one women of site 1B were reported with *Schistosoma* eggs after histopathological examination. None of them were positive in the lavage *Schistosoma* PCR, one showed *Schistosoma* DNA in urine and two of them in feces. On the other hand, in the South Africa study *Schistosoma* DNA was detected in the lavages of all 8 Pap smears with detectable *S. haematobium* eggs and the DNA levels of these 8 were significantly higher than the lavage DNA levels of those women negative in the cytology (Mann-Whitney U = 33.5, n=394, $p<0.001$).

In the low endemic region of Madagascar (site 3A) *Schistosoma* DNA was demonstrated in lavage in 1 of the 4 women where eggs were seen in the cytology smear, while in the selected *S. haematobium* infected women from site 3B, *Schistosoma* DNA was demonstrated in 28 of the 41 women where eggs were seen with cytology. Again lavage DNA levels of these women where eggs were seen in the Pap smears were significantly higher than the lavage DNA levels of those women negative in the cytology (Mann-Whitney U = 990.0, n=117, $p<0.001$).

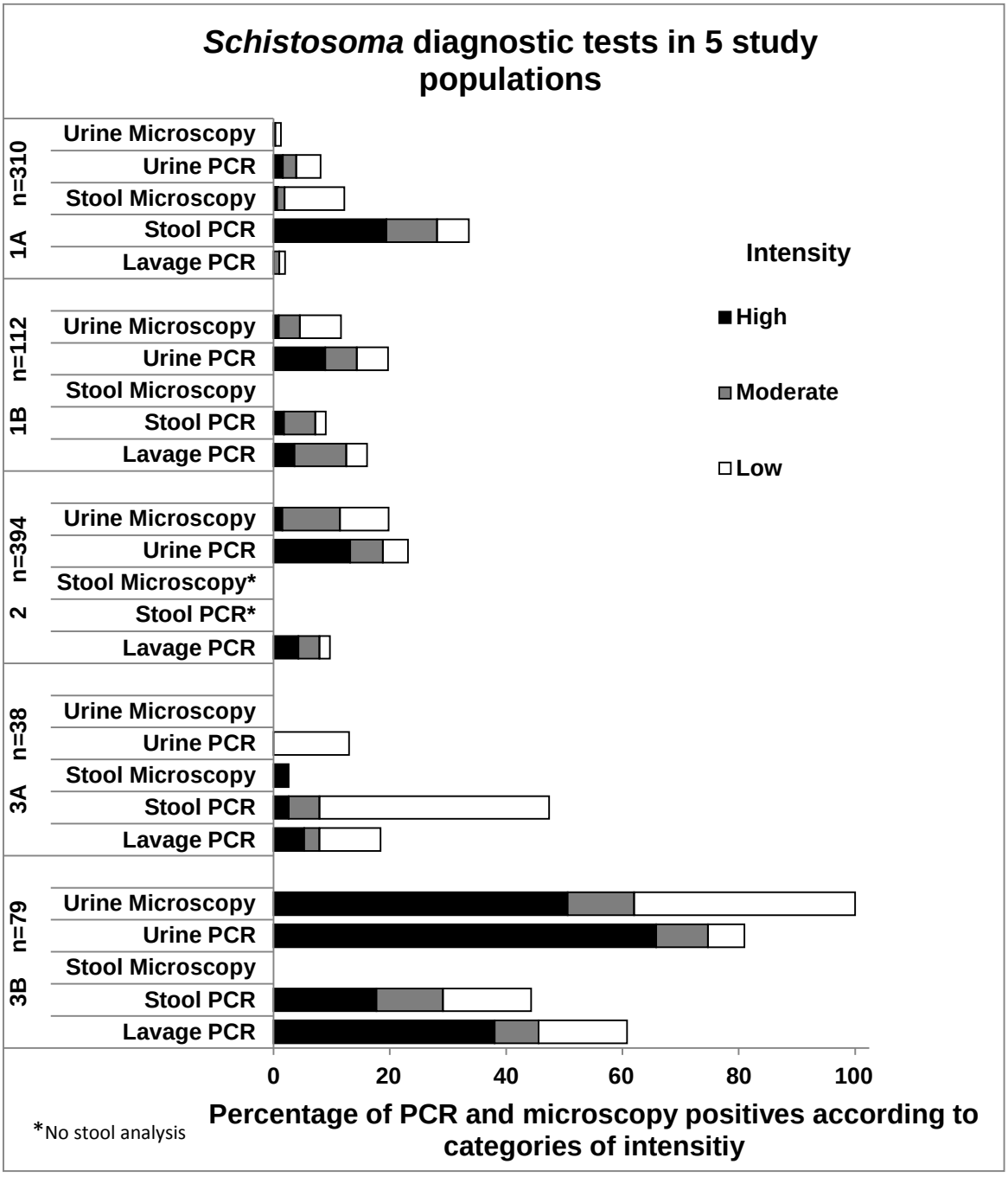

Figure 2. Summary of *Schistosoma* diagnostics according to DNA loads and egg intensities among the 5 study populations. Intensity in urine samples was defined as follows; high intensity >50 ova per 10mL, moderate intensity as 10-50 ova per 10mL and low intensity as <10 ova per 10mL. High intensity of eggs in stool samples was defined as >400 eggs per gram (epg), moderate intensity as 100-400epg and low intensity as <100epg. The *Schistosoma* DNA loads were defined as high-intensity (Ct<30), medium-intensity (30≤Ct<35) or low intensity (35≤Ct<50). Region 1A and 1B represent the *S. mansoni* and *S. haematobium* endemic areas in Tanzania respectively, region 2 represents the *S. haematobium* endemic area in South Africa and Regions 3A and 3B represent the low and high *S. haematobium* endemic regions of Madagascar respectively. Details of the populations are given in the text.

DISCUSSION

The quest for more sensitive and specific diagnostic tools in the diagnosis of FGS continues as more information on this neglected entity unfolds. It is clear that non-invasive FGS diagnosis among women who are also at risk for HIV needs to complement colposcopy in the rural settings.

PCR in urine and stool. While traditional microscopy is cheaper and more readily available, it also poses challenges like observer variation and decreased sensitivity in detecting low intensity infections. The *Schistosoma* PCR as detected amongst the study areas in urine and stool samples showed higher detection rates generally, in comparison to urine and stool microscopy respectively, as has been noted previously (Obeng *et al.* 2008; Cavalcanti *et al.* 2013). While PCR has better specificity and sensitivity, the major disadvantage is the cost, and field applicability (Cavalcanti *et al.* 2013). Another limitation of the PCR assay used in this study is that it is not able to distinguish between *S. haematobium* and *S. mansoni*.

US vs FGS. Urinary tract involvement in FGS has been studied and it has been reported that women could have urinary schistosomiasis with or without genital schistosomiasis (Poggensee *et al.* 1998); (Swai *et al.* 2006). While urine microscopy and urine PCR might show improved sensitivity and specificity for the detection of schistosomiasis, it might not be the best tool to assess genital schistosomiasis. In the South African study, despite the correlation of urine PCR and CVL PCR, some cases were positive in CVL only, supporting the notion that schistosomiasis could occur in the genital tract only, this raises a question about whether urine PCR is the best indicator of FGS (Pillay submitted 2015). However, in rural areas where resources are lacking, and gynaecological analysis is not always feasible, urine may be a more practical sample to obtain although not ideal.

Comparison of CVL results among 5 regions. *Schistosoma* DNA in CVL was detected in all 5 study sites, although providing variable results among the regions. In this comparative study

the CVL results correlated to the Pap results (despite the prevalence in Pap smears being generally much lower, except in Madagascar- Region 3B) and should be considered as an additional diagnostic tool for FGS at a population level. CVL results were much more sensitive than the Pap smear results in all of the regions except one (Region 1A).

***S mansoni* and FGS.** While there is evidence that *S. haematobium* is the causative agent for FGS, little is known about the effect of *S. mansoni*. The *S. haematobium* worm is known to prefer residence in the pelvic venous plexuses therefore the ova tend to become lodged in urogenital tissues, while *S. mansoni* prefers the portal venous system and is usually found in the intestinal tract (Colley *et al.* 2014). It has been suggested that while *S. mansoni* ova are usually lodged in the liver or intestines, this parasite can also be found in the genital tract (Feldmeier *et al.* 1998).

In regions in Africa, it has been found that there is overlap between the geographical areas in which both species are endemic and at risk for co-infections (Gryseels *et al.* 2006). There has however been limited information about mixed infections. In a cross-sectional study conducted among two communities in Senegal it was found that 53% had mixed infections (Meurs *et al.* 2012). In an FGS study conducted in Tanzania *S. mansoni* was reported to be found in 13% of the women (Poggensee, Feldmeier and Krantz 1999). In the few recent case reports from Brazil, detection of *S. mansoni* in the fallopian tubes and ovaries has been described (Arruda *et al.* 2007; Cavalcanti *et al.* 2011; Gonçalves Amorim *et al.* 2014). In the present study, two thirds of the women from the *S. mansoni* endemic region who had positive *Schistosoma* CVL, also had positive *Schistosoma* DNA in their stool samples. This could support the finding that *S. mansoni* might be responsible for FGS especially in endemic regions as indicated by the case reports (Poggensee, Feldmeier and Krantz 1999; Cavalcanti *et al.* 2011).

CONCLUSION

In conclusion, *Schistosoma* PCR in urine and stool samples may be better diagnostic tools especially for mapping disease at population level, in light of the costs and logistics. CVL PCR is also a valuable tool for diagnosing genital schistosomiasis since it is more sensitive than conventional Pap smears. The potential for *S. mansoni* being implicated in FGS should not be underestimated especially in populations at risk for HIV as there are implications, from the association that has been reported in several studies. Further research towards developing a species specific point of care diagnostic tool that can be applied to urine, stool and cervical mucus is recommended for field applicable diagnosis of schistosomiasis to enable appropriate patient management.

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7.0 CHAPTER SEVEN

7.1 Paper 4

Liquid-based cytology as a tool for a more precise diagnosis and risk- assessment of cervical squamous cell atypia among young women from *Schistosoma* and HIV endemic populations in South Africa.

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In manuscript.

The purpose of writing this paper was to investigate further the value of laboratory tools for FGS. In this study we compared traditional Pap smears to liquid-based cytology (LBC) samples and found that there was a better cellular quality using LBC sampling. In this research it was found that more than two thirds 567(68.1%) of the population of young women had cervical squamous atypia. The atypia was also significantly associated with urogenital schistosomiasis. Possible risk factors for cervical atypia and schistosomiasis are also reported.

Young women aged 16-23 years from rural high schools in the north coast of KwaZulu-Natal were invited to participate in a study. Risk factors were identified through a questionnaire. Cytology Pap smears and Liquid based cytology (LBC) samples were investigated for *Schistosoma* ova and squamous cell atypia among 833 young women at baseline. Urine microscopy was done for the identification of *Schistosoma* ova. HPV genotyping was conducted in the LBC supernatant in a selection of 10 cases that were cytologically diagnosed with high grade squamous intraepithelial lesion (HSIL).

My role in this research was to conduct the cytology, and based on the results from the initial study among young women, I facilitated the change from traditional Pap smears to LBC sampling. This change in sampling improved the diagnostic quality of the results. LBC sampling is not only advantageous for cytology but DNA analysis is possible in the LBC residual. From a public health perspective, we consider including a risk analysis of the cervical atypia using HPV subtyping since this information could inform the future management of young women with cervical atypia. We have also proposed a sampling triage which consists of LBC cytology and HPV and possibly *Schistosoma* DNA analysis using the residual LBC sediment. This triage for women at risk for schistosomiasis and cervical atypia is novel and to our knowledge it has not been done among *Schistosoma* endemic populations.

Paper 4

Liquid-based cytology as a tool for a more precise diagnosis and risk-assessment of cervical squamous cell atypia among young women from *Schistosoma* and HIV endemic populations in South Africa.

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Key words: Female Genital Schistosomiasis, cervical squamous cell atypia, liquid-based cytology, HPV genotyping, risk assessment.

List of abbreviations

ASCUS	atypical squamous cells of undetermined significance
DNA	deoxy ribonuclease
CIN	cervical intraepithelial lesion
FGS	female genital schistosomiasis
HPV	human papillomavirus
HIV	human immune deficiency virus
PCR	polymerized chain reaction
LBC	liquid-based cytology
SIL	squamous intraepithelial lesion
LSIL	low grade squamous intraepithelial lesion.
HSIL	high grade squamous intraepithelial lesion
SCA	squamous cell atypia
S haematobium	*Schistosoma haematobium*
STIs	sexually transmitted infections

ABSTRACT

Background: Globally, Africa has the highest prevalence of HIV, schistosomiasis and cervical cancer. Female genital schistosomiasis (FGS) is hypothesized to be associated with both HIV and cervical cell atypia. We wanted to explore the correlation between schistosomiasis and cervical squamous cell atypia in a hot and humid rural area. We additionally wanted to compare two diagnostic methods for cytological evaluations [Pap smears and Liquid-based cytology (LBC)] and to explore DNA analysis in the residual LBC sample as a tool for risk assessment for high-risk HPV types. The residual LBC sample also has the potential to be used to test for *Schistosoma* DNA

Methods: In this cross sectional study, young women aged 16-23 years from rural high schools in KwaZulu-Natal were invited to participate in the study. Risk factors were identified through a questionnaire. Cytology Pap smears and LBC samples were investigated for *Schistosoma* ova and squamous cell atypia in 833 women at baseline. Urine microscopy was done for the identification of *Schistosoma* ova. In a small sub-population, HPV genotyping was conducted using residual LBC samples in a selection of 10 cases that were cytologically diagnosed with high grade squamous intraepithelial lesion (HSIL).

Results: The participants were sexually active from the mean age of 18 (SD 9.97), and more than half of them had at least one child. The majority (92.4%) relied on rivers as their primary water source. The *Schistosoma* prevalence detected cytologically and via urine microscopy was 12 (1.4%) and 178 (21.4%), respectively. Squamous cell atypia was detected in 567 (68%) young women of which 19 (2.3%) were diagnosed with HSIL. There were significant associations between the participants who were positive for any squamous cell atypia and those who had *S. haematobium* ova in Pap smears (OR= 5.6, *p=0.005*; 95% CI 1.6-21.0) and *S. haematobium* eggs in urine OR= 2.9, 95% CI 1.72 - 4.99, *p=0.005*). Majority (85.4%) (*p*=0.000) of the LBC samples were suitable for assessment as compared to 97.6% (*p*=0.000) of Pap smears which were sub-optimal. HPV genotyping done on the 10 HSIL cases using the residual LBC revealed a spectrum of HPV types, some of them high-risk. The samples were adequate for DNA analysis despite being stored at ambient temperatures for 12-18 months.

Conclusion: A significant association exists between urogenital schistosomiasis and squamous atypia in this young population. More than half of the women in this study population had cervical atypia and a small proportion also had high grade cervical lesions. Better diagnostic specimens were obtained using LBC sampling compared to Pap smears. Importantly, HPV genotyping in the residual LBC samples worked well in the 10 cases diagnosed as HSIL, many revealing high-risk HPV infections. In a single LBC sample, cervical cytology with identification of sexually transmitted infections, cervical atypia and HPV DNA-genotyping can

thus be used for risk assessment in the identification of which patients should have follow-up. In the wake of decreasing costs of LBC this might be a cost effective and sustainable solution for targeted intervention in rural Africa. Further research is needed to see if the LBC residual sample also could be used for testing *Schistosoma* DNA.

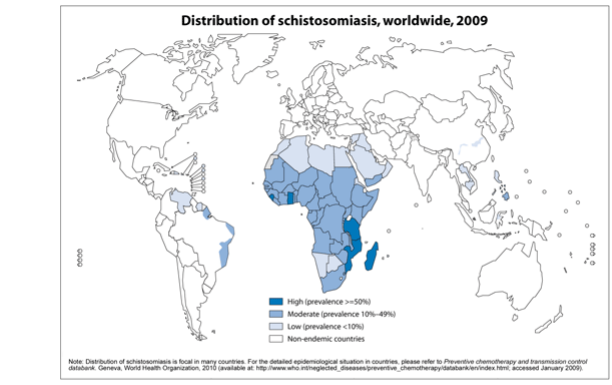
INTRODUCTION

Genital lesions in women caused by schistosomiasis have been identified for many years (Madden 1899; Youssef, Fayad and Shafeek 1970; Berry 1976; Poggensee and Feldmeier 2001; Kjetland *et al.* 2005). When ova of the parasite *Schistosoma haematobium* become lodged in the genital mucosa, they cause immunological and tissue changes. Female Genital Schistosomiasis (FGS) causes sandy patches, mucosal vascular abnormalities and contact bleeding that may render women more prone to acquiring HIV (Kleppa *et al.* 2014; Norseth *et al.* 2014).

Human papillomavirus (HPV) has a high prevalence in young women and is one of the major causes of cervical cancer (Schiffman *et al.* 2007). In *Schistosoma* endemic areas, it has been suggested that schistosomiasis could be a risk factor for acquisition and maintenance of HPV, thus being a co-factor for the development of cervical cancer, a major health burden in developing countries (Prabhakaran and Brown 2004; Kjetland *et al.* 2009). Africa has the highest global distribution of HIV, schistosomiasis and cervical cancer as is shown in Figure 1; (Organisation 2014; WHO 2015; World Health Organisation 2015).

It is suggested that schistosomiasis is mostly acquired in childhood. Associated changes in the cervical mucosa have been hypothesized to facilitate the development of cancer through early transmission of HPV (Poggensee and Feldmeier 2001). Diagnosing FGS among young women is challenging since the direct diagnosis with tissue biopsies are not applicable in young women who are also at risk for HIV in rural endemic areas.

The aim of this study was to explore the relationship between squamous cell atypia (SCA) and schistosomiasis in a young HIV endemic population, highlight the risk factors and investigate diagnostic tools that can be used in low resource areas. As part of that, we also wanted to compare traditional Pap smears and liquid-based cytology (LBC) and explore the possibilities of using HPV genotyping in residual LBC samples for risk analysis.



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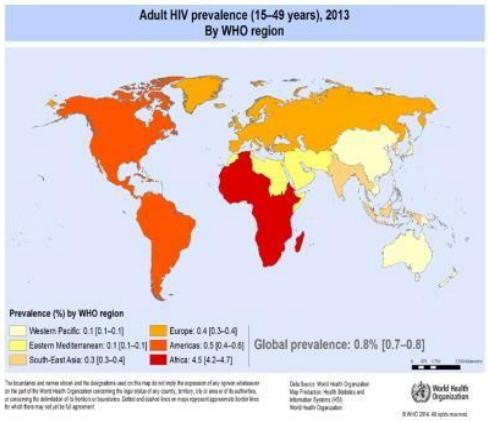

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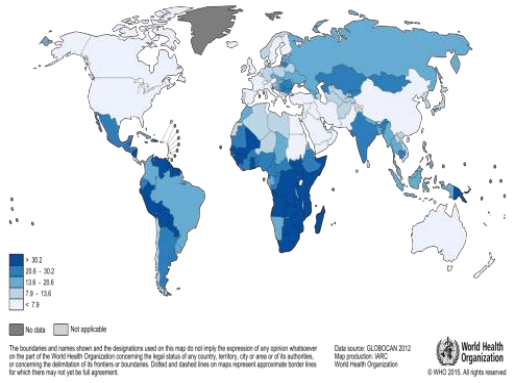

C

Figure1. Three maps showing global distributions of the following entities: (A) Schistosomiasis –showing mostly a moderate prevalence in most of Africa, (B) HIV- adults-15-49 years and (C) Cervical cancer incidence. Africa has the largest overlap and distribution of all three disease entities. (Organisation 2014; WHO 2015; World Health Organisation 2015).

MATERIALS AND METHODS

In a *Schistosoma* endemic area on the north coast of KwaZulu-Natal, high school females were invited to participate in a cross-sectional study as described previously (Kleppa *et al.* 2015; Randjelovic *et al.* 2015). The detailed description of the sampling and ethical considerations are described elsewhere (Holmen *et al.* 2015). Consenting participants underwent a semi-structured face-to-face interview in the local language isiZulu and urine samples for microscopy were collected and processed locally as described previously, prior to gynaecological examination (Pillay *et al.* 2014); (Pillay 2015- submitted). Traditional Pap smears were collected by scraping a wooden spatula in the cervix and the fornices, then spray-fixed using a commercial cytological fixative for preservation in the first period in 510/833 participants. Based on the initial limited quality of cytology results, Liquid-based cytology (LBC) using the SurePath™ (Franklin Lakes, NJ, USA) was used for the remaining 323/833 samples.

The cytology screening was done blinded to any of the other laboratory results or clinical findings. Smears were examined microscopically in a systematic manner for all cytological entities that are expected to be found in cervical smears (Solomon *et al.* 2002). The diagnosis of *Schistosoma haematobium* was based on finding intact ova within shells, empty shells with terminal spines and calcified or dead ova (Berry 1966). Epithelial dysplasias were graded according to the Bethesda System (Solomon and Nayar 2004) and reported as described elsewhere (Pillay 2015- submitted). Ten HSIL samples were selected for HPV genotyping using the residual LBC sample that had been stored at ambient temperature for 12-18 months. The HPV genotyping was conducted at the Norwegian HPV Reference Laboratory in Akershus University Hospital using the WHO-validated hybridization based Luminex protocol (Schmitt *et al.* 2006; Söderlund-Strand, Carlson and Dillner 2009) which is used in the surveillance of the effectiveness of the HPV vaccine in Norway. The method detects the following 37 HPV types: 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 67, 68, 69, 70, 73, 74, 81, 82 ,83, 86, 87, 89, 90, 91. In addition two genetically distinct versions

of HPV35, 58 and 68 are detected, respectively. A separate β-globin PCR was used for the detection of human DNA to check the sample adequacy.

Statistical analyses:

IBM SPSS 20.0 (Chicago, Illinois, USA) was used for statistical analysis. Descriptive statistics were used in the explorative study. Categorical variables were summarized by frequency and percentage and presented using bar charts. For the diagnostic tests the continuous variable output was given for the positive cases only. Inferential statistics were done using the Fisher's Exact Test. The odds and risk ratios and the corresponding 95% confidence intervals were determined for 2 × 2 cross-tabulations. All *p*-values were two-tailed values and *p*-values less than 0.05 were considered as significant.

RESULTS

Study Population: The flow of participation in which 833 young women gave urine samples, underwent gynaecological examinations and had cytology performed (Figure2). Traditional Pap smears were done in 510/833 participants; the remaining 323/833 had LBC samples collected. Table 1 shows the risk factors reported in the questionnaire and that 742 (89%) of these young women reported water contact with rivers.

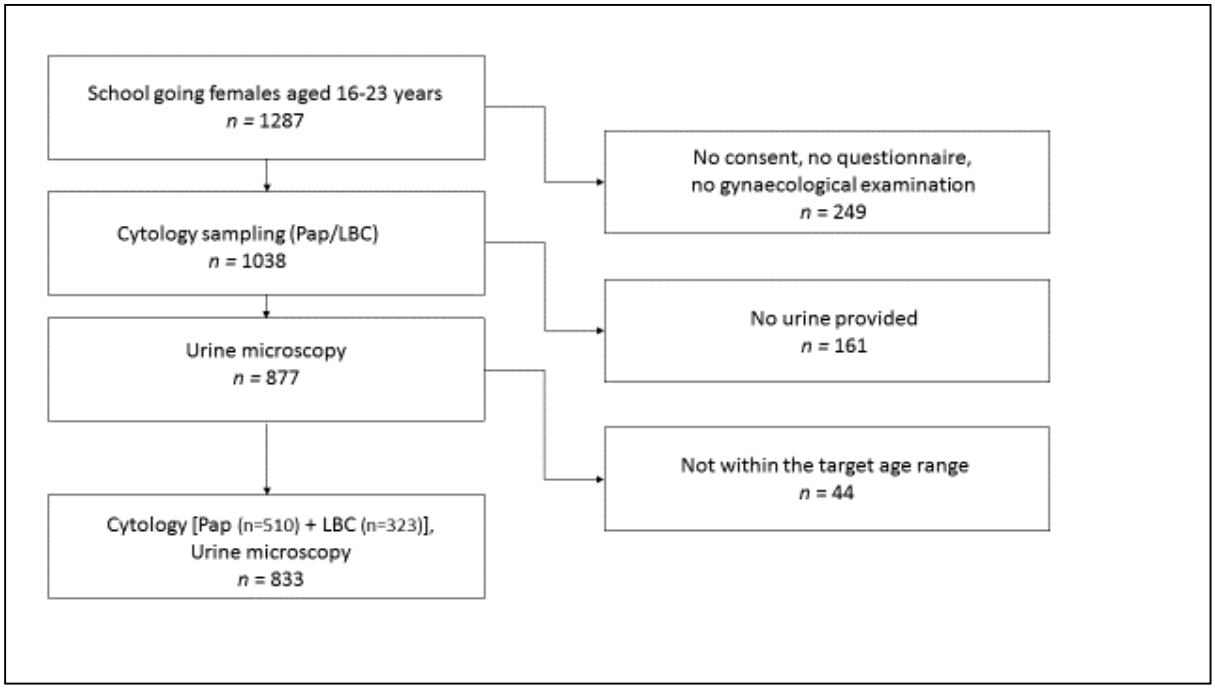

Figure2. Flow chart detailing study participation and adherence among the participants

Table1: Participant characteristics

Characteristics	Number	(%)	Standard Deviation
Study population	833		
Age (years)			
Mean	19		1.78
Reported in interview			
Mean age of sexual debut	18		9.97
Number of children alive			
None	288	(34.6)	
One	528	(63.0)	
Two	7	(1.0)	
Types of risk water body contact:			
None	30	(3.6)	
River	770	(92.4)	
Dam	38	(4.6)	
Standing water	6	(0.7)	
Several types	17	(2.0)	
Treated for Bilharzia Previously Baseline			
No	317	(38.1)	
Yes	151	(18.1)	
Unsure	365	(43.8)	

Cytology: Of the Pap smears, 97.6% (498/510) (p=0.000) were sub-optimal and for the LBC samples 85.4% (267/323) (p=0.000) were satisfactory for evaluation. The most common reasons why Pap smears were sub-optimal for cytological assessment included the following: thick smears, poor preservation and cells obscured by blood. The squamous cell atypia detected via traditional Pap smears was 271/833 (32.5%) as compared to that detected using LBC sampling which was 296/833 (35.3%). *Schistosoma* ova were detected in 12 (1.4%) cases via cervical cytology (Table 3). Five of the 12 cases were seen in Pap smears and 7 in LBC preparations. In the total population of 833, *S. haematobium* ova were detected in only 5/510 (0.01%) cases using Pap smears compared to 7/ 323 (2.2%) LBC samples. Of the 12 cases that were *Schistosoma* positive on cytology, 5 (41.7%) were negative on urine microscopy. Ten of the 12 (83.3%) young women with cytologically detected *Schistosoma* ova reported rivers as

their main source of water contact. Only 3 (25%) of them reported having received treatment for schistosomiasis before. Squamous cell atypia was diagnosed in 10/12 (83.3%) young women with *Schistosoma* ova in their Pap smears.

Table 3 Twelve Cases positive for *Schistosomiasis* by cytology compared to urine Microscopy, squamous cell atypia, participant age and cytology sampling type

n	**Urine microscopy Ova per40x field**	**Cytology Schistosoma Positive Baseline**	**Specimen type**	**SCA baseline**	**Follow up**	**Specimen type**	**Cytology Schistosoma Positive Follow-up**	**SCA follow up**	**Age**
1	Negative	Positive	Pap	NEG	NO	n/a	n/a	n/a	18
2	Negative	Positive	Pap	ASCUS	NO	n/a	n/a	n/a	18
3	Negative	Positive	LBC	LSIL	NO	n/a	n/a	n/a	17
4	Negative	Positive	LBC	ASCUS	NO	n/a	n/a	n/a	22
5	Negative	Positive	LBC	LSIL	NO	n/a	n/a	n/a	21
6	Positive (4.00 Ova)	Positive	Pap	NEG	NO	n/a	n/a	n/a	20
7	Positive (3.00 Ova)	Positive	Pap	ASCUS	NO	n/a	n/a	n/a	20
8	Positive (2.50 Ova)	Positive	LBC	ASCUS	NO	n/a	n/a	n/a	23
9	Positive (5.50 Ova)	Positive	LBC	LSIL	NO	n/a	n/a	n/a	17
10	Positive (140.5 Ova)	Positive	LBC	ASCUS	NO	n/a	n/a	n/a	16
11	Positive (27.5 Ova)	Positive	LBC	LSIL	YES	LBC	POS	LSIL	19
12	Positive (20.00 Ova)	Positive	Pap	ASCUS	YES	LBC	POS	HSIL	19

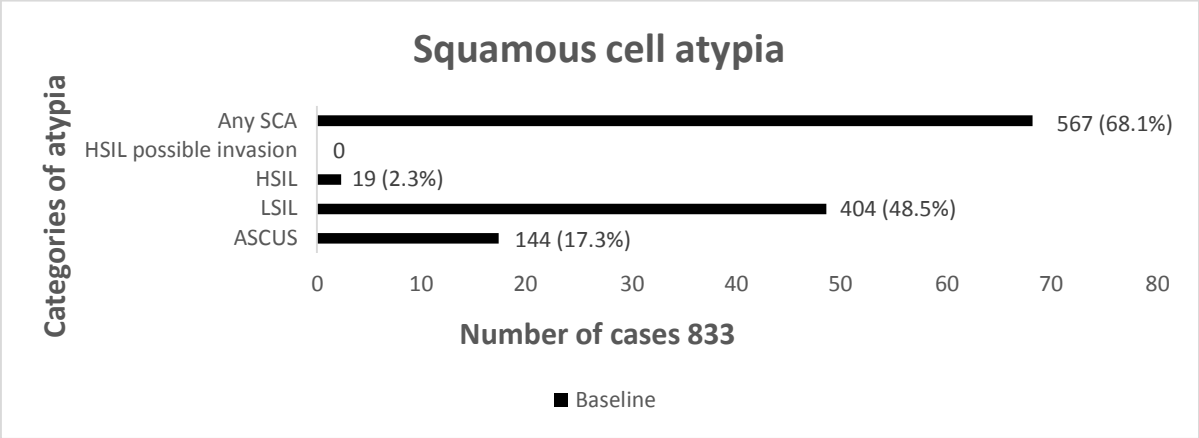

Figure 3. Distribution of squamous cell atypia (SCA) overall and as per specified categories high grade squamous intraepithelial lesion (HSIL), low grade squamous intraepithelial lesion (LSIL) and atypical cells of unknown significance (ASCUS)] in young women aged 16-23.

There was a significant association between the participants who were positive for any squamous atypia and those who had *S. haematobium* eggs in Pap smears (OR= 5.8, 95% CI 1.61-21.00, *p=0.005*) or *S. haematobium* eggs in urine (OR= 2.9, 95% CI 1.72 - 4.99, *p=0.005*) at baseline. Most of the squamous atypia was in the low grade squamous intraepithelial lesion (LSIL) category which refers to active HPV infection or CIN1 was found at baseline (Figure 3). Nineteen (1.4%) of the young women were diagnosed cytologically with HSIL. HPV genotyping results from 10 LBC samples with cytologically detected HSIL are shown in Table 4. Among the 10 HSIL cases *Schistosoma* ova were not detected via cytology, but 4/10 (40%) were however positive for urinary schistosomiasis.

Table 4. HPV Genotyping, age and urine microscopy results among 10 cases of HSIL

Age	Urinary Schistosomiasis	HPV Genotype
22	Positive	51, 53
16	Positive	6, 18, 66
16	Negative	6, 16
17	Positive	33, 35
18	Negative	16, 40, 59
19	Positive	18, 31
19	Negative	82
20	Negative	35, 68, 82
20	Negative	18, 31, 58, 66
23	Negative	16,82

Urine microscopy: The urinary schistosomiasis prevalence was 178/833 (21.4%), with a mean egg count of 7.2 eggs/10mL and the range was 0.01-680.50 eggs per 10mL. More than 50 eggs/10mL were found in 23/178 (13.0%) and less than 50 eggs/10mL were found in the remaining 155/178 (87.0%). There was a significant association between the two testing methods used: *Schistosma* detection in cytology and urinary schistosomiasis (OR = 5.3, p = 0.005; 95% CI 1.66 – 16.97).

DISCUSSION

FGS, cervical cancer and HIV affect women and girls in developing countries. Evidence suggesting the link between HIV and schistosomiasis are discussed in several studies (Mbabazi *et al.* 2011; Jourdan 2013; Kleppa *et al.* 2014).The link between schistosomiasis and cervical cancer has been more challenging to establish.

Population characteristics and risk factors: In South Africa the highest mortality from cervical cancer occurs among Black women (De Abreu, Horsfall and Learmonth 2013). Based on their general characteristics of environment, age, poverty, lack of knowledge of risk factors and lack of knowledge on appropriate health care, our study population appeared no different from other South African rural women who experienced several barriers towards cervical cancer screening. This results in women mainly being diagnosed in advanced stages of cervical cancer highlighted earlier (Learmonth, van Vuuren and De Abreu 2015).

Sexually transmitted HPV infection has been implicated in almost 100% of invasive cervical cancers (Walboomers *et al.* 1999). Other established risk factors include early onset of sexual activity, high number of lifetime sexual partners, parity, young age at full term pregnancy, use of oral contraceptives, immunosuppression and smoking (Berrington and Green 2007). More than half of our study population had at least one child which indicates that there were engaging in unprotected sex. The majority of atypia was found in the LSIL category which comprises active HPV (koilocytosis) and histologically CIN1. It is known that HPV infections with or without cytological dysplasia can resolve spontaneously (Stanley 2006).

In developed countries, screening of young women is therefore a controversial topic since there may be a tendency to over-treat (Szarewski and Sasieni 2004). Infections with the high risk/carcinogenic genotypes of HPV more often lead to cervical cancers, including types 16, 18, 31, 39, 45, 52 and 58 (Walboomers *et al.* 1999).

The International Agency for Research on Cancer (IARC) has defined the following 12 types as carcinogenic (meaning that they have *sufficient* evidence for carcinogenicity) 16, 18, 31, 33,

35, 39, 45, 51, 52, 56, 58, 59 and types 26, 53, 66, 67, 68, 70, 73, and 82 have been identified as the group that has *limited* evidence for carcinogenicity (International Agency for Research on Cancer 2007). In the present study a challenge was to decide on the course of further management of cervical atypia in 68% of young women who are also at risk for HIV and schistosomiasis. It was therefore decided from a public health perspective to evaluate the carcinogenicity of HPV through using HPV genotyping among a subsample of 10 HSIL cases using the residual LBC samples.

Conventional Pap smears versus LBC: LBC is an advanced method since cells are collected and placed into a liquid preservative medium. The sample is further processed in the laboratory using an automated technique whereby the cells are concentrated into a 1cm circular area on the slide (Maharaj and Munthree 2007). This process is advantageous compared to conventional cytology since excess background material like blood and mucus are removed. When the cytology preparation has been made, further testing on the residual LBC sample is possible (Hutchinson *et al.* 1999). A previous study in which HPV DNA was analyzed using 21 day old residual LBC samples showed successful results (Dixon *et al.* 2010). Our samples were stored 12 to 18 months. In the present study, there was concern about the viability of the LBC samples since they were stored for an extended period in ambient temperatures. Despite this, the samples proved adequate for HPV DNA analysis. It was also of concern that of the 19/833 (1.4%) young women having HSIL, all 10 who were typed for HPV had one or more of the carcinogenic types.

Cytology detection of schistosomiasis: The detection of schistosomiasis using cytology was low as found previously in similar studies (Feldmeier, Helling-Giese and Poggensee 2001; Poggensee and Feldmeier 2001); (Pillay submitted 2015). The diagnostic yield using LBC however was higher than conventional Pap smears. Inclusion of LBC sampling improved the diagnostic quality of the cytology results in comparison to traditional Pap smears. This could contribute to the higher detection rate of cervical cell atypia. While an association of squamous

atypia with genital schistosomiasis in Pap smears and with urinary schistosomiasis was found, this should be viewed in context. The relationship between schistosomiasis and cervical cancer is complex, and while there could be a strong association among these entities, in this study it was not possible to prove causality or eliminate the confounders. Previous studies have shown that women with HIV have an increased risk for HPV infection and invasive cervical cancer compared to HIV negative women (Manzini 2001; Sasco *et al.* 2010).

CONCLUSIONS AND PUBLIC HEALTH PERSPECTIVE

LBC had several advantages over conventional Pap smears in this study. LBC sampling improved cytology diagnostic quality and yield and rendered the opportunity for additional molecular testing, shown by the HPV genotyping in a small subset. It is of interest to explore if *Schistosoma* DNA testing could be additionally done in the residual LBC stored at ambient conditions. If that can be done, such a diagnostic triage would greatly benefit health care possibilities in low resource areas. The disadvantage of LBC sampling is that it costs considerably more than conventional Pap smears and requires some automation for further processing. Despite the disadvantages, collecting LBC samples especially among studies conducted in rural settings, where opportunities for repeat Pap smears and follow up are challenging, might be more effective and of value in enhancing the diagnosis and identification of high-risk HPV DNA for further management.

Cytology was useful in revealing the extent of squamous atypia among this young population who is not routinely screened; a limitation is that it was not feasible to confirm these results using histology. While the solution to institute screening among a younger age group may not be practical considering that the current national South African cervical screening programme is finding it a challenge to implement among older women (Denny, Quinn and Sankaranarayanan 2006). HPV vaccination is also an option for consideration especially if targeting primary school aged girls (Anorlu 2008; Moodley *et al.* 2013). More research is

required on the prospect of screening women younger than the age of 30 especially if they have multiple risk factors such and have early onset of sexual activity in developing countries.

HPV genotyping is potentially beneficial as a complementary diagnostic indicator in identifying carcinogenic types and this can inform the further management of young women with HSIL. The long term benefit for endemic populations is to be educated about schistosomiasis and cervical cancer risks and given options for appropriate health care in order to reduce the prevalence of these preventable diseases.

In this study, we have shown that residual LBC material can be used for HPV genotyping in a non-frozen state, and we postulate that the same material could be used as a triage for *Schistosoma* PCR together with cytological analysis. In rural areas it would be beneficial to investigate cervical atypia and sexually transmitted infections together with HPV and *Schistosoma* DNA analysis, for risk assessment and management of cervical atypia and antischistosomal treatment.

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8.0 CHAPTER EIGHT

8.1 Paper 5-Short Communication

Preliminary findings of the effect of prior treatment with Praziquantel on squamous atypia are described in young women at risk for female genital schistosomiasis. Pavitra Pillay, Myra Taylor, Hashini N Galappaththi-Arachchige, Borghild Roald and Eyrun F Kjetland.

In manuscript.

The purpose of writing this short communication is to consider the study objective on the effect of anti-schistosomal treatment with Praziquantel on squamous cell atypia (SCA). At the onset of the research it was postulated that the mass drug administration (MDA) would be rolled out in the study communities and that we could measure the effect of cervical atypia in relation to mass treatment at baseline and post treatment. The MDA did not occur as planned since there were many logistical and community issues that were beyond the control of this study. Instead MDA was done in a few schools at the time of writing and it was not possible to determine who had received treatment.

My role in addressing this aspect of the research was to conduct the analysis of the cytology results at baseline. In order to attempt to address the research question, it was decided to use information pertaining to prior anti-schistosomal treatment received from the questionnaire data.

The findings allude to a possible benefit of prior treatment with less atypia found in the group that reported prior treatment, however the results are preliminary and were insufficient to completely address this objective. Further research on the effect of anti-schistosomal treatment is required.

Paper 5: Short Communication

Preliminary findings on the effect of prior treatment with Praziquantel on squamous atypia in young women at risk of female genital schistosomiasis

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Key words: Female genital schistosomiasis, Praziquantel, effects of treatment, genital lesions.

List of Abbreviations

HIV	human immunodeficiency virus
FGS	female genital schistosomiasis
SCA	squamous cell atypia
LBC	liquid-based cytology
LSIL	low grade squamous intraepithelial lesion
HSIL	high grade squamous intraepithelial lesion
WHO	World Health Organization
MDA	mass drug administration
HPV	human papillomavirus
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
SADOH	South African Department of Health

ABSTRACT

Background: Efforts to eradicate schistosomiasis using the anti-schistosomal drug Praziquantel have been undertaken over many decades, yet we are still faced with an estimated 260 million people at risk in Africa. Female genital schistosomiasis (FGS) is hypothesized to increase the risk of HIV and cervical cell atypia. Anti-schistosomal treatment could potentially reduce morbidity and prevalence of other diseases such as HIV infection, and perhaps, cervical lesions. Preliminary findings on self-reported anti-schistosomal treatment are described in relation to schistosomiasis and squamous cell atypia (SCA).

Methods: Young women aged 16-23 years from rural high schools in KwaZulu-Natal were included. Urine microscopy was undertaken and a questionnaire was administered. Cytology Pap smears and Liquid based cytology (LBC) samples were investigated for schistosomiasis and SCA from 833 young women.

Results: The participants were sexually active from a mean age of 18 years, more than half had at least one child 528 (63%) and the majority 770/833 (92.4%) relied on rivers as their primary water source. Only 151 (18.1%) of the young women reported having taken anti-schistosomal treatment previously. The *Schistosoma haematobium* ova detected cytologically and in urine microscopy was 12 (1.4%) and 178 (21.4%) respectively. SCA was detected among 567 (68%) of which, 19 (2.3%) were diagnosed with high grade squamous intraepithelial lesion (HSIL). Of those with *S. haematobium* in cytology and urines, 9/12 (75%) and 104/178 (58%) reported no previous treatment (or unsure) for schistosomiasis. The findings were non-significant. None of the participants who had HSIL had been treated for schistosomiasis previously.

Conclusion: Of the group that recalled having received Praziquantel treatment there was a lower prevalence of SCA and s*chistosomiasis* than among those who were unsure of prior treatment or not previously treated, the sample size was however small and further research is required.

INTRODUCTION

Urogenital schistosomiasis, affects approximately 260 million people from Africa (World Health Organisation 2015).The anti-schistosomal drug Praziquantel has been in use for decades yet schistosomiasis remains a public health burden (King 2015). Female genital schistosomiasis (FGS) is caused by damage to mucosal surfaces by the parasite *Schistosoma haematobium*. It is hypothesized that alterations to the genital mucosa could make women more susceptible to acquiring HIV infection as well as cervical atypia (Jourdan 2013); (Kjetland, Leutscher and Ndhlovu 2012) One report indicates that genital lesions are prevented or resolve if anti-schistosomal treatment is taken in the early years as opposed to in adulthood (Kjetland *et al.* 2008).

The World Health Organization (WHO) set goals to treat 75% of school age children in endemic countries by 2010 (World Health Organisation 2015). In response, a mass drug administration (MDA) pilot programme was implemented in KwaZulu-Natal, South Africa in the period 1997 to 2001 by the South African Department of Health (SADOH) (Johnson and Appleton 2005).This programme resulted in a (95.3%) reduction in ova excretion and heavy infections were also reduced (Johnson and Appleton 2005). The national policy of annual MDA has however not been implemented although occasional initiatives such as Ugu District Department of Health in 2012 sometimes provide local, limited MDAs.

The SADOH has yet to implement its policy from 2008 and the Integrated School Health Programme from 2012 for treatment of helminth infections (Department of Health and Basic Education 2012); (Department OF Health 2008). One of the reasons for the lack of MDA programme implementation in South Africa is the high cost of brand Praziquantel, which has been calculated as 50 times higher than the costs of generic anti-schistosomal treatment that is offered for free to all in other countries in Africa (Berge *et al.* 2011). Introduction of generic drugs in South Africa require that the donor embarks on a costly, bureaucratic process for

approval from the South African Medicines Control Council who will not accept medication accredited in foreign institutions (Berge *et al.* 2011).

Praziquantel is effective in killing adult schistosomal worms and administered as a single dose of 40mg/kg. Previous studies have found that in *Schistosoma* infections that are untreated or treated too late could have serious implications for health and the social well-being (King, 2015). Viable or dead ova trapped in tissues of the genito-urinary tracts can cause pathology in both males and females (Gryseels *et al.* 2006; Leutscher *et al.* 2008; Stecher *et al.* 2015).

FGS has been associated with secondary infertility, menstrual disorders and ectopic pregnancies but the effect of treatment has not been explored (Leutscher *et al.* 1998; Poggensee and Feldmeier 2001; Kjetland *et al.* 2006). In a non-blinded case control study among adults (Magak *et al.* 2015). In the present study among young females we sought to explore the effect of self-reported prior anti-schistosomal treatment on young women with squamous cell atypia.

MATERIALS AND METHODS

This was a cross sectional study with a nested prospective study in an area where high schools had been offered treatment by the Department of Health. Females aged 16-23 years were invited for gynaecological investigations, the ethics, methodology for sample collection and processing have been described previously (Holmen *et al.* 2015; Kleppa *et al.* 2015); (Pillay *et al* submitted 2015). Figure 1 shows that 833 underwent gynaecological examinations and Pap smears, gave urine samples and answered an interview administered questionnaire. This included a question in isiZulu (the local language), "Have you ever been treated for Bilharzia (using the local word "isichiene" the local word for Bilharzia), response: (yes/no).

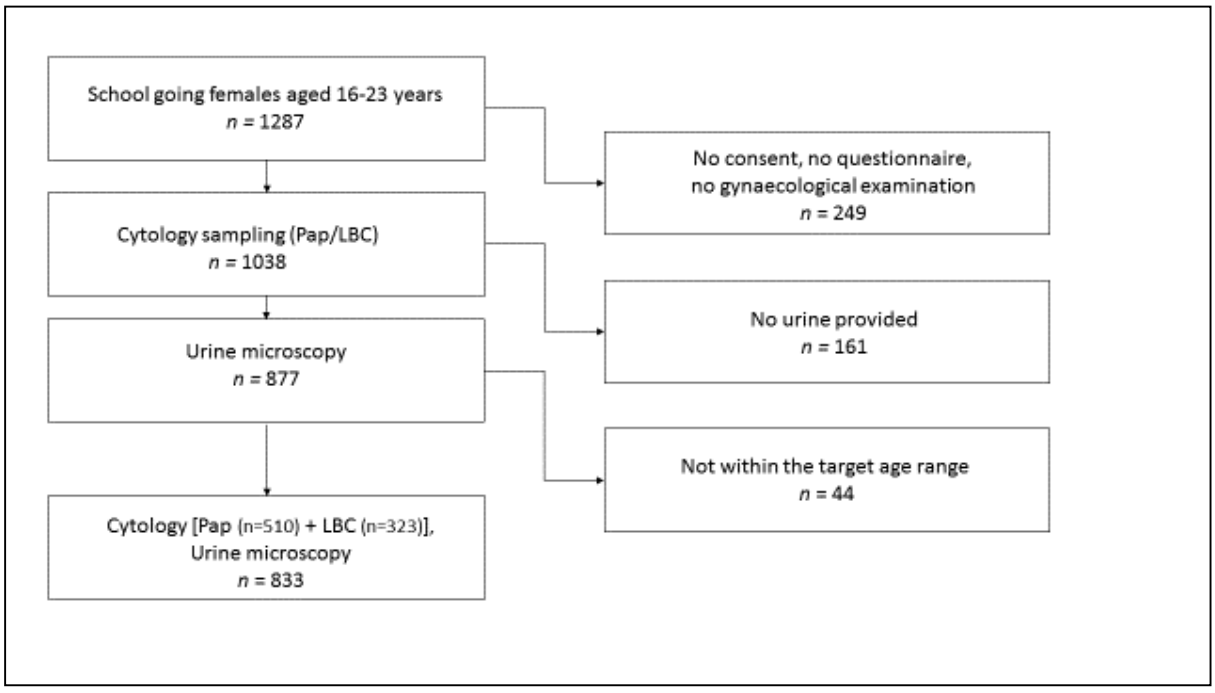

Figure 1. Flow of participation showing baseline and follow up

RESULTS:

All participants were recruited from schools where Praziquantel drug administration had been offered previously, but only 151/833 (18.1%) reported ever having received treatment (Table 1). The participants reported being sexually active from a mean age of 18 years (SD 9.97), and more than half 528 (63%) had at least one child and the majority 770 (92.4%) relied on rivers as their primary water source. The *Schistosoma* prevalence detected cytologically and via urine microscopy was 12 (1.4%) and 178 (21.4%) respectively. Squamous cell atypia was detected among 567 (68%) of which, 19 (2.3%) were diagnosed with high grade squamous intraepithelial lesion (HSIL). Of the participants who were positive for schistosomiasis in Pap smears, only 3/12 (25%) young woman had reported receiving prior anti-schistosomal treatment. Of those who had *Schistosoma* ova detected in urine only 74/178 (41.5%) had been treated previously. Squamous atypia was found among 104/567 (19.0%) of the women who reported prior anti-schistosomal treatment (Figure 2)

Table 1: Number of participants who reported prior anti-schistosomal treatment

	Number	(%)
Treated for Bilharzia *n*= 833		
No	317	(38.1)
Yes	151	(18.1)
Unsure	365	(43.8)

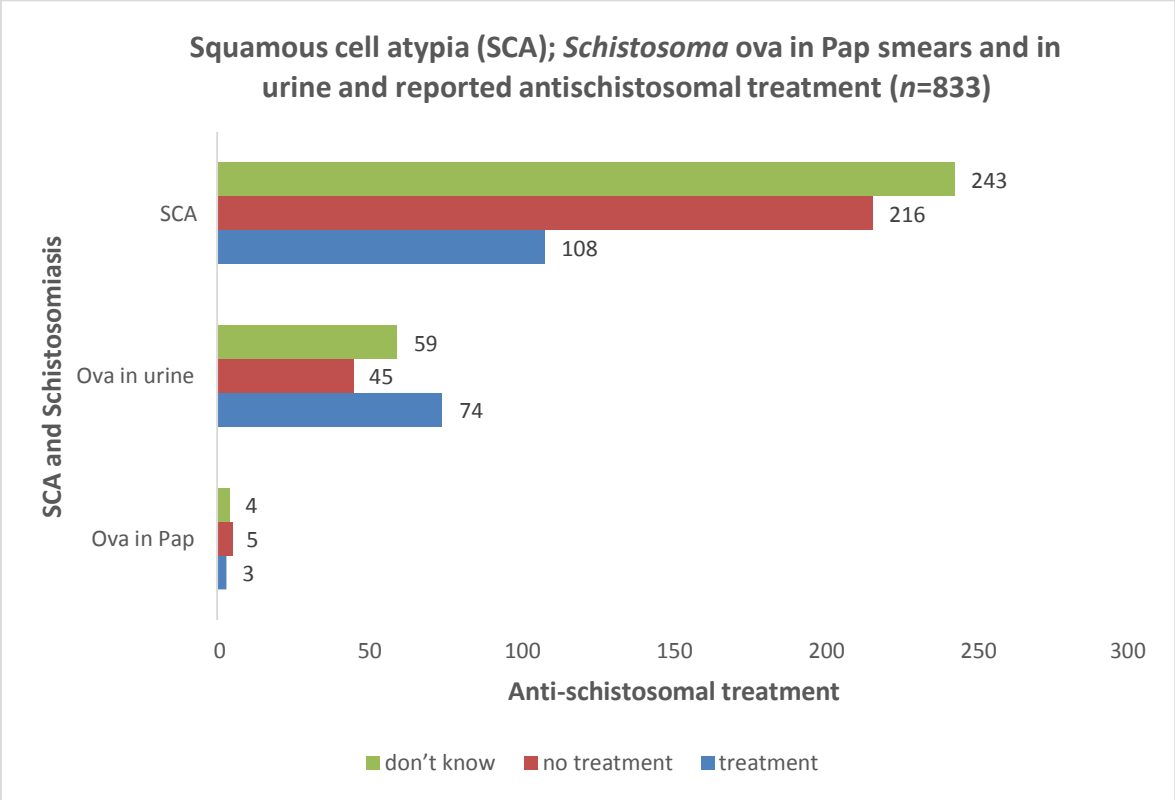

Figure 2. Comparing the occurrence of SCA and Schistosomiasis as detected in Pap smear cytology and urine microscopy among participants who reported prior antischistosomal treatment, no treatment or were unsure of prior treatment.

DISCUSSION

The prevalence of squamous cell atypia and schistosomiasis among the young women who had reported that they received prior anti-schistosomal treatment was significantly lower than amongst those who did not report prior treatment. The women in our study population had high levels of both schistosomiasis and SCA. Known contributing factors are the age of sexual debut which was not unusual in this population. More than two thirds of these young school going women had at least one child, indicating unprotected sex. In young women who had received prior treatment, none had HSIL, and it may be possible that prior treatment in these young women could have influenced this.

The exact timing of the anti-schistosomal treatment was not known. Furthermore the diagnostic tools were inadequate. HPV DNA genotyping would have provided information on this co-infection as an ultimate cause of the atypia. Furthermore Schistosoma PCR would have supported the diagnosis. A further limitation to this study was the low uptake of mass treatment, at 43% as reported previously (Randjelovic *et al.* 2015).

Furthermore, not all women seen at baseline came for a follow-up visit. In some instances contact details provided by participants had changed or were incorrect, or participants had moved to another area. The findings may represent a Type 2 error due to the small number of women who reported having received prior treatment.

These are preliminary findings and the confounders have not been fully explored. The MDA did not occur at the planned time due to rejection of donated generic Praziquantel from the WHO. It was therefore not possible to measure the effect of Praziquantel on genital lesions at the time of writing. Further, the information on prior Praziquantel treatment was only the self-reported information

CONCLUSION

In order to implement MDA programmes, it is necessary to first complete mapping which entails identifying the prevalence of schistosomiasis, once this is done then targeted mass drug administration can occur and should reach the 75% coverage envisioned by the WHO. Diagnostic are required during the different phases of MDA (Utzinger *et al.* 2015). Political will must be there to ensure that participants are not only given a once off dose of anti-schistosomal drugs but repeated treatment options need to be considered to prevent reinfection, and to ensure sustained elimination of this parasitic infection (King 2015).

Further investigation on the effects of adequate anti-schistosomal treatment on genital lesions is warranted and several rounds of treatment may be necessary (Kjetland *et al.* 2008). Furthermore, the role of genital schistosomiasis on the infection and persistence by human papillomavirus (SCA) should be explored. The extremely high prevalence of young women with squamous cell atypia requires both further research and public health interventions, in the risk assessment and for further management of squamous cell atypia.

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9.0 CHAPTER NINE

9.1 Unpublished Cytology Results

Other micro-organisms identified cytologically, but not included in the written manuscripts are listed in Table 1.4. *Trichomonas vaginalis, Bacterial vaginosis* and Herpes simplex virus are sexually transmitted infections in addition to HPV that was found among the young women.

Table1.4 Cytologically detected micro-organisms among the young women from the south and north coast regions

Characteristics	Number	(%)	Standard Deviation
Study population south coast region	394		
Schistosoma haematobium	8	(0.2)	0.14
Trichomonas vaginalis	47	(11.9)	0.32
Bacterial vaginosis	133	(33.8)	0.47
Candida albicans	53	(13.5)	0.34
Lactobacillus	108	(27.4)	0.45
Herpes simplex virus	1	(0.2)	0.50
Study population north coast region	833		
Schistosoma haematobium	12	(1.4)	0.48
Trichomonas vaginalis	59	(7.1)	0.26
Bacterial vaginosis	250	(30)	0.46
Candida albicans	99	(11.9)	0.32
Lactobacillus	403	(48.4)	0.50
Herpes simplex virus	7	(0.8)	0.91
Ascaris lumbricoides	6	(0.7)	0.85

10.0 CHAPTER TEN

10.1 Synthesis

The hypothesis that young women from *Schistosoma* endemic populations in our study are at risk for HPV and squamous cell atypia of the cervix is confirmed. We found there was an association between the squamous cell atypia and schistosomiasis. Diagnostic tools for FGS namely, Pap smear and LBC cytology; *Schistosoma* PCR in urine and cervico-vaginal lavage; and urine microscopy were used and compared to investigate this. The full effect of mass treatment was not possible to investigate since the roll-out of mass treatment had not occurred at the time of writing, however preliminary results of reported prior treatment and the effect on cervical squamous atypia are considered. In each investigation conducted in this study, the public health effects have been discussed and recommendations have been made with respect to reducing the burden of FGS and SCA.

Prevalence of SCA and other cytopathology in the presence of FGS among young women:
Among the young women in our study population SCA was detected using cytology samples with 27% of SCA found in the south coast (Paper 2) and 68% using two different methods of detection in the north coast (Paper 4). The prevalence of schistosomiasis in both regions was focal using microscopy it was 21.4% in the north coast and 19.7% in the south coast. Paper 2 shows that squamous atypia was not associated with schistosomiasis but this could represent a Type 2 error, since the prevalence of schistosomiasis, as found by Pap smear cytology and *Schistosoma* PCR was quite low. Paper 4 shows that there was a significant association between squamous atypia and schistosomiasis, determined by Pap smear and LBC cytology. It was not possible to establish in young women with both genital schistosomiasis and cervical squamous cell atypia which disease acted as a catalyst for the other. Previous studies are contradictory, some studies indicate that there is an association but there are also studies which have found no association between schistosomiasis and cervical atypia. It has been hypothesized that the damage to mucosal linings in genital schistosomiasis could make young women more prone to HPV infection (Kjetland *et al.* 2009a).

In South Africa it was reported that women have a 1 in 35 lifetime risk of acquiring cervical cancer (Ferlay *et al.* 2010). Cervical cancer is one of the few cancers that can be prevented if detected and treated early. Regular screening for cervical cancer using Pap smears, has resulted in a decline in cervical cancer among many developed countries, however this cancer is reported to be the second most common cancer among South African women (Moodley *et al.* 2006). Most of the squamous cell atypia in our study was in the LSIL category; it was also concerning that a few cases of HSIL (7/394 in the south coast and 19/833 in the north coast) were found among these young women. Among a subsample of 10 HSIL cases from the north

coast region, HPV genotyping revealed one or more of the identified high-risk or carcinogenic types of HPV **(Paper 4)**. HPV infections have the highest prevalence among young women at the onset of sexual activity, in their teens, 20s, or 30s and cervical cancer is estimated to take about 20 years after the initial HPV infection to develop (Cuzick *et al.* 2008). HPV infections are also known to regress spontaneously even amongst those who have SIL (Walboomers *et al.* 1999). In our study population, young women may face an additional health burden of HIV (Directorate Epidemiology 2013). Studies in women with pre-cancerous cervical lesions and HIV infections have shown that lesions tend to persist and progress to invasive cancer at a much faster rate (Moodley *et al.* 2006; Adam *et al.* 2008). An additional exacerbating fact is that most women are unaware of cervical cancer or the importance of screening and usually present when in the advanced stages of cancer. It has been reported that women in Sub-Saharan Africa lose the most years of life to cervical cancer compared to than other cancers (Anorlu 2008).

In an FGS study conducted among Tanzanian women, additional STI's that were found to be associated with gynaecological damage pre and post antischistosomal treatment, included Chlamydia, syphilis, gonorrhoea, trichomoniasis and cervical warts (Downs *et al.* 2013). In the present study, it was found that the young women in both the north and south coast had the following infective agents in their cytology samples, *Trichomonas vaginalis, Bacterial vaginosis, Candida albicans, Lactobacillus* and *Herpes simplex virus* in addition to HPV. STI's and schistosomiasis within the lower genital tract have been suggested as agents which favour the acquisition of HIV, and although many studies in Africa usually consider the effects of most STI's in HIV acquisition, the effects of schistosomiasis seems to be a neglected entity (Kjetland *et al.* 2014).

It is not known how early FGS occurs and in this study among 10-12 years old girls the aim was to explore this **(Paper1)**. It was also not possible to directly identify squamous atypia among the young girls as gynaecological examinations are not ethical. Information pertaining to genital symptoms was acquired using self-reported questionnaire data (Hegertun 2013). The inclusion of the following genital symptoms, were regarded as an indirect marker for atypia in the genitals: discharge, bleeding, ulcers, tumours and pain. It was found that 35% of the girls had genital symptoms even before the onset of sexual activity (Hegertun *et al.* 2013). On comparing these genital symptoms with the *Schistosoma* PCR results a significant association between the two entities was found at the school level **(Paper1)**. This was the first study among pre-pubertal girls where an association between schistosomiasis and genital symptoms was found at the school level and these findings support previous studies in which there has been the suggestion that genital *Schistosoma* infections occur in early childhood (Poggensee and Feldmeier 2001; Prabhakaran and Brown 2004; Kjetland *et al.* 2009a).

In (**Papers 2 and 4)** we report on the risk factors identified in both our study areas, where it was found that the mean age of sexual debut among the young women was 16 and 18 years respectively. Of the young women in the study sample, in the South coast 41% had one child while 63% had one child in the north coast. The young women came from rural areas where poverty and unemployment is rife, and in both study areas, rivers were the most common water source reported by 71% in the south coast and 94% in the north coast respectively. When comparing the overall prevalence of squamous atypia and schistosomiasis, it was found that the north coast region had a higher prevalence of these two entities as compared to the south. The following are thus potential risk factors; having at least one child, water contact with rivers infested with schistosomiasis, and young age at sexual debut.

Our findings could be the result of other social and cultural challenges that young women in South Africa face. It has been reported that teenage pregnancy is a common occurrence and that sexual activity commences in the mid-teenage years, where boys particularly have multiple sexual partners (Jewkes *et al.* 2001). Young women in South Africa have little power over their sexual encounters, which are sometimes forced or coerced (Maharaj and Munthree 2007). Other factors such as poverty and lack of education are factors which contribute to the disempowerment of young women in negotiating their sexual encounters (Manzini. Ntsiki 2001). The finding of atypia with the potential to become invasive cancer among a young non-routinely screened population should not be underestimated.

Early diagnostic tools for FGS

Since it is known that invasive sampling techniques to detect FGS in the lower genital tract of young women who may be also at risk for HIV and other sexually transmitted infections is not a possibility, this study investigated the role of *Schistosoma* PCR in urine and cervico-vaginal lavage samples. Urine microscopy was used to compare the PCR results. *Schistosoma* ova were also detected using Pap smears and LBC samples.

Schistosoma PCR testing among 708 girls revealed that this test was significantly associated with single urine microscopy, and since there was a considerably large sample from which the *Schistosoma* PCR analysis was run, these findings are in support of *Schistosoma* PCR in cervico-vaginal lavage being used as a standardized diagnostic technique to identify populations at risk for FGS. The additional association with genital symptoms indicates that FGS exists among the girls in the study population. **(Paper1).**

Among the participants from the south coast, *Schistosoma* PCR in urine and cervico-vaginal lavage samples were done and these results compared to urine microscopy **(Paper 2)**. All three testing methods for *Schistosoma* identification were significantly associated. There was a higher prevalence of schistosomiasis among cervico-vaginal lavage samples 107/394 (27.1%) than in the Pap smears 8/394 (2.0%) and the low sensitivity of Pap smears was consistent with a previous study (Feldmeier, Helling-Giese and Poggensee 2001). In this study in 3/8 (37.5%)young women there was evidence of schistosomiasis in the genital tract being seen in Pap smears and in cervico-vaginal lavage without being found in the urinary tract. This is an important finding since it suggests that urine microscopy alone may not be the best indicator for FGS.

In the light of the low detection rate of schistosomiasis using traditional Pap smears it was decided to use an alternative method of collecting cytological material from the cervix, namely LBC sampling **(Papers 2 and 4)**. Using LBC cytology resulted in an improvement in the preservation and diagnostic quality of the sample and the detection of schistosomiasis was slightly improved using LBC sampling. This was a novel method of cytological analysis in an FGS study, since all previous studies have used the conventional Pap smears (Feldmeier, Helling-Giese and Poggensee 2001; Randrianasolo *et al.* 2015). Using the LBC sampling also brought in the possibility of adding a novel triage of testing for FGS populations using diagnostic cytology and molecular testing for HPV DNA and *Schistosoma* PCR **(Paper 4).** The use of complementary HPV genotyping in identifying the following 37 HPV types: 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 67, 68, 69, 70, 73, 74, 81, 82 ,83, 86, 87, 89, 90, 91 will be considered in a follow-up study to inform further management among the young women with cervical atypia.

In a South African study that was conducted by the National Health Laboratory Services, investigating the cost effectiveness of LBC against conventional cytology it was found that the LBC results were comparable to conventional cytology based on the detection of CIN2 or higher lesions detected. Additionally it was reported that within the public sector the cost per slide for a conventional Pap smear was R64 compared to R85 for an LBC slide (de Jager *et al.* 2013). The findings of comparable sensitivities in detecting cytological atypia, are in contrast to the findings of the present study and they do not take into consideration the benefits of additional testing for HPV. In research settings, where there are limited opportunities for repeat smears and in order to ensure that many of the diagnostic tests are done with minimal sampling would thus favour LBC sampling, despite the cost difference when compared to conventional Pap smears.

In **Paper 3**, we compare the diagnostic performance of non-invasive laboratory tests across five regions from three countries in Africa which are endemic for schistosomiasis (*S. haematobium* or *S. mansoni*). In this study 903 cervico-vaginal lavage *Schistosoma* PCR assays were completed collectively, and the results from this analysis confirmed that the PCR assay has a higher sensitivity in detecting genital schistosomiasis than other techniques like cytology. There was also generally a higher *Schistosoma* detection rate among the urine and stool PCR assays compared to urine and stool microscopy. Since the PCR assay was able to detect schistosomiasis in the young women who had low DNA loads, such an assay could be useful in low endemic areas. The drawback of the PCR assay is that it was unable to differentiate between the *S. haematobium* and *S. mansoni* and the assay that we used is not field applicable. Samples for PCR analysis also have to be stored in frozen conditions to ensure optimal results.

Effect of treatment on early stages of genital disease

The precise effect of the antischistosomal drug Praziquantel on genital tract lesions is still under investigation, although prior studies have inferred that early treatment may help in the resolution of lesions and that lesions among women remain unchanged with treatment (Richter 2000; Kjetland *et al*. 2008) The genital lesions identified in FGS include sandy patches, abnormal blood vessels, contact bleeding and rubbery papules (Norseth *et al.* 2014; Randrianasolo *et al.* 2015). In a study among Tanzanian women it was concluded that early and frequent doses of Praziquantel should be administered to populations at risk, to help prevent the possible long term effects of genital schistosomiasis (Downs *et al*. 2013).

In this study, there were delays in the acquisition of the anti-schistosomal drug Praziquantel as well as delays in the anticipated rollout of the MDA programme among schools in the study area. These challenges were beyond the control of the research team. Due to this it was not possible at the time of writing to fully explore the effects of treatment on genital lesions. This objective is considered in **Paper 5 -Short Communication,** where questionnaire data pertaining to whether participants had received prior treatment were compared to squamous atypia. The findings revealed that women who had received prior treatment had overall less squamous cell atypia, and that of the 12 cases with HSIL that were detected cytologically, none of these women had received prior treatment. The other results were variable and it was not possible to draw any solid conclusions about the effect of treatment.

While there are other risk factors like early sexual debut, number of sexual partners, pregnancy at an early age, it is established that HPV types 16 and 18 are associated with almost 100% of invasive cervical cancers (International Agency for Research on Cancer 2007).

9.2 Limitations

Mostly we report cross-sectional studies that cannot inform about the direction of the associations between for example FGS and SCA.

For *Schistosoma* PCR assays to be performed ensuring optimal DNA preservation samples must be stored at -80^{0}C once collected. In our study, when the initial PCR analysis was done on urine samples and cervico-vaginal lavage samples they were stored a week at 4^{0}C and thereafter stored for several months at -80^{0}C before being transported under frozen conditions to the Netherlands. It was not possible to store them directly at -80^{0}C since there were logistical challenges with the freezers. Despite this, significant associations between the Schistosoma PCR tests and microscopy were found. It is possible that the performance of the PCR tests could have been slightly compromised due the initial challenges with storage.

When Pap smears were collected in the south coast study area the preservation of the smears was not optimal for most of the samples. The clinicians and research assistants who were collecting the samples were made aware of the importance of proper fixation of smears and they made efforts to improve on the collection procedure, but with limited success. It is possible that the smear preservation was altered due to the sequence in which sampling was done since the cervico-vaginal lavage samples were collected prior to the Pap smears. In collecting lavage samples it is possible that the saline used could have altered the mucosal surface of the cervix, thereby causing the Pap smears to be poorly preserved. Despite Pap smears being sub-optimal a diagnosis was made where possible, and later LBC sampling was introduced. The LBC samples showed improved diagnostic quality and also offered the possibility of using further DNA testing to enhance the diagnostic techniques in FGS.

The slow MDA among the study participants was a limitation. At the onset of the main research project it was planned that research would occur simultaneously with the roll-out of antischistosomal treatment which was co-ordinated by the Department of Health. This process, however had faced many setbacks, among the most important was the late release of funds for the MDA, there was also insufficient collaboration between all sectors, like health, education, water and sanitation. One of the major reasons for poor or limited implementation of MDA in South Africa is the high cost of Praziquantel tablets, and the challenges with getting cheaper generic drugs registered with the Medicines Control Council in South Africa. Using the self-reported questionnaire data on prior receipt of Praziquantel treatment to assess the effect of treatment is a form of recall bias. This is a limitation since some of the participants could have

answered this question incorrectly because the response is dependent on memory. The accuracy of the recall was not possible to measure and therefore these results must be treated with reserve. If MDA had occurred as planned, assessing the effect of treatment on genital lesions would have been more accurate if young women were followed over time, using biomarkers instead.

Despite these limitations, it is promising to note that at a recent meeting held on the 9th of November 2015 with the following stakeholders, the research project team, District Health Managers and School Health Nurses, a representative from the WHO and gynaecologists, doctors and nurses from local district clinics and hospitals, a resolution toward the implementation of MDA was pledged and agreed to by all stakeholders. The research project members had also presented some of the health concerns around the impact of FGS to raise awareness and to promote the importance of providing anti-schistosomal treatment to those at risk.

Further difficulties occurred with the tracking of the young rural women after the baseline analysis since often original details provided by participants had changed or were sometimes incorrect, or participants had moved to another area and it was difficult to trace them. Some participants also refused follow up and treatment.

This study focussed only on girls and young women who are at risk for schistosomiasis. Male genital schistosomiasis (MGS) is also an important neglected entitythat was not considered. It has been reported that among males, urogenital schistosomiasis peaks around the second decade, additionally, young men are also prone to STIs around this time of their lives as well (Leutscher *et al.* 2008). It has been reported that diagnosing MGS is also challenging, since like genital schistosomiasis, the effects of MGS may be undetected or misdiagnosed (Leutscher *et al.* 2008). Further research is required on MGS and the possible effects with HIV transmission as well as other STI's, since the information on MGS is limited (Stecher *et al.* 2015).

11.0 CHAPTER ELEVEN

11.1 Conclusions and Recommendations

In conclusion, this research has revealed that girls and young women in *Schistosoma* endemic areas are at risk for cervical squamous cell atypia. Contributing risk factors for young women from rural areas include poverty since in both study areas the majority relied on rivers as their main water source as well as age at sexual debut and having at least once child. As a result, the neglect in the identification and management of schistosomiasis and cervical squamous cell atypia may in part contribute to the high prevalence rates for invasive cervical cancer and HIV in KwaZulu-Natal. While this study was unable to prove a causal relationship between schistosomiasis and cervical cell atypia, it must be noted that both these entities are prevalent amongst our young female population and it is important for policy makers to ensure that this is addressed. Bearing in mind the fact that women in Sub-Saharan Africa lose most life years to cervical cancer than any other cancer, health promotion should be targeted at girls and young women to ensure that they are at least aware of the consequences of risky sexual behaviour.

Schistosomiasis continues to be a problem affecting many girls and young women in KwaZulu-Natal. The focus of this study was on the female sex and did not include males, and those using contaminated water sources who are also at risk of schistosomiasis infections.

An essential step in addressing this health problem is to get support and involvement from communities, but for this to occur, people need to be made aware of these diseases. A programme in which young girls and boys in high schools are identified and trained to help impart knowledge to others in their communities may be an option to consider, since in this way, youth are not only educated, but they are empowered by being given some responsibility in communities to help improve health status. In KwaZulu-Natal the school health teams visit schools and can inform both educators and learners (Randjelovic *et al.* 2015). It is also recommended that while health education can be spread though schools, it would also be appropriate for youth health clinics to be set up so that youth can seek appropriate health care that includes testing for infections more easily.

Schistosomiasis is a focal disease which depends on the distribution of the snail species *Bilinus.* The use of real-time PCR as a method to determine the prevalence of schistosomiasis in a large volume of samples to improve the targeting of the MDA using Praziquantel to treat the infection and reduce or prevent the morbidity caused by schistosomiasis, is promising.

While it was not possible to directly diagnose FGS among young 10-12 year old girls, their reported symptoms suggest early FGS infections. There is the possibility of using "non-invasive" sampling devices like the use of a sleeve that is placed into a girl's underwear to collect vaginal discharge for PCR analysis. This analysis should include both Schistosomiasis and HPV and further research into devising and implementing such a device needs to be considered. Regular MDA with Praziquantel must be implemented depending on the prevalence. In light of the high prevalence of invasive cervical cancer among South African women, policy makers need to ensure the regular implementation of HPV vaccination among young girls.

Among the older cohort, it was also found that non-invasive diagnostic tools like LBC cytology and *Schistosoma* PCR in cervico-vaginal lavage samples together with HPV DNA testing could improve the detection of genital schistosomiasis. While traditional cytology was found to be limited, LBC sampling showed an improved detection rate and there is room for further research investigating the use of Schistosoma PCR testing in residual LBC samples. Further research comparing the clinical data including the colposcopy findings is recommended.

It was not possible to fully investigate the effect of antischistosomal treatment on genital lesions among the participants however, cervical atypia among young women who are at risk also for HIV is of major concern. It might be argued that finding cervical atypia among a young population could not be too concerning since most cervical atypia caused by HPV infections tend to clear within 24-36 months. However the findings of one or more high risk HPV in the sub-sample of young women with HSIL is of concern. Prior studies have concluded that cervical atypia can persist and progress to invasive cancer much quicker among women with HIV. KwaZulu-Natal has a high prevalence of HIV amongst this age group (Directorate Epidemiology 2013).

Cervical cancer is the second most common cancer in South Africa, and while invasive cancer is known to occur in women over the age of 30, the precise impact of HIV and schistosomiasis on cervical atypia is not fully understood. The reported genital symptoms among girls and presence of cervical atypia among the young women from our *Schistosoma* endemic populations, warrants immediate and repeated mass drug administration. , Especially among young children, girls and young women as a priority to help reduce the negative consequences of FGS. While it not be feasible to implement cervical screening among young women below

the age of 30, it is important for policy makers to be aware of the possible impact FGS and HIV on cervical atypia, and to design health literacy with these disease dynamics included.

In KwaZulu-Natal, the number of school health teams has been increased and these teams will be informed about the results of this study. With emphasis on an increase in awareness of the problem of schistosomiasis the association with cervical atypia and the importance of anti-schistosomal treatment and early diagnosis and monitoring of atypia. In a province with endemic schistosomiasis, and high prevalence of HIV and high SCA, health literacy concerning these diseases is essential and the Department of Health has a critical role in increasing awareness.

In terms of South Africa's Constitution and Bill of Rights everyone has the right to health and clean water and adequate sanitation is an essential component. This has yet to be achieved and advocacy is required to ensure that these basic requirements are met and that sources of water are not contaminated. An intersectoral campaign by government departments is urgently required to achieve this.

This study was undertaken in KwaZulu-Natal, but five of South Africa's provinces are endemic for schistosomiasis and it is a disease prevalence in many Sub-Saharan countries. The results of this study indicating the association of schistosomiasis and SCA emphasise the relevance of early and regular MDA.

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APPENDIX 1

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23 June 2015

Mrs Pavitra Pillay
15 Waverton Road,
Berea,
Durban. 4001

Dear Mrs Pillay

PROTOCOL: Female Genital Schistosomiasis (FGS) as an epidemiological risk factor for squamous cell atypia and genital disease in a longitudinal cohort of young women in KwaZulu-Natal. REF: BF057/11

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 24 August 2015
Expiration of Ethical Approval: 23 August 2016

I wish to advise you that your application for Recertification dated 02 June 2015 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be ratified by a full Committee at its next meeting taking place on **14 July 2015.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

APPENDIX 2

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23 June 2015

Mrs Pavitra Pillay
15 Waverton Road,
Berea,
Durban. 4001

Dear Mrs Pillay

PROTOCOL: Female Genital Schistosomiasis (FGS) as an epidemiological risk factor for squamous cell atypia and genital disease in a longitudinal cohort of young women in KwaZulu-Natal. REF: BF057/11

I wish to advise you that your correspondence dated 15 August 2013 requesting approval of Amendments for the above mentioned study has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee.

This approval will be ratified at the next meeting to be held on **14 July 2015.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

APPENDIX 3

Female genital schistosomiasis (FGS) as an epidemiological risk factor for squamous cell atypia and genital disease in a longitudinal cohort of young women in KwaZulu Natal

Information and request for participation in the research project

We, the Universities of Oslo in Norway and KwaZulu-Natal, are doing research on Bilharzia

To find out if and how Bilharzia affects young women.

To investigate if treatment works better in the young.

Why you are being asked to participate

We know that treatment kills the Bilharzia worms and works for urinary disease. Previous research has shown that treatment did not work properly for genital damage in older adult women. Now we wish to test it in young adults. We ask you

- because you may have had contact with the parasite in the water
- because you are still young
- because we hope to protect you from genital damage from Bilharzia.

Your decision

Participation in the study is entirely voluntary and you may withdraw at any time. You may ask any questions and we will try to answer. You do not have to give a reason if you do not wish to participate. Your treatment now or in the future will not be affected by your decision. You may also interrupt any investigation as you wish. Approximately 2000 young women may participate.

If you are pregnant you cannot join the study, but you are welcome to participate in the study two months after giving birth.

Some of the investigations we do can be done at a hospital, but others are not yet available in South Africa and some samples may therefore be sent overseas but at no cost to you.

Consequences for you

Participating in this study will mean that

- you will give urine, stool and have blood tests, and an interview will be done
- you will have gynaecological investigations for diseases. The gynaecological examination will last about 20 minutes. If there is reason to do more tests during the examination you will be asked for permission
- you will be taken to the VIBE youth clinic by a female driver
- samples will be tested for cancer, infections and HIV, and you decide if you want the results or not
- you will receive treatment for Bilharzia as recommended by the World Health Organisation
- you might be asked to participate for a period up to 2 years
- if we discover special problems during the interview or the investigation we will either refer you or treat you
- you will lose half a day from school to participate in the study

Gynecological examination

You will have an ordinary gynaecological investigation to look for genital Bilharzia and other genital problems by experts. The procedure is uncomfortable and it is better if you relax. The examination will be done by a female doctor with a female nurse/assistant present in the room. The doctor will explain the procedure to you before the examination.

Risks

The tablets for Bilharzia may have side effects; some will feel sick for a couple of days. Some may get a rash, diarrhoea, or vomit. People with many worms feel worse as the worms die.'

Benefits

You will receive treatment for any disease we discover at no charge and be referred if necessary. Your Bilharzia worms will die and you will feel healthier.

How your samples and personal data are taken care of

The information that we collect in research will provide new information about Bilharzia in women and we will share this information with health workers who need this. However no one will ever know about your personal information. Samples and the interview will be analysed and stored ***without*** your name on it. Anything with your name is stored separately. This will be done both before and after treatment so that the investigators can see if the disease is better. The specimens will be investigated in the best laboratories ***without*** your name. Samples might be sent to other countries for analysis by experts. If you agree to participate in the study, you also give permission for this. All information will be stored securely. The samples and information will only be used to study Bilharzia together with women's diseases, risks for cancer and HIV for up to 20 years. It will not be used for other purposes. The Principal Investigators and the doctors of the study are formally responsible for

the security. These have access to the address file. If important information is discovered during the study we will make sure that you are informed, if you wish.

Who approved the project

The project has been reviewed and approved by the KwaZulu-Natal Department of Health and ethical committees in both South Africa and Norway.

Economy

The study is financed by research grants from abroad. There are no plans for collaborations with industry, nor plans for commercialization. The researchers involved in the study have no personal financial gain in connection with this study.

Project Management/More information

If you have any questions regarding the study, please feel free to contact the Principal Investigator and project manager: Dr M Taylor 031 2604499 or 2661592.

Contact details in case you have problems. We will also contact the person below in case of adverse events: Biomedical Research Ethical Committee

tel.: (031) 260 4769; fax: (031) 260 4609; e-mail: BREC@ukzn.ac.za

The participants of the study: Dr M Taylor, Dr J Kvalsvig 031 260-4499 (w) 2661592 (h)

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Dr Elisabeth Kleppa 079 194-2652

Your rights

If you agree to participate in the study, you have the right to access all personal information we have registered about you. You have the right to correct any faulty information. You may at any time withdraw from the study without having to explain your reasons. This will not affect your normal access to healthcare and treatment in any way. If consent is withdrawn, you may request material/ information to be destroyed/ deleted.

Consent to participate in the research project

You have been informed about the study by _____

Participation in the study is based on voluntary, informed consent. You are free to ask for any additional information. If you, after having received all the information you deem necessary, wish to participate in the study, you must sign this consent form

I, _____ *(name in capital letters), confirm that I have received written information about the study and have had the opportunity to ask for additional information, and that I will participate in the project.*

*Signature*_____*Date*_____

(Signed by the project participant) *(Dated by the project participant)*

Participation in other project, which, what happens there? _____

_____*Year:* _____

If you would like information about the study as we go along, how do you wish to be contacted?

_____

_____

_____

_____

If you were invited in the weekend or during school holidays would you be allowed to come? _____

Where should we pick you up? (home/ school/ clinic/ other) __

APPENDIX 4

TEEN QUESTIONNAIRE

Teen-15 id |__|__|__|__|
Interviewer _____
Interview date dd/ mm/ yy ____/____/_____

Please use a circle or tick where there are pre-entered responses QC
BY_____(sign, red pen)

Isiqalo: Igama lami.....ngisebenzela iVIBE project njengoba kwakushiwo ngaphambilini. Ngiphinde ngikubonge ngokunginikeza imvume yokuthi ngisebenze nawe. Ngithanda ukugcizelela amanye amaphuzu ngaphambi kokuthi siqale.
Yonke lembuzongxoxo imayelana nesichenene sesibeletho sabesifazane kanti futhi yonke into esizoyixoxa izohlala phakathi kwethu..Ayikho imibuzo elungile nengalungile. Unelungelo lokuyimisa impendulo ngxoxo noma isiphi isikhathi. Ukusebenzisana kuyoba intokozo / *Introduction: I am .....and I am working for the VIBE project as mentioned during the informed consent procedure. Thank you again for giving me permission to work with you. I would like to emphasize some points before we start.*
All the questions below are related to Female Genital Bilharzia and everything discussed here will be confidential. There are no wrong or right answers. You are free to stop the interview at any time. Your cooperation will be appreciated.

1) **Isikole** / school: _____
2) **Igama lesikole sebanga eliphansi ophuma kuso** / Name of the primary school you came from_____
3) **Uyasebenza na? njenge?**/Are you working? As? _____
4) **Iminyaka** / Age |__|__|
5) **Wazalelwaphi?** / Where were you born ?_____
6) **Inkolo** / Religious affiliation [Christian] [Muslim] [Shembe] [Leganyana] [Methodist] [other_____

	Yebo / Yes	Cha / No	NA	**Uma kungu yebo hlobo luni lwezinkomba? /** If yes what kind of symptoms?
a) **Unaso isifo sesifuba somoya? /** Do you have asthma?				
b) **Kukhohona imithi othi uma uyiphuza ube nokungaphatheki kahle? /** Do you have any allergies to medication? (Penicillin, Cotrimoxazole or Bactrim)				
c) **Kukhona ukudla othi uma ukudlile kungakuphathi kahle? /** Do you have any allergies to food? (fruit/seafood/nut)				

7) **Ulimi lwasekhaya** / Home Language [Zulu] [Xhosa] [English] [Other_____

ALLERGY

8)

MENSTRUATION

9) Ugcine nini ukuya esikhathini? / LMP (First day of last menstural period): ____/____/_____dd/mm/yy or NA

10) Waqala uneminyaka emingaki ukuya esikhathini? / Age of your first menstruation? |__|__|

11) Ubanaso isilumo kangangokuba kumele uhlale ekhaya? / Do you normally have painful periods so that you have to stay home from school? [yebo] [cha]

12) Ubanaso isilumo esikwenza ufise ukuthatha amaphilisi ezinhlungu? / Do you normally have painful periods so that you wish to take painkillers? [yebo] [cha]

13) Indlela yokuhlela / Contraceptive method:

a) **Uyajova?** / Are you on contraceptive injection? [yebo] [cha]

b) **Amaphilisi okuhlela** / contraceptive pills [yebo] [cha]

14) Uya ngendlela efanayo nyanga zonke / Are your periods regular [yebo] [cha] [NA= Post Partum / if injectable contraceptive / pills]

15) Uya izinsuku ezingaki esikhathini? / How many days does your period normally last? |__|__| [NA= Post Partum / if injectable contraceptive / pills]

16) Kuye kwenzeke wophe ungalindele? / Do you have times of unexpected bleeding or spotting in between your normal periods? [yebo] [cha] [NA= Post Partum / if injectable contraceptive / pills]

17) Ujwayele ukopha kakhulu kuze kube namahluli? / Do you normally have periods with heavy bleeding and clots? [yebo] [cha] [NA= Post Partum / if injectable contraceptive / pills]

PREGNANCY

18) Usukhulelwe kangaki sekukonke? / How many times have you been pregnant? | __|__|

19) Bangaki abantwana abaphilayo? / How many children are alive? | __|__| [NA=99]

Month Year	Abortion = 1. Miscarriage =2. Birth =3	Trimester: 1= 1st (before 3 mnths) . 2= 2nd (between 3 to 6 mnths). 3=3rd (6 to 9 months)	Complications, please describe

URINE AND BILHARZIA

20) Wake walashelwa isichenene nini?/ Have you ever been treated for Bilharzia, when?

Never |__| OR [age] |__|__|1st time |__|__|2nd ime |__|__|3rd time

21) Urinary symptoms:

	Wake waba nayo inkinga noma yiphi ngokuchama njenge: / Have you ever had any problems with urination like:	**Esontweni eledlule /** This last week	**Kudala phambilini /** Sometime before	**Akukaze /** Never
a.	**Zinhlungu uchama** / Pain when you urinate			
b	**Ukuzwa sengathi ufuna ukuchama esikhaleni noma uhlezi unganyakazi uze ucishe uzichamele** / Sudden urge to urinate causing a leak, even when sitting still [urge incontinence]			
c.	**Ukuzwa sengathi ufuna ukuchama esikhaleni noma unganyakazi ngaphandle kokuthi ucishe uzichamela** / Sudden urge to urinate, even when sitting still [urge] (no leakage)			
d.	**Iconsi lomchamo uma ugxuma, ukhwehlela noma uhleka** / Drop of urine if you jump, cough or laugh [stress incontinence]			
d.	**Umchamo obomvu** / Red urine			

WATER CONTACT

22) Manje ngizokubuza ngezinhlobo zezinto ozenzayo noma owake wazenza ngamanzi, uzenza noma wazenza kangakanani, uhlala isikhathi esingakanani emanzini nokuthi umzimba uthintana kangakanani namanzi. /Now I will ask you what kind of water activity you have or have had, how often you do them, for how long you stay in the water and how much of your body that is in contact with the water:

a. **Umfula**/river **Amadamu**/dam **Amanzi amile**/standing water. **Amanzi avela kulezizisuka**/ water from these sources. **None**
b. **Igama lomfula noma idamu olisebenzisayo njengamanje? /** What is the name of your current waterbody? Name of river / dam etc:

   _____

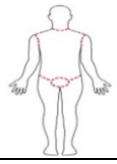
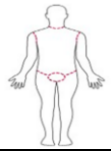
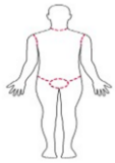
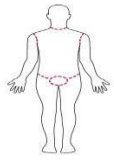
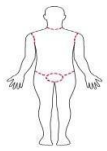
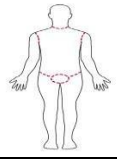
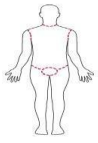
c. **Ingabe abanye esikoleni sakho basebenzisa lamanzi? Bangaphezu kwe 10___ noma bangaphansi kwe 10___Abekho___/** Do others in your high school use this waterbody? More than 10 people ------less than 10 ----- None
d. **Kwakunjalo nisebancane? Yebo Chaza uma kuhlukile/** Is that the same as in childhood ? If different: _____

23) Kukhona isikhathi empilweni yakho owake wahlangana nalamanzi? [yebo] **[cha]**
Is there a period in your life when you had water contact? (make crosses for each period)

|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 yrs

24) Usuke wathintana namanzi omfula noma idamu ngonyaka odlule (2013) noma kulonyaka (2014)? / Did you have contact with the river or dam in 2013 or 2014? [yebo] [cha]. **Uma kungu Yebo sicela uchaze:** / If yes, please explain:

	Kangaki/ *How often?* **Nsukuzonke** */Daily (4)* **Kujwayele** */Often (3)* **Kwesinye isikhathi** */Sometimes (2)* **Kuthukela/ qabukela** / *Rarely (1)* **Ngeke** / *Never (0)*	**Uke uhlale kangakanani / Uhlala kangakanani emanzini? /** *For how long did/ do you stay in the water?* **Ngaphezulu kuka 5 h /** *More than 5 h (4).* **3-5 Amahora /** *3-5 hours (3).* **Ngaphansi kwamahora amathathu /** *Less than 3 hours (2).* **Kuze kube yimizuzu ewu-60 /** *Up to 60 minutes (1)*	**Umzimba wake wawathinta / uwathinta kangakanani amanzi ngesikhathi wenza lezizinto** */How much of your body was/is in contact with water during this activity?*
Uke noma wake wadlala/ ukubhukuda?/ Did or do you play / swim?			
Uke noma wake wawasha / wageza?/*Did or do you wash / bathe?*			
Uke noma wake wazihlanza izingubo? / *Did or do you do laundry?*			
Uke noma wazihlanza izingubo zokulala? /Did or do you wash blankets?			
Uke noma wake wawakha amanzi?/*Did or do you collect water?*			
Uke noma wake wadoba? /Did or do you *fish?*			
Uke uwele umfula zize izinyawo zakho zithintwe amanzi?/*Did or do you ever cross the water , so your feet become wet?*			

SYMPTOMS AND EVENTS

25) Kubukeka kunjani ukuphuma ngaphansi kuwena? / How is your current discharge?

a) Kumbala muni/ Colour 1 2 3 4 5 6 7 8 [NA=99] (please circle)

b) Okusagazana / Trace of blood 0 1 2 3 4 5 6 [NA=99]

26)

	Wake wezwa ukungaphatheki kahle esithweni sakho sangasese njengo: / Have you previously felt any discomfort in your private parts like**:**	**Esontweni eledlule /** This last week	**Kudala phambilini** Sometime	**NA /** Never
a.	**Ukuluma** / Itch			
b.	**Ukushisa / Ukushoshozela** / Burn / Sting			
d.	**Isilonda /** Sore / ulcer			
e.	**Isimila / isigaxa** / Lump / tumour			
f.	**Okuphumayo okusagazana /** Bloody discharge			
g.	**Okushubile/ Okusasigaxana okuphumayo** Thick/ lumpy discharge			
h.	**Okusamanzi okuphumayo /** Watery discharge			
i.	**Iphunga elingajwayelekile /** Abnormal smell			
j.	**Okuphumayo okunombala ongajwayelekile /** Abnormal coloured discharge			
k.	**Kukhona osuke wakushutheka esithweni sakho sangasese njenge nsipho?** / Did you put substances inside your vagina? Such as soap ?			
l.	**Wake wagcatshwa esithweni sangasese noma waxilongwa ngezinsimbi ezicijile** / Ever had genital cutting or used a sharp instrument in the genital area (not episiotomyand not previous Pap smear)			

27) Usuke wabanazo izinhlungu esinyeni? [yebo] [cha] Uma kungu Yebo sicela uchaze: /
Have you ever experienced abdominal pain? If YES, please explain:

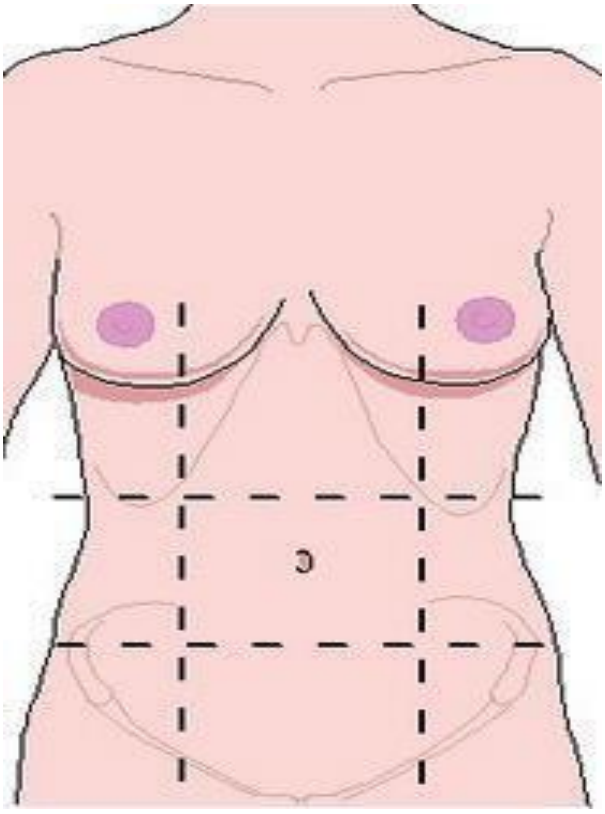
WHERE (abdominal location, see figure)?

WHEN (activity)?

HOW OFTEN? (This last week or sometime)

RA: Plese write the code(s) directly in the correct quadrant(s) in the figure

Activity	**This last week** (last 7 days)	**Sometime**
Menses	M2	M1
During sex	DS2	DS1
After sex	AS2	AS1
Urination	U2	U1
Other	X2	X1
Anytime	A2	A1



CONTRACEPTION & STDs

28) **Uke wakusebenzisa okulandelayo kuleli sonto eledlule** / Have you used the following the last week(the last 7 days): **Ikhondomu yabesilisa** / male condom [yebo] [cha]
29) **Uyazi ukuthi ziyini izifo zocansi STI?** / Do you know what an STI is? [yebo] [cha] [angazi]
STD izifo ezithelelana ngokocansi, zibonakala kube khona okuphumayo okungajwayelekile, ukuluma, kube bomvu, amaqhubana noma isilonda esithweni sangasese /STI's are diseases that you get through sexual contact, and symptoms include an abnormal vaginal discharge, itchiness, redness, lumps or sores in your private parts.

30) **Wake walashelwa izifo zocansi ngenyanga edlule?** / Have you been treated for an STI the last 4 weeks? [yebo] [cha] [angazi]
31) **Ucabanga ukuthi unaso isifo socansi njengamanje?** / Do you think you have an STI now?

Kungani Why? _____

32) **Usulashelwe kangaki izifo zocansi?** / How many times treated for an STI? |___|___|
33) **Usuke wathola ukulashelwa noma isiphi isifo socansi komunye umtholampilo emuva kokuba ulana? /** Have you received any STI treatment since you were at this clinic last time? [yebo] [cha] [angazi]. If yes, when? ____/____/_____dd / mm / yy

INTERCOURSE

34) **Waqala uneminyaka emingaki ukwenza ucansi?** / What was your age when you had sex for the first time? |___|___|
35) **Uma ubanezinhlungu wenza ucansi chaza ukungaphatheki kahle kwakho: /** If you have pain during sex, please describe the discomfort:

    a. **Ubuhlungu obujulile** / Deep / thrusting pain [yebo] [cha] [NA if no sex the last 6 months]
    b. **Ubuhlungu uma kungena** / Superficial / upon entering [yebo] [cha] [NA if no sex the last 6 months]

36) **Hloboluni locansi owake walwenza? /** What kind of sex have you had?
    c. **Ukuphathaphatha isitho sangasese kuphela** / Petting [yebo] [cha]
    d. **Ukusoma** / Thigh sex [yebo] [cha]
    e. **Olokukhotha isitho sangasese** / Oral [yebo] [cha]
    f. **Olwasesithweni sowesifazane** / Vaginal [yebo] [cha]
    g. **Olwasembotsheni yokuzikhulula** / Anal [yebo] [cha]
    h. **Ugcine nini ukwenza ucansi**? / When was the last time you had sex? ____/____/_____dd / mm / yy
    i. **Uyisebenzisile Icondom ugcina ukwenza ucansi? /** Did you use a condom the last time you had sex? [yebo] [cha]
37) **Ucabanga ukuthi kujwayelekile ukopha kancane emva kokwenza ucansi /** Do you think it is normal to have a little bleeding after sex? [yebo] [cha] [angazi]
38) **Uke wabona wopha emva kocansi?** / Have you ever seen bleeding after sex? [yebo] [sometimes / ngesinye isikhathi] [cha]
39) **Uyalwenza ucansi ngesinye iskhathi uma usesikhathini** / Do you sometimes have intercourse during your menstrual period [yebo] [cha]
40) **Uke uzizwe ucindezelekile ukwenza ucansi nomuntu ngenxa yezipho/imali akunike yona?** / Have you ever felt pressured to have sex, because of the gifts or money you have been given ? [yebo] [cha]
41) **Wake wahlukunyezwa ngokocansi noma waphoqwa ukwenza ucansi** / Have you ever been sexually abused or forced to have intercourse? [yebo] [cha] IF YES:

42) **Sekuke kwenzeka kulezinsuku ezintathu ezidlule?** / Has it happened the last 3 days? [yebo] [cha] [NA]

Ngiyadabuka ukuzwa lokho, ngiyazi kunzima kuwena ukukhuluma ngalokhu. Kuwumthwalo onzima kakhulu ukuwuthwala ngokwakho. I am so sorry to hear that, that must have been very hard. I know it is very difficult for you to talk about this. It's a very heavy load to have to carry on your own. **Ukhona osuke wakhuluma naye ngalokhu.** Have you talked to anyone about it? [yebo] [cha]
Ungathanda ukukhuluma nomaluleki ngaloludaba? / Would you like to talk to a mentor about it? [yebo] [cha]

RELATIONSHIP AND HIV STATUS
Ngaphambi kokuba uphendule imibuzo elandelayo sifuna ukukuqinisekisa ukuthi lolulwazi luyimfihlo futhi angeke lwatshelwa noma ubani. Imibuzo elandelayo imayelana nobudlelwane kanye nesimo sakho sesandulela ngculazi. Ngiyazi eminye yalemibuzo inzima ukuyiphendula kodwa ngicela usize wenze okusemandleni akho. Lemibuzo siyibuza wonke umuntu./ Before you answer the next questions, we want to assure you that the information you give will not be told to anyone. The next questions are about relationships and HIV status I know some of these questions are hard to answer but please do your best. We are asking the same questions to everybody.

43) a) Wayeneminyaka emingaki umaqondana wakho omdala kunabo bonke? / How old was your oldest partner? |__|__|
b) Uke waba nomaqondana osokiwe? / Have any of your partners been circumcised? **(Loku kuchaza ukuthi inyama yesitho sowesilisa sangasese isusiwe futhi ikhanda laso liyabonakala ngaso sonke isikhathi /** This means that the foreskin has been removed and the penis head is visible at all times)

44) Uma ucabanga wake waba nomaqondana onesandulela ngculazi / Do you think you have had an HIV positive partner ? [yebo] [cha] [angazi]

Umaqondana / Partner	Current	former (1)	former (2)	former (3)	former (4)	former (5)	former (6)	former (7)
Yebo								
Cha								
Angazi								
Not applicable								

45) Uke waba nnmaqondana odla imishanguzo / Have you ever had a partner who is taking ARV's? [yebo] [cha] [angazi]
46) Unaye umaqondana njengamanje? / Do you have a steady partner at the moment? [yebo] [cha]
47) Usuwenze ucansi nabantu abangaki empilweni yakho? / Lifetime sexual partners? | __|__|
48) Ulale nabantu abangaki ngenyanga edlule? / Number of sexual partners you have had the last month(The last 30 days?) |__|__|
49) Ezinyangeni ezintathu ezedlule ugcina ukwenza ucansi waphuza utshwala noma izidakamizwa ? / In the past 3 months; have you had sexual intercourse under the influence of alcohol or drugs? [yebo] [cha]
50) Wake wahlolelwa isandulela ngculazi / Have you been tested for HIV? [yebo] [cha]
51) Ungakhululeka ukungitshela ngesimo sengculazi, ingabe unaso isandulela ngculazi? Would you feel comfortable telling me your status, do you have HIV? [yebo] [cha] [angazi] [Patient declined information]

UMA KUNGU YEBO: Ngiyaxolisa ukuzwa lokho, kufanele ukuthi kunzima. / IF YES: I am sorry to hear that, it must be hard for you.

52) Waze nini ngesimo sakho? / When did you get to know / / mm / yy

53) Ukhona osuke wamutshela ngalokhu? / Have you told anyone about it? [yebo] [cha

54) Ngabe CD4 count uyayihlola? / Is your CD4 count monitored? [yebo] [cha]

a) Uma kuwuyeboibingakanani CD4 count? / If yes, last CD4 count? |__|__|__|

b) Nini? / When? ___/___/ ___mm / yy

55) Uyayithatha imishanguzo? / Are you taking ARV's? [yebo] [cha]

56) Ungathanda ukuthi sinihlelele iqembu lokululekana nabanye abafundi abanesimo esifana nesakho?/Would you like us to organise a support group for HIV positive learners and would you join? [yebo] [cha] [angazi]

Wahlolwa watholwa unegciwane lengculazi futhi neCD4-count ayibhekwa. Ingabe lokhu kuyiko? / You have been tested positive for HIV and your CD4-count is not monitored. Is this correct?

Uma kungu YEBO: Sibona ukuthi uhlolisiswe ngokushesha. Ngakho ke, uzothola incwadi evela kuthina ezokusiza ukuthi uxhumane nomtholampilo./ If yes: We recommend that you have a thorough investigation as soon as possible. Therefore, you will receive a letter from us that will help you to contact your clinic.

ALCOHOL / IZIDAKAMIZWA / DRUGS

Imibuzo elandelayo ingophuzo oludakayo. Izimpendulo onginika zona angeke zatshelwa muntu. / The following questions are about drugs. The answers you give will not be told to anybody.

57) Ingabe abangani bakho bayazisebenzisa izidakamizwa? / Do your friends use drugs? [yebo] [cha] [angazi]

58) Uyazisebenzisa izidakamizwa? / Do you use drugs ? [yebo] [cha]

59) Wake wasebenzisa okunye noma okungaphezulu kwalokhu okulandelayo? / Have you ever used one or more of the following

a) **Insangu** / dagga [yebo] [cha]
b) **Yibensin** / bensin [yebo] [cha]
c) **Okunye 1** / Other 1[specify] _____
d) **Okunye 2** / Other 2 [specify] _____
e) **Izidakamiswa ezijovwayo** / Injectable drugs? [yebo] [cha]
f) **Yiglue** / glue [yebo] [cha]
g) **Umgwinyo** / ecstacy [yebo] [cha]

FAMILY AND LIVING

60) Qala ngomdala kunabobonke endlini: / Start with the oldest in the household:

Relation: M = mother. F= father. G= Legal guardian. B = brother. S = sister. Gm = grandmother. Gf = grandfather. U = uncle. A = aunt. C = cousin. Sf = Stepfather. Sm = Stepmother. Ss = Stepsister. Sb =Stepbrother. FR- friend, H=housekeeper. Ch= Child (son/daughter of study subject). Y= Husband / boyfriend of study subject. O = other. Ni = Niece. Ne=Nephew. X=study subject						
Ubani ohlala kulendlu yakini? Who lives in your house?	**Isilinganiso seminyaka** App. age	**Umsebenzi** Work		**Umfundi** Student		**Izinga lemfundo eliphezulu [Ayikho / Ephansi / Ephezulu / Ephakeme]** Top education (0=None) (1=Primary) (2=High school) (3=Tertiary) (10=don't know)
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	

INFORMATION ABOUT CLINICAL FINDINGS

Uma ucwaningo luthola lezizifo ezilandelayo ungafuna ukwazi?/ If the study discovers the following diseases, do you want to know:

1. **Izifo ezithathelana ngokocansi zingadala ukulimala kwenqondo, ukungabatholi abantwana noma umdlavuza olaphekayo. Ungafuna ukwazi uma unalezozifo /** Some sexually transmitted diseases may cause brain damage, infertility and treatable cancer. You want to know if you have such a disease [yebo] [cha] [angazi]

2. **Igciwane lengculazi liyathibeka futhi ungaphila isikhathi esideuze uguge uma wazi ngesimo sakho. Uyafuna ukwazi? /** HIV is treatable and you can live until you get old if you have your result. You want to know [yebo] [cha] [angazi]

3. **Ezinye izifo ezingatholakala ocwaningweni.Uyafuna ukwazi? /** Other diseases that maybe discovered. You want to know [yebo] [cha] [angazi]

Do you have any questions for me?

Is there anything you want to talk about?

LONG TERM TRACKING DATA PAGE

Person 1

1. **Isibongo /** Surname[s] _____
2. **Amagama** / First name[s] _____
3. **Isidlaliso / Isithakazelo / Noma elinye igama** / Other names _____
4. **Isikole** / school: _____Grade: |___|___| Section:|___
5. **Ubani uthishawakho manje?** / Who is your current teacher ?_____
6. **Usuku lokuzalwa** / D.o.b. ____/____/____dd/ mm/ yy **Wazalelwaphi?** / Where_____
7. **Uhlala kuphi isikhathi esiningi? [Ikheli lala uhlala khona] /** Where do you live most of the time? Physical address _____
8. **Ujwayele ukulala kangakanani lapha? [sonke isikhathi] [ingxenye yesikhathi] /** How often do you sleep here? [All the time] [Most of the time]
9. **Ikheli leposi** / Postal address _____
10. **Inombolo kamakhalekhukhwini** / Cell |___|___|___| - |___|___|___|___|___|___|___|
11. **Inombolo yocingo lwasekhaya** / Landline |___|___|___|-|___|___|___|___|___|__|___|
12. Email: _____
13. **Kungani ubelapha isikhathi esiningi?** / Why are you here most of the time?

    _____
14. **Ubani okunakekelayo lapha? [igama]** / Who is looking after you here? [name]

    _____
15. **Igama lombheki /** Name of a guardian_____sex [M][F]

16. **Ubuhlobo /** Relation (see below)_____
17. **Ngubani ongumninimuzi? /** Who owns the house?_____
18. **Ngubani oyinhloko yekhaya? /** Head of the household?_____
19. **Uyasebenza na? njenge?/**Are you working? As? Where ?_____

Person 2

20. **Ingabe ikhona enye indawo ohlala kuyo ngezimpelasonto, amaholide noma ezinye izinsuku?** Another place where you stay (eg weekends,holidays,other)? [**yebo] [cha**]
21. **Ujwayele ukulala kangakanani lapho? ngezinye zezinsuku zesikole ngezimpelasonto amaholide okunye [chaza] /** / How often do you sleep there? [some school days] [weekends] [holidays] [other_____
22. **Ikheli lapho ovakashela khona** / Physical address _____

    _____

    _____
23. **Ikhelileposi /** Postal address_____

    _____
24. **Inombolo kamakhalekhukhwini** / Cell |___|___|___|-| | | | | | | |

    _____
25. **Inombolo yocingo lwasekhaya** / Landline |___|___|___|-|___|___|___|___|___|__|___|
26. **Kungani uhlala lapha ngesinye isikhathi?** / Why do you stay here sometimes? _____

27. **Ubani okunakekelayo lapha? [igama] /** Who is looking after you here? (name) sex [M] [F]
28. **Ubuhlobo umama / umalume / umngani etc.** / Relation (see below) _____
29. **Ngubani ongumninimuzi?** / Who owns the house?_____
30. **Ngubani oyinhloko yekhaya?(ubuhlobo) /** Head of the household? (Relationship)

_____

Asisoze sanikeza omunye umuntu imininingwane yakho kodwa kungenzeka sifune ukukuthola. Singabuza bani? / We will never give information about you to anyone, however we may want to find you. Who should we ask?

Person 3

31. **Sicela usinike igama lomunye umuntu osondelene naye esingamthinta uma singakwazi ukukuthola ocingweni lakho. /** Please give the name of another close person we can contact if we can`t get hold of you
32. **Igama? /** Name _____sex [M] [F]
33. **Ubuhlobo** / Relation (see below)_____
34. **Kungabe uhlala endaweni efanayo njengeyakho /** Does he / she live in your area? [yebo] [cha]
35. **Ikheli lapho ehlala khona** / Physical address *(may be skipped)*_____

_____

36. **Inombolo kamakhalekhukhwini** / Cell |___|___|___|-| | | | | | | | _____
37. **Inombolo yocingo lwasekhaya** / Landline |___|___|___|-|___|___|___|___|___|_ _|___|

Person 4

38. **Uma ungase ube nohambo noma usuke kulendawo, ubani ongaba nemininingwane yakho yokukuthinta [ngaphandle kwalena engenhla]?** / If you were to travel or move away, who in your class or in school would have your contact details [other than the above]?
39. **Igama** / Name_____sex [M] [F]
40. **Ubuhlobo umama / umalume etc** / Relation (see below) _____
41. **Ikheli lapho ahlala khona** / Physical address *(may be skipped)*_____

_____

42. **Inombolo kamakhalekhukhwini** / Cell |___|___|___|-| | | | | | | | _____
43. **Inombolo yocingo lwasekhaya** / Landline |___|___|___|-|___|___|___|___|___|_ _|___|

Relation: M = mother. F= father. G= Legal guardian. B = brother. S = sister. Gm = grandmother. Gf = grandfather. U = uncle. A = aunt. C = cousin. Sf = Stepfather. Sm = Stepmother. Ss = Stepsister. Sb =Stepbrother. FR- friend, H=housekeeper. Ch= Child (son/daughter of study subject). Y= Husband / boyfriend of study subject. O = other. Ni = Niece. Ne=Nephew. X=study subject

Id. no: |___|___|___|___| **Girls Questionnaire** **Reducing**
Bilharzia Project

REDUCING BILHARZIA PROJECT

APPENDIX 5

NB: Please use a tick where there are pre-coded responses
Name of Interviewer:_____ **Date:** |___|___|___|
day month year

A. Personal data page

1. Isibongo */ Surname(s)*

_____

Amagama */ First name(s)*

_____

Nickname/praise names/other names_____

2. Uneminyaka emingaki?/ *How old are you? Age (years)* |___|___|

3. Wazalwa nini? */When were you born?* |___|___|___|
day month year

4. Wazalelwaphi? /Where were you born?

_____

School: _____*Area:*

_____

Grade: |___|___| *Section:* |___|

5. Ubani igama likathisha wakho kulonyaka?/ *What is the name of your class teacher this year?*

_____

6. Uhlala kuphi isikhathi esiningi? (Ikheli lala uhlala khona) */Where do you live most of the time? (Physical address)*_____

_____

7. Ujwayele ukulala kangakanani lapha? sonke isikhathi ☐ **ingxenye yesikhathi** ☐ /
How often do you sleep here? (All the time / Most of the time)

8. Ikheli leposi */ Postal address*

_____

a. Inombolo kamakhalekhukhwini/*Cell phone number*
|___|___|___| -|___|___|___|___|___|___|___|

b. Inombolo yocingo lwasekhaya/*Landline number* |___|___|___| -
|___|___|___|___|___|___|___|

9. Kungani ubelapha isikhathi esiningi? */ Why are you here most of the time?*

_____

a. Ubani okunakekelayo lapha? (igama)/ Who is looking after you here? *(name*)

_____ ☐☐

b. Igama lombheki */Name of a guardian*_____sex M/F

c. Ubuhlobo umama ☐ **/umalume /umngani** ☐ **omunye (chaza).** */ Relation (mother /uncle /friend /other (specify)*_____

d. Ngubani oyinhloko yekhaya? */ Who is the head of the household?*_____

***e*. Ngubani ongumninimuzi?** */Who owns the house?*

_____

10. Ingabe ikhona enye indawo ohlala kuyo ngezimpelasonto, amaholide noma ezinye izinsuku? yebo ☐ **cha** ☐ **Uma kungu CHA → 11/** */Is there another place where you stay on weekends, holidays or other days? (Yes, no) (IF NO →11)*

a. Ujwayele ukulala kangakanani lapho? ngezinye zezinsuku zesikole ☐ **ngezimpelasonto** ☐ **amaholide** ☐ **okunye (chaza)** */How often do you sleep there?(some school days,weekends, holidays, other –explain))*

_____

b. Ikheli lapho uhlala khona */ Physical address*

_____

___

_____

___

c. Ikhelileposi */ Postal address*_____

_____

___

d. Inombolo kamakhalekhukhwini/*Cell phone number*
|___|___|___||___|___|___|___|___|___|___|

e. Inombolo yocingo lwasekhaya /*Landline number* |___|___|___||___|___|___|___|___|___|___|

f. Kungani uhlala lapha ngesinye isikhathi? */ Why do you stay here sometimes?*

_____

____

g. Ubani okunakekelayo lapha? (igama)/ *Who is looking after you here? (name)*

_____ sex F/M ☐

h. Ubuhlobo umama ☐ **/umalume** ☐ **/umngani** ☐ **etc.** */ Relation (mother /uncle /friend etc.other (specify)*

_____

i. Ngubani oyinhloko yekhaya?(ubuhlobo) */ Who is the head of the household?(Relationship)*

_____

____

***j*. Ngubani ongumninimuzi?** */ Who owns the house?*

_____

11. Unaso esinye isihlobo esisondele kuwe esihlala kwenye indlu? yebo ☐ ☐

cha **uma kung CHA → 12 /** *Do you have any other close relative living in another household? (Yes, no) (If no go to 12)*

a. Ubani igama? */What is the name* _____-

_____

b. Ubuhlobo umama ☐ **/umalume** ☐ **etc** */ Relation (mother/uncle etc)*

_____

____

c. Ingabe uhlala endaweni efanayo njengeyakho yebo ☐ **cha** ☐ */Does he/she live in the same area as you? (Yes, no)*

d. Ikheli lapho uhlala khona */ Physical address*

_____

_____

____

e. Ikhelileposi */ Postal address*
_____
_____
____
f. Inombolo kamakhalekhukhwini */Cell phone number*
|___|___|___|-|___|___|___|___|___|___|___|
g. Inombolo yocingo lwasekhaya */ Landline number*
|___|___|___| -|___|___|___|___|___|___|___|

12. Uma ungase ube nohambo noma usuke kulendawo, ubani ongaba nemininingwane yakho yokukuthinta (ngaphandle kwalena engenhla)?*/If you were to travel or move away, who would have your contact details (other than the above)?*

a. Igama*/Name*
_____
b. Ubuhlobo umama☐ /umalume ☐ etc */ Relation mother/uncle etc*
_____
____
c. Ikheli lapho ahlala khona */ Physical address*_____
_____
____
d. Ikhelileposi */ Postal address*
_____
_____
____
e. Inombolo kamakhalekhukhwini*/Cell phone number*
|___|___|___|-|___|___|___|___|___|___|___|
f. Inombolo yocingo lwasekhaya */ Landline number* |___|___|___| -
|___|___|___|___|___|___|___|
13a. Ubani osayine ifomu lakho lemvume? (ubuhlobo, igama) */ Who signed your consent form? (relation, name)*
_____
b. Kungani kunguyena? */ Why this person?*
_____

14. Awukakamusho/yiphathi u/ekamama wakho noma ubaba wakho okuzalayo.
*/*You haven't yet mentioned your biological mother and/or biological father.
a. Ingabe umama wakho usaphila? Yebo ☐ cha ☐ angazi ☐ */Is your mother still alive? (Yes, no, DK)*
b. Ingabe ubaba wakho usaphila? Yebo ☐ cha ☐ angazi ☐ */Is your father still alive? (Yes, no, DK)*
Uma kungu CHA: Ngiyadabuka ukuzwa ukuthi umama noma ubaba wakho usashona. Ngizokubuza ngomzali osaphilayo.*/ I'm sorry to hear that your mother or father has passed away. I am going to ask you about the parent who is alive.*
c. Uma kungu CHA kubo bobabili abazali yiya ku15.*/ If No for both parents go to 15.*

14d.	**Igama likamama okuzalayo /** *Biological mother*	**Igama likababa okuzalayo /** *Biological father*
i) Unako ukuthintana nomama/ubaba wakho? *Do you have contact with your mother/father?*	**yebo**☐ **cha** ☐ **akwenzeki** ☐ yes no NA	**yebo** ☐ **cha** ☐ **akwenzeki** ☐ yes no NA
ii) Ingabe umama/ubaba wakho uhlala eduze kwala uhlala khona?	**yebo** ☐ **cha**☐ **akwenzeki**☐ yes no NA	**yebo** ☐ **cha** ☐ **akwenzeki** ☐ yes no NA

Does your mother/father live near you?		
iii) Ingabe yiliphi izinga lemfundo eliphezulu likamama/baba wakho? */What is your mother/father's top education?*	**Ayikho imfundo esemthethweni** ☐ No formal education **Imfundo ephansi** ☐ Primary school **Imfundo ephezulu** ☐ High school **Imfundo ephakeme** ☐ Tertiary **Angazi** ☐ Don't know	**Ayikho imfundo esemthethweni** ☐ No formal education **Imfundo ephansi** ☐ Primary school **Imfundo ephezulu** ☐ High school **Imfundo ephakeme** ☐ Tertiary **Angazi** ☐ Don't know
iv) Ubani igama likamama/baba wakho? */What is your mother/father's name?*		
v) Lithini ikheli likamama/baba wakho? */What is your mother/father's address?*		

e. Ingabe ubaba wakho uhlala nawe ekhaya? Yebo☐ cha ☐Uma kungu CHA → 15a)*/Does your father live **with you** at home? (Yes, no) **(If no → 15a)***

f. Ingabe ubaba wakho uyakusiza ngomsebenzi wakho wesikole uma udinga usizo? Yebo☐ cha ☐ */Does your father help you with schoolwork if you need help? (Yes, no)*

g. Ingabe ubaba wakho uke akujezise ngokwenza okungalungile? Yebo☐ cha ☐
/Does your father ever punish you for doing wrong? (Yes, no)

h. Ingabe lokho kuyakuvimba/gwema ukuthi ungakwenzi futhi? Yebo ☐cha ☐
/Does that stop you from doing it again? (Yes, no)

i. Ungasho uthi ubaba wakho unomthetho oqinile? Yebo ☐ cha ☐
/Would you say that your father is too strict? (Yes, no)

j. Ingabe unomthetho oqinile kunobaba womngane wakho? Yebo ☐ cha ☐ ☐ angazi
/Is he stricter that your friends' fathers? (Yes, no, D/K)

15a. Ingabe umama wakho uhlala nawe ekhaya? Yebo☐ cha ☐ Uma kungu CHA → */Does your mother live **with you** at home? (Yes, no) **(If no → 16a)***

b. Ingabe umama wakho uyakusiza ngomsebenzi wakho wesikole uma udinga usizo? Yebo ☐ cha ☐ */Does your mother help you with schoolwork if you need help? (Yes, no)*

c. Ingabe umama wakho uke akujezise ngokwenza okungalungile? Yebo ☐ cha☐
/Does your mother ever punish you for doing wrong? (Yes, no)

d. Ingabe lokho kuyakuvimba/gwema ukuthi ungakwenzi futhi? Yebo ☐cha ☐
/Does that stop you from doing it again? (Yes, no)

e. Ungasho uthi umama wakho unomthetho oqinile? Yebo ☐ cha ☐ */Would you say that your mother is too strict? (Yes, no)*

f. Ingabe unomthetho oqinile kunomama womngane wakho? Yebo ☐cha ☐ ☐ angazi */Is she stricter that your friends' mothers? (Yes, no, D/K)*

16a. Ngubani omandla ekukunakekeleni?
Who is your main caregiver?

M = mother		F = father		B = brother		S = sister	
Gm = grandmother		Gf = grandfather		U = uncle		A = aunt	
C = cousin		Sf = step-father		Sm = step-mother		Sb= step-brother	
Ss= step-sister		N = none		O = other (please list)			

16b. Ingabe uyakusiza ngomsebenzi wakho wesikole uma udinga usizo? Yebo ☐ cha☐ */Does she/he help you with schoolwork if you need help? (Yes, no)*

16c. Ingabe uke akujezise ngokwenza okungalungile? Yebo ☐cha ☐ */Does she/he ever punish you for doing wrong? (Yes, no)*

16d. Ingabe lokho kuyakuvimba/gwema ukuthi ungakwenzi futhi? Yebo ☐ **cha** ☐
IDoes that stop you from doing it again? (Yes, no)
16e. Ungasho ukuthi unomthetho oqinile? Yebo ☐ **cha** ☐ */Would you say she/he is too strict? (Yes, no)*
16f. Ingabe unomthetho oqinile kunombheki womngane wakho? Yebo ☐ **cha** ☐ **angazi** ☐ */Is he stricter that your friends' caregiver? (Yes, no, D/K)*

B. Family and living

1. Usuhlale isikhathi esingakanani lapha? |__|___|*/How long have you lived here? (years)*
2. Wake wahlala edolobheni? Yebo ☐**cha** ☐ **angazi** ☐ */Have you ever lived in a city? (Yes, no, D/K)*
Uma kungu CHA yiya ku Q5: *If no :go to Q5*
Uma kungu YEBO:*/If yes:*
3. Wawuneminyaka emingaki ngesikhathi uhlala edolobheni? */ How old were you when you lived in the city? (D/K)* **|__|__|/ angazi** ☐
4. Wahlala isikhathi esingakanani lapho? Isikhathi esingaphansi konyaka ☐ **1-5 iminyaka** ☐ **ngaphezulu kuka 5 weminyaka** ☐ */For how long did you live there?(Less than 1 year, 1-5 years, more than 5 years)*

5. Qala ngomdala kunabobonke endlini:/*Start with the oldest in the household:*

'	**Isilinganiso seminyaka** *Approximate age*	**Umsebenzi Yebo, cha,** *Work (Yes/No)*		**Umfundi (Yebo, cha,)** *Student (Yes/No)*		**Izinga lemfundo eliphezulu (Ayikho/Ephansi/ Ephezulu/Ephakeme)** *Top education (0=None/1=Primary/ 2=High/3=Tertiary)*
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	
		Yes	No	Yes	No	

M = mother
F= father
B = brother
S = sister
Gm = grandmother
Gf = grandfather
U = uncle
A = aunt
C = cousin
Sf = Step-father
Sm = Step-mother
Ss = Step-sister
Sb =Step-brother
O = other (friend, housekeeper etc)

OC = Our Child
Ne = nephew
Ni = niece

6. Ngubani omandla ekunihlinzekeni/pheni ekhaya? (igama nobuhlobo) */Who is the main provider in the household? (name and relation)*

_____
_____

C. Ukuthinta amanzi / *Water contact*

Manje ngizokubuza imibuzo ngokuthinta amanzi.
Now I will ask you some questions about water contact.

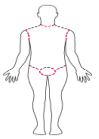
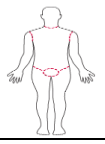
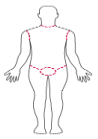
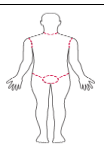
1. Uwatholaphi amanzi okuphuza? emfuleni empompini/womphakathi esiphethwini esivikelekile esiphethwini esingavikelekile kuxubene empompini ongaphakathi */Where do you get drinking water? (river, stand pipe/communal stand pipe, protected spring, unprotected spring, mixed, indoor tap)*

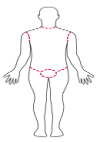
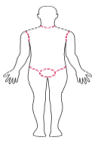
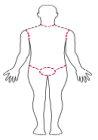
2. Ingabe amantombazane ekilasini lakho ayabhukuda emfuleni noma edamini ezinsukwini ezishisayo? yebo cha angazi */ Do girls in your class swim in the river or dam on hot days?(Yes, no, D/K)*

3. Ingabe umngane wakho omkhulu uyakwenza lokho? yebo ☐ cha☐ angazi ☐
/ Does your best friend do this?(Yes, no, D/K)

4. Uyakwenza wena? yebo ☐ cha☐ */ Do you do this? (Yes, no)*

5. Manje ngizokubuza ngezinhlobo zezinto ozenzayo ngamanzi, nizenza kangakanani, isikhathi eside kangakanani osihlala emanzini nokuthi uthintana kangakanani umzimba wakho namanzi? */Now I will ask you what kind of water activity you do, how often you do them, for how long you stay in the water and how much of your body that is in contact with the water.*

	Kangaki/ *How often?* **Daily (4)***/Daily (4)* **Kujwayele (3)***/Often(3)* **Kwesinye isikhathi (2)** */Sometimes (2)* **Kuthukela/qabukela (1)** */ Rarely (1)* **Ngeke (0)** */ Never (0)*	**Uhlala kangakanani emanzini? /** *For how long do you stay in the water?* **Ngaphezulu kuka 5 h (4)** *More than 5 h (4)* **3-5 Amahora (3)/** *3-5 hours (3)* **Ngaphansi kwamahora amathathu (2)** *Less than 3 hours (2)* **Kuze kube yimizuzu ewu-60 (1)** *Up to 60 minutes (1)*	**Umzimba uwathinta kangakanani amanzi ngesikhathi wenza lezizinto** */How much of your body is in contact with water during this activity?*
Umfula/river **Amadamu**/dam **Amanzi amile**/standing water **Amanzi avela kulezisuka**/water from these sources			
Uyadlala / Uyabhukuda?/Do you play / swim?			
Uyawasha / uyageza *Do you wash / bathe?*			
Uyazihlanza izingubo?/*Do you do laundry?*			
Uyazihlanza izingubo zokulala? /Do you wash blankets?			

Uyawakha amanzi? */Do you collect water?*			
Uyadoba?/Do you *fish?*			
Uke uwele emanzini?*/Do you ever cross the water?*			

D. Wena nomndeni wakho */ You and your family*

1. Imibuzo elandelayo ingobudlelwane nomndeni wakho kanye nontanga bakho. Ngizokufundela isitatimende kumele ucabange ngesitatimende ungitshele uma kungekona, ingxenye ingekona, ingxenye iyiqiniso noma iqiniso/ *The next questions are about your relationships with your family and peers. I will read out a statement, and you must think about the statement and tell me if it's false, partly false, partly true or true.*

1.	**Isitatimende /** *Statement*	**Akukona**/ *False*	**Ingxenye Ayikona**/ *Partly false*	**Ingxenye yiqiniso**/ *Partly True*	**Iqiniso**/ *True*
1a.	**Kukhona abantu obaziyo emndenini wakho abenza zinto ukukujabulisa**/ *There are people you know amongst your family who do things to make you happy*				
1b.	**Kukhona abantu obaziyo emndenini wakho abakwenza uzizwe uthandeka**/ *There are people you know amongst your family who make you feel loved*				
1c.	**Kukhona abantu obaziyo emndenini wakho ongathembela kubo noma kwenzekani***/There are people you know amongst your family who can be relied on no matter what happens*				
1d.	**Kukhona abantu obaziyo emndenini wakho abangabona ukuthi uyanakekelwa uma udinga lokho** / *There are people you know amongst your family who would see that you are taken care of if you needed to be*				
1e.	**Kukhona abantu obaziyo emndenini wakho abakumukela njengoba unjalo** */There are people you know amongst your family who accept you just as you are*				

1f	**Kukhona abantu obaziyo emndenini wakho abakwenza uzizwe uyingxenye ebalulekile emindenini yabo.***/There are people you know amongst your family who make you feel an important part of their lives*				
1g.	**Kukhona abantu obaziyo emndenini wakho abakuxhasayo nabakuqguqguzelayo.***/There are people you know amongst your family who give you support and encouragement*				

NB: Siza ufunde isitatimende 2 kuzozonke izitatimende ezingezansi.*/NB: Please read this for all statements below:*

2. Uma umbheki/mzali wakho ekucela ukuba uhlale ekhaya uwashe ngesikhathi engekho bese: */If your caregiver asked you to stay at home and do the washing while she/he was out, and then:*

	Isitatimende / *Statement*	**Yebo /** *Yes*	**Cha /** *No*
2a.	**Abangani bakho bakucele ukuthi uphumele emnyango uyodlala nabo ungahamba?***/Your friends asked you to come out and play with them, would you go?*		
2b.	**Abangani bakho bakucele niyobhukuda emfuleni nabo ungahamba?***/Your friends asked you to go swimming in the river with them, would you go?*		
2c.	**Umhambi akucele ukuthi uye naye esitolo niyothola amaswidi ungahamba?***/ A stranger asked you to go to the shop with him to get sweets, would you go?*		

NB: Siza ufunde isitatimende 3 kuyoyonke imibuzo yesitatimende ngasinye*/NB: Please read statement 3 for questions below each statement*

3	**Ingabe umbheki/mzali wakho usuke esekhaya uma usekhaya** */Is your caregiver at home when you are home:*	**Ngeke** *Never*	**Ngesinye isikhathi** *Sometimes*	**Izinsuku eziningi** *Most days*	**Nsukuzonke** *Every day*
3a.	**Emini** */ In the daytime?*				
3b.	**Ebusuku** */ At night?*				
3c.	**Ngempelasonto** */On the weekend?*				

NB: Siza ufunde isitatimende 4 kuyoyonke imibuzo yesitatimende ngasinye*/NB: Please read statement 4 for questions below each statement*

4	**Ingabe umbheki/mzali wakho uyathanda ukwazi /** *Does your caregiver want to know*	**Ngeke** *Never*	**Ngesinye isikhathi** *Sometimes*	**Izinsuku eziningi** *Most days*	**Nsukuzonke** *Every day*
4a.	**Ukuthi kuqhubeka kanjani esikoleni?***/How you are getting on at school?*				
4b.	**Ukuthi uyaphi uma nizikhipha nabangani bakho?***/Where you are going when you go out with friends?*				
4c.	**Ukuthi ubani ozikhipha naye?***/Who you go out with?*				

E. Ezempilo / *Health*

Imibuzo elandelayo ingezempilo yakho.*/The next questions are about your health.*

1. Ingabe uyazi siyini isichenene? yebo ☐ cha ☐ anginasiqiniseko ☐*/Do you know what Bilharzia is? (Yes, no, unsure)*

Isichenene yisifo ongasithola ngokuthinta amanzi angcolile.*/Bilharzia is an infection you can get through contact with infected water.*

2. Ingabe kukhona emndenini onaso noma owake wabanesichenene yebo ☐ cha Angazi ***/ Has anyone in your family ever had Bilharzia?***

3. Wake waba naso isichenene? yebo cha angazi */ Have you ever had Bilharzia? (Yes, no, D/K)*

Uma kunguyebo:*/If yes:*

4a. Wake walashelwa isichenene phambilini? yebo ☐ cha ☐*/Have you ever been treated for Bilharzia before?Yes, no)*

Uma kungu CHA yiya ku Q5*/If No go to Q5*

Uma kungu YEBo yiya ku4b*/ If Yes go to 4b.*

4.b Walashelwa nini isichenene (iminyaka) |___|__| |___|___| |___|___| */When were you treated for Bilharzia (age)* 1st time 2nd time 3rd time

Ngaphambi kokuba uphendule imibuzo elandelayo sifuna ukukuqinisekisa ukuthi lolulwazi luyimfihlo futhi angeke lwatshelwa noma ubani. / *Before you answer the next questions, we want to assure you that the information you give will not be told to anybody.*

5. Unako ukukhwehlela, inkinga yokuphefumula, isifo esiqhubekayo noma ukukhubazeka? Siza chaza*/Do you have a cough, breathing problems, a chronic disease or a disability? Please describe.*_____

_____

_____

6. Ingabe uyaye uye kohlolwa emtholampilo noma kudokotela? yebo ☐cha ☐ akwenzeki ☐ */ Do you regulary go to a clinic or a doctor? (Yes, no, NA*)

Ingculazi yisifo esithathelana ngegazi noma ngokuhlangana ngokocansi nasobisini lwebele. /*HIV is a disease that is transmitted through blood, sexual contact and breast milk.*

7. Wake wezwa ngengculazi? yebo ☐ cha ☐ */Have you heard about HIV before? (Yes, no)*

8. Usuke wahlolelwa ingculazi? yebo ☐ cha ☐ angazi ☐ */Have you ever been tested for HIV? (Yes, no, DK)*

9. Ingabe uyazi ukuthi unayo ingculazi? yebo ☐ cha☐ angazi☐ */Do you know if you have HIV?(Yes, no, DK)*

Uma kungu CHA yiya ku Section F*?If No go to Section F*

Uma kunguYEBO: Ngiyadabuka ukuzwa ukuthi unengculazi kodwa kukhona imishanguzo emihle yazo zonke lezizifo eziqhubekayo. */(If yes:) I am so sorry to hear that you have HIV, but nowadays there is good treatment for all of these chronic diseases.*

10. Kukhona obheka izinga lakho leCD4-count emtholampilo? yebo ☐cha ☐ angazi☐ *I is there someone at the clinic monitoring your CD4-count? (Yes, no, DK)*

F. Okuphathelene nezitho zokuzala nomchamo */ Genitalia and urine*

Mhlawumbe uyazi ukuthi abesifazane bayopha zinyanga zonke. Lokhu kubizwa ngokuthi ukuya esikhathini. Ungaya esikhathini kusukela eminyakeni eyisikhombisa (7) kuya kwengamashumi amabili (20) ubudala */ Maybe you know that women bleed every month. This is called menstruation. You can start your menstruation from 7 up to 20 years of age.*

1. Usuqalile ukuya esikhathini? Yebo ☐ cha ☐ */Have you started menstruating? (Yes/no)*

Id. no: |___|___|___|___| **Girls Questionnaire** **Reducing**
Bilharzia Project

Uma kungu YEBO yiya kuQ2*/If No go to Q2* **Uma kungu CHA yiya kuQ4***/If No go to Q4*
2. Waqala nini ukuya esikhathini |__|__|(iminyaka)*/ When did you first get menstruation(age)*
3. Uma kunguyebo, ingabe uya esikhathini njalo nje?/ Yebo ☐ cha ☐*/If yes, do you get your menstruation regularly?Yes, no*

SEBENZISA ICHART YEMIBALA BESE UFAKA INAMBA YOMBALA kuColour 2a No 2b
USE COLOUR CHART AND INSERT ONE COLOUR NUMBER FOR 2a AND ONE COLOUR NUMBER FOR 2b.

4	**Wake waba nakho okuphumayo noma okusagazana ungekho esikhathini ungasiza ukhombe ukuthi kumbala muni** / *Have you ever had discharge or trace of blood when it's not your menstruation and can you please point out the colour.*	**Njalo ngesonto** *Every week*	**Njalo ngenyanga** *Every month*	**Kanye** *Once*	**Akukaze** *Never (0)*	**Njalo (3) kwesinye isikhathi (2) akukaze (0)** */Always(3) sometimes (2) never (0)*
4a.	**Okuphumayo** /*Discahrge (Grade 1-8)*					
4b.	**Okusagazana***/Trace* of blood *(Grade 1-6)*					
4c.	**Ingabe kunephunga (njalo, kwesinye isikhathi, akukaze)** *Does it smell (always, sometimes, never)*					

Emantombazaneni isitho sangasese sinezimbobo/gudu ezintathu. Imibuzo elandelayo igxile ikakhulu embotsheni yesibili ebizwa ngokuthi yinkomo (Isitho sangasese sowesifazane)*/In girls the private parts consist of three openings. The next questions focus mostly on the second opening, called the vagina.*

5	**Wake wezwa ukungaphatheki kahle esithweni sakho sangasese njengo:** */Have you ever felt any discomfort in your private parts like:*	**Esontweni eledlule** *This last week*	**Kudala phambilini** *Sometime before*	**Akwenzeki** */Never*
5a	**Ukulunywa** */ Itch*			
5b	**Ukushisa/Ukushoshozela** */ Burn/Sting*			
5c	**Uzozo/isilonda** */ Sore (ulcer)*			
5d	**Isimila/isigaxa** */ Lump (tumour)*			
6	**Wake waba nayo inkinga noma yiphi ngokuchama njenge:***/Have you ever had any problems with urination like:*	**Esontweni eledlule** */This last week*	**Kudala phambilini** */Sometime before*	**Akwenzeki** */Never*
6a	**Zinhlungu uchama** */ Pain when you urinate*			
6b	**Ukuzwa sengathi ufuna ukuchama esithubeni** */Sudden urge to urinate*			
6c	**Iconsi lomchamo uma ugxuma, ukhohlela noma uhleka** */ Drop of urine if you jump, cough or laugh*			
6d	**Umchamo obomvu** */ Red urine*			

G. Wena nabangani bakho - Ubuhlobo */ You and your friends – Relations* **Manje ngizokubuza imibuzo ngabangane bakho nokuhlobana kwenu. Lezi yizitatimende, ngizokufundela izitatimende kufuneka ungitshele ukuthi akukona, ingxenye ayikona, ingxenye iyiqiniso, iqiniso.** */Now I will ask you some questions about your friends and relationship. These are statements, and I will read the statement for you, and you must tell me if it is false, partly false, partly true or true.*

1	**Isitatimende** */Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
1a.	**Kukhona abantu obaziyo kubangane bakho abenza izinto ukukujabulisa**/*There are people you know amongst your friends who do things to make you happy*				
1b.	**Kukhona abantu obaziyo kubangani bakho abakwenza uzizwe uthandeka.** /*There are people you know amongst your friends who make you feel liked.*				
1c.	**Kukhona abantu obaziyo kubangani bakho abangathenjwa noma kwenzakalani.** /*There are people you know amongst your friends who can be relied on no matter what happens.*				
1d.	**Kukhona abantu obaziyo kubangane bakho abangabona ukuthi unakekelekile uma udinga lokho**/*There are people you know amongst your friends who would see that you are taken care of if you needed to be*				
1e.	**Kukhona abantu obaziyo kubangane bakho abakwenza uzizwe uyingxenye ebalulekile ezimpilweni zabo** /*There are people you know amongst your friends who make you feel that you are an important part of their lives*				
1f.	**Kukhona abantu obaziyo kubangani bakho abakumukela njengoba unjalo**. / *There are people you know amongst your friends who accept you just as you are.*				
1g.	**Kukhona abantu obaziyo kubangani bakho abakuxhasayo nabakugqugquzelayo.** /*There are people you know amongst your friends who give you support and encouragement.*				

H. Okudakayo */ Alcohol*

Imibuzo elandelayo ingophuzo oludakayo. Izimpendulo onginika zona angeke zatshelwa yinoma ubani./*The following questions are about alcohol. The answers you give will not be told to anybody.*

1. Umbono kabani owazisa kakhulu mayelana nezophuza (ukusebenzisa utshwala) (abazali noma ogogo ☐/ababheki ☐ abafowenu ☐ /odadewenu abangani abanye abadala /*Whose opinion do you value most about the use of alcohol? (parents, grandparent, guardian, brother, sister, friend, other person, who (relation).*

___

2. Wake wabuphuza utshwala? Yebo cha akukhompendulo *IHave you ever drunk alcohol? Yes, no, NR*

Uma kungu YEBO yiya ku Q3*/If Yes go to Q3*

Uma kungu CHA → izitatimende ngotshwala emva kukaQ5*/If NO → statements about alcohol after Q5*

3. Yisiphi isikhathi sokuqala uzwa utshwala? (iminyaka) */When was the first time you tasted alcohol? (age)* . |__|__|

4. Ubuphuza utshwala ezinyangeni ezintathu ezedlule? yebo cha akwenzeki */Have you been drinking alcohol the past 3 months? (Yes, no, NA)*

Manje ngizokufundela izitatimende ngotshwala, kumele ungitshele uma isitatimende singekona, ingxenye ingekona, ingxenye iyiqiniso noma iqiniso / *Now I will read you some statements about alcohol, and you have to tell me if the statement is false, partly false, partly true or true.*

5	**Isitatimende** */Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
5a.	**Uma udakwa utshele abangani bakho bangacasuka baphoxeke.** / *If you got drunk and you told your friends, they would be angry and disappointed.*				
5b.	**Abangani bakho bakhuluma kakhulu ngokungabinesidingo sokuphuza utshwala.** / *Your friends talk a lot about the need to not drink alcohol.*				
5c.	**Wena nabangani bakho niyagqugquzelana ukuthi ningaphuzi (utshwala).** / *Your friends and you encourage each other not to drink.*				
5d.	**Ucabanga ukuthi akulungile ukudakwa.** / *You think it's bad to get drunk.*				
5e.	**Uma udakwa abangani bakho bangakukhathalela baqiniseke ukuthi uphephile.** / *If you got drunk, your friends would care and make sure you were safe.*				
5f.	**Wake wadakwa** /You were once drunk.				
5g.	**Ungathanda ukuthi uke udakwe** /You would like to get drunk.				

Id. no: |___|___|___|___| **Girls Questionnaire** **Reducing**
Bilharzia Project

I. Izidakamizwa */ Drugs*

1. Ingabe abangani bakho bayazisebenzisa izidakamizwa? yebo cha angazi */ Do your friends use drugs? (Yes, no, DK)*

2. Uyazisebenzisa izidakamizwa? yebo cha */ Do you use drugs?(Yes, no)*

3.Wake wasebenzisa okunye noma okungaphezulu kwalokhu okulandelayo? */ Have you ever used one or more of the following*	**Insangu** dagga	**Yiglue** glue	**Yibensin** bensin	**umgwinyo** ecstacy	**Okunye 1** Other 1 (specify)	**Okunye 2** Other 2 (specify)
	Yebo ☐ Cha ☐	Yebo ☐ Cha ☐	Yebo ☐ Cha ☐	Yebo ☐ Cha ☐		

J. Ukuziphatha ngokocansi */ Sexual behaviour*

Imibuzo elandelayo ingokuziphatha ngokocansi nokucabangayo wena nabangani bakho ngocansi. / Ngiyazi eminye yalemibuzo inzima ukuyiphendula kodwa ngicela usize wenze okusemandleni akho. */The next questions are about sexual behaviour and what you and your friends think about sex. I know some of these questions are hard to answer, but please do your best.*

1. Umbono kabani owazisa kakhulu mayelana nokuziphatha kwezocansi ? abazali umkhulu nogogo umbheki umnewenu udadewenu umngani omunye muntu (ubuhlobo nengane) */ Whose opinion do you value most with regards to your sexual behaviours? (parent, grandparent, guardian, brother, sister, friend, other person,(relationship with the child)*

_____

2. Hloboluni locansi owake walwenza? */ What kind of sex have you had?*

2a	**Ukuphathaphatha isitho sangasese kuphela** */ Petting*	Yebo	Cha
2b	**Ukusoma** */ Thigh sex*	Yebo	Cha
2c	**Olokukhotha isitho sangasese** */ Oral*	Yebo	Cha
2d	**Olwasesithweni sowesifazane** */ Vaginal*	Yebo	Cha
2e	**Olwasembotsheni yokuzikhulula** */ Anal*	Yebo	Cha
2f	**Alukho** */ None*	Yebo	Cha

Uma kungu YEBO yiya kuQ3*/If Yes go to Q3*
Uma kungu CHA kwelesitho sangasese sowesifazane noma olwesitho sokuzikhulula yeqela embuzweni 10 / *If no vaginal or anal, jump to* question 10.

3. Wawungakanani ngesikhathi wenza ucansi lwesitho sowesifazane okokuqala ngqa? (iminyaka) */ How old were you when you had vaginal sexual intercourse for the first time?(age)*

4. Empilweni yakho usuwenze ucansi nabantu besilisa abangaki? (inamba) */ During your life, with how many males have you had sexual intercourse?(number)*

5. Ezinyangeni ezintathu ezedlule usuwenze ucansi nabantu besilisa abangaki? |__|__|(abesilisa)/ *During the past 3 months, with how many males did you have sexual intercourse? (males)*

6. Uke waphuza uphuzo oludakayo noma izidakamizwa ngaphambi kokuba wenze ucansi ngesikhathi ugcina? ☐yebo ☐cha ☐NA */ Did you drink alcohol or use drugs before you had sexual intercourse the **last time?**(Yes, no, NA)*

7. Esikhathini sokugcina wenza ucansi ikhona indlela owayisebenzisayo ukuvikela ukukhulelwa? (khetha impendulo ibe yinye) / *The **last time** you had sexual intercourse, did you use a method to **prevent pregnancy**?*

7a	**Ayikho indlela eyasetshenziswa**	*No method was used*	Yebo	Cha
7b	**Amaphilisi okuhlela**	*Birth control pills*	Yebo	Cha
7c	**Amakhondomu**	*Condoms*	Yebo	Cha
14d	**Depo-Provera (umjovo wokuvikela inzalo)**	*Depo-Provera (injectable)*	Yebo	Cha
14e	**Ukukhipha (ngaphambi kokuqeda)**	*Withdrawal*	Yebo	Cha
14f	**Ezinye izindlela (yisho)**	*Other method (specify)*	Yebo	Cha
14g	**Anginasiqiniseko**	*Not sure*	Yebo	Cha

8. Wake wakhulelwa? yebo cha */Have you ever been pregnant? (Yes, no)*
9. Uma kunguyebo: *If yes*: **Usumasu/khulelwe kangaki** [__] / *How many pregnancies have you had? (number)*
10. Ingabe kukhona owake wakunika imali, izinto noma wakwenzela okuthile ngokwenza ucansi?yebo cha / *Has someone ever given you money, things or favours for having sex?*
11. Ingabe kukhona owake wakuphoqa ukuba wenze ucansi? yebo cha */Has someone ever forced you to have sex? (Yes, no)*
Uma kunguYEBO: Ngiyadabuka ukuzwa ukuthi kukhona owake wakuphoqa ukuba wenze ucansi naye. Okuningi sizokhuluma ngaloku kamuva engxoxwenimbuzo yethu. */If yes: I'm sorry to hear that someone has forced you to have sex. We will talk more about this later in the interview.*

Yikuphi kulezizitatimede ezilandelayo mayelana nemibono yezocansi okuxhaswa ngabangani bakho? Siza ukhethe isitatimende ESISODWA / *Which of the following statements regarding sexual ideas is supported by your friends? Please choose **one** statement:*	
Uma wenza ucansi akumele usebenzise ikhondomu NOMA */If you do have sex, you should not use a condom at all OR*	☐
Uma wenza ucansi kumele usebenzise ikhondomu kuphela nomuntu ongamazi NOMA */If you do have sex, you should use a condom only with someone you do not know OR*	☐
Uma wenza ucansi kumele usebenzise ikhondomu ngasosonke isikhathi NOMA / *If you do have sex, you should use a condom every time OR*	☐
Akumele nhlobo wenze ucansi / *You should not have sex at all*	☐

Imibuzo elandelayo ingawe nabangane bakho indlela abayiyo ngokwenza ucansi oluphephile. Ngizokufundela izitatimende ungaphendula ukuthi akukona, ingxenye ayikona, ingxenye iyiqiniso, iqiniso. */The following questions are about **you and your friends' attitudes towards practicing safe sex**. I will read out a statement and you can answer if it's false, partly false, partly true or true.*

12.	**Isitatimende** */Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
12a.	**Uma wenze ucansi ngaphandle kwekhondomu utshele abangani bakho bangacasuka baphoxeke.** / *If you had sex without a condom and you told your friends, they would be angry and disappointed.*				

12b.	**Abangani bakho baxoxa kakhulu ngokuzithiba noma ukwenza ucansi oluphephile njengokusebenzisa ikhondomu.** */Your friends talk a lot about the need to abstain or practice 'safe' sex i.e. use a condom.*				
12c.	**Wena nabangani bakho niyagqugquzelana ngokuzithiba noma nenze ucansi oluphephile njengokusebenzisa ikhondomu.** / *Your friends and you encourage each other to abstain or practice 'safe' sex i.e. use a condom.*				
12d.	**Uma umngani wesifazane azi ukuthi wenze ucansi uzikhiphile angeke abanandaba ukuthi uyisebenzisile noma awuyisebenzisanga ikhondomu.** / *If a female friend knew that you had sex on a date, she wouldn't care if you had used a condom or not.*				
12e.	**Uma umngani wesilisa azi ukuthi wenze ucansi uzikhiphile angeke abanandaba ukuthi uyisebenzisile noma awuyisebenzisanga ikhondomu.** / *If a male friend knew that you had sex on a date, he wouldn't care if you had used a condom or not.*				
12f.	**Uma ucabanga ukuthi omunye wabangani bakho angase alingeke enze ucansi ungabagqugquzela ukuthi bazithibe noma basebenzise ikhondomu.** / *If you think that one of your friends may be tempted to have sex, you would encourage them to abstain or use a condom.*				

K. Wena nekusasa lakho / *You and your future*

Imibuzo elandelayo ingendlela ozizwa ngayo ngekusasa. */The following questions are about your feelings and* ***attitude towards the future.***

1. Ezinyangeni ezingu 12 ezedlule uke wazizwa udangele uphelelwa yithemba cishe zonke izinsuku amasonto amabili noma ngaphezulu kulandelana ingangokuthi wayeka ukwenza ojwayele ukukwenza? yebo cha akwenzeki / *During the past 12 months, did you ever feel so sad or hopeless almost every day for two weeks or more in a row that you stopped doing some usual activities?*

2. Manje ngifuna sikhulume ngemizwa yakho esontweni eledlule. Ngizokufundela izitatimende kumele uphendule uma kungekona, ingxenye ingekona, ingxenye iyiqiniso noma iqiniso. / *Now I want to talk about your feelings the last week. I will read out a statement and you have to answer if it's false, partly false, partly true or true.*

	Isitatimende/*Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
2a	**Ungamane udele ngoba akukho ongakwenza ukuzenzela izinto kangcono.** / *You might as well give up*				

	because there is nothing you can do about making things better for yourself.				
2b	**Uma izinto zizimbi uyasizwa ukwazi ukuthi angeke zahlala zinjalo unomphela***/When things are going badly, you are helped by knowing that they cannot stay like that forever*.				
2c	**Ulindele ukuphumelela ngokufunayo ngengomuso.** /*In the future, you expect to succeed in what you want.*				
2d	**Ikusasa lakho kuwena libukeka limnyama lingenathemba.** / *Your future seems dark and hopeless to you.*				
2e	**Ikusasa lakho kuwena linokungacaci futhi liyakungabazisa .** /*The future seems vague and uncertain to you.*				
2f	**Ulilangazelele/phokophelele/ ikusasa lakho.** / *You look forward to your future*.				
2g	**Akusizi ukuzama ukuthola noma yini oyifunayo ngoba vele angeke uyithole***/There's no use in really trying to get anything you want because you probably won't get it.*				

3. Imibuzo elandelayo ingendlela ozizwa ngayo ngawe. Ngizofunda isitatimende kumele uphendule ukuthi akukona, ingxenye ayikona, ingxenye iyiqiniso noma iqiniso. / *The following questions are about **how you generally feel about yourself**. I will read out a statement and you have to answer if it's false, partly false, partly true or true.*

	Isitatimende*/Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
3a	**Uzizwa ungumuntu obalulekile.** / *You feel you are a person of worth.*				
3b	**Uzizwa ungumuntu onezinto ezinhle zeqophelo (izenzo).** / *You feel you have many good qualities*.				
3c	**Kukonke uzizwa ukuthi uyisahluleki** */All in all, you feel that you're a failure.*				
3d	**Uzwa ukuthi uyakwazi ukwenza izinto njengabanye abantu abaningi.** / *You feel you are able to do things as well as most other people.*				
3e	**Uzwa ukuthi awunakho okungakanani ongabaneqholo ngako.** /*You feel you do not have much to be proud of.*				
3f	**Sekukonke ugculisekile ngawe.** / *On the whole you are satisfied with yourself*.				
3g	**Ufisa sengathi ungaba nokuzihlonipha okuthe xaxa.***/You wish you could have more respect for* yourself.				

4. Izitatimende ezilandelayo zingendlela owenza ngayo uma ubhekene nezingqinamba. Emva kokuba sengizifundile lezizitatimende, siza uphendule uma kungekona, ingxenye ingekona, ingxenye iyiqiniso noma iqiniso.*/The next statements are about* ***how you react when facing difficulties****./ After I've read the statement, please answer if it's false, partly false, partly true or true.*

	Isitatimende*/Statement*	**Akukona** *False*	**Ingxenye Ayikona** *Partly false*	**Ingxenye yiqiniso** *Partly True*	**Iqiniso** *True*
4a	**Uma unenkinga ucabanga ukuthi ungayixazulula.** / *When you have a problem, you think you can solve it.*				
4b	**Uma omunye ekuphikisa ungazithola izindlela zokuthola okufunayo.** / *If someone opposes you, you can find the ways to get what you want.*				
4c	**Kulula kuwena ukugxila ezinhlosweni zakho futhi ufeze izinjongo zakho.** / *It is easy for you to stick to your aims and accomplish your goals.*				
4d	**Uyazethemba ukuthi ungamelana nezimo ongazilindele.** / *You are confident that you could deal efficiently with unexpected events.*				
4e	**Ungaxazulula izinkinga eziningi uma ungenza imizamo efanele.** / *You can solve most problems if you invest the necessary effort.*				
4f	**Ungabeka umoya phansi uma ubhekene nezinkinga ngoba ungathembela ekwazini ukubukela.** / *You can remain calm when facing difficulties because you can rely on your coping abilities.*				
4g	**Uma ubhekwe yinkinga ujwayele ukuthola izixazululo eziningi.** / *When you are confronted with a problem, you can usually find several solutions.*				

<u>L. Okunye ukubamba iqhaza</u> */ <u>Further participation</u>*

Sithanda ukukubonga ngokubamba kwakho iqhaza kulokhu. Sizama ukusiza abanye ngalolucwaningo. */ We want to thank you for participating in this. We are trying to help others by this research.*

2. Singathanda ukuba sithintane nawowonke amantombazane ngokuzayo.Ungasisiza ukugcina umkhondo wabangani bakho? yebo☐ cha ☐ NA☐ */ We would like to contact all the girls in the future. Would you help us keep track of your friends? (Yes, no, NA)*

3. Ingabe uyathanda ukungenela olunye ucwaningo lapho sikuhlola khona futhi? Lokhu kuzosisiza sifunde okuningi ngesichenene ☐bo ☐cha ☐ NA
/ Are you interested in joining an extra project where we take a few more tests from you? This will help us to learn more about Bilharzia.(Yes, no, N/A)

Summery of questions with a star attached: (Read only the part(s) that corresponds with what the participant mentioned earlier on)
Ngesikhathi sengxoxombuzo ngikubuze imibuzo enzima ukuba uyiphendule. Ngiyabonga ngezimpendulo zakho ezithembekile. Ungitshele ukuthi:*/During the interview I've asked you questions that are hard to answer. I thank you for your honest answers. You told me that:*

- **4. Wahlolwa watholwa unegciwane lengculazi futhi neCD4-count ayibhekwa. Ingabe lokhu kuyiko?***/ You have been tested positive for **HIV** and your CD4-count is not monitored. Is this correct?*

  Uma kunguYEBO: Sifuna ukuthi umbheki wakho azi ngalokhu, sibonisa ukuthi uhlolisiswe ngokushesha okukhulu. Ngakhoke, uzothola incwadi evela kithina ezokusiza wena nombheki wakho ukuba nithintane nomtholampilo.*/If yes: We want your caregiver to know about this, and we recommend that you have a thorough investigation as soon as possible. Therefore, you will receive a letter from us that will help you and your caregiver to contact a clinic.*

- **5. Usuke wahlangabezana nokungaphatheki kahle esithweni sakho sangasese. Singathanda ukukusiza ngaloko. Lokho kusho ukuthi kumele uthintane nomthalampilo uthole okokwelashwa ukuze kuphele lokhu kungaphatheki kahle.***/You have experienced **discomfort in your private parts**. We would like to help you with this. That means you should get in contact with a clinic and get the necessary treatment to take away the discomfort.*

- **6. Uye waphoqwa ukwenza ucansi. Akekho onemvume yokuba akuphoqe ukuthi wenze ucansi naye, futhi ngiyadabuka kakhulu ukuzwa ukuthi usuke wahlangabezana nalokho. Uyafisa ukukhuluma ngalokho nomunye osebenza nathi? Uma ungakakulungeli ukuba ukhulume ngako, ungathola inombolo yocingo ongasithinta kuyo uma ushintsha umqondo.***/You have been **forced to have sex**. No one is allowed to force you to have sex with them, and I'm sorry to hear that you've experienced this. I know it is very difficult for you to talk about this. It's a very heavy load to have to carry on your own. And I wonder if you would like some help from somebody… We would like to offer you …… If you're not ready to talk about it now, you can get a phone number that you can call if you change your mind.*

APPENDIX 6

LIST OF ORAL/POSTER PRESENTATIONS

BRIGHT Scientific Day: Ramsgate Durban. South Africa. 12/11/2015. An oral presentation entitled: Liquid Based Cytology as a diagnostic tool for a more precise risk- assessment of schistosomiasis and cervical squamous cell atypia among young women from *Schistosoma* and HIV endemic populations in South Africa. Pillay P, Taylor M, Galappaththi-Arachchige HN, Zulu SG, Roald B, Kjetland EF. Durban University of Technology, South Africa, [2] University of KwaZulu Natal, South Africa, [3] Oslo University Hospital, Norway

International Scientific Workshop on Neglected Tropical Diseases: Female Genital Schistosomiasis and its impact on HIV/AIDS. Magaliesburg, South Africa. 28/01/2015. An oral Presentation entitled: Cytology and PCR as diagnostic tools for Female Genital Schistosomiasis in a population of young women aged 16-21years from rural South Africa. P Pillay[1, 2], M Taylor[2], SG Zulu[2], SG Gundersen[3], E Kleppa [4], K Lillebo[4], EF Kjetland[2, 4], L van Lieshout[5], B Roald [4]. Durban University of Technology, South Africa, [2] University of KwaZulu Natal, South Africa, [3] University of Agder, Norway [4] Oslo University Hospital, Norway, [5] Leiden University Medical Center, The Netherlands

16th International Congress on Infectious Diseases held in Cape Town, South Africa from April 2 to 5, 2014. Poster Presentation title: Schistosoma PCR among high school girls in South Africa as a complimentary diagnostic tool for Female Genital Schistosomiasis (FGS) Authors: **P Pillay[1,2]**, M Taylor[2], SG Zulu[2], SG Gundersen[3], E Kleppa [4], K Lillebo[4], EF Kjetland[2,4], EAT Brienen[5], L van Lieshout[5] Affiliations: [1] Durban University of Technology, South Africa, [2] University of KwaZulu Natal, South Africa, [3] University of Agder, Norway [4] Oslo University Hospital, Norway, [5] Leiden University Medical Center, The Netherlands

9th Public Health Association of South Africa (PHASA) 25-27TH September 2013. Poster Presentation title: The use of PCR and extensive urine microscopy in the diagnosis of Schistosomiasis among schoolgirls in KwaZulu Natal. **[1]P Pillay**, [2]M Taylor, [2]SG Zulu, [3]J.J. Verweij, [3]P Hoekstra, [3]EAT Brienen[3], [4,5]SG Gundersen, [6]E Kleppa, [6]E F Kjetland, [3]L van Lieshout. Affiliations: [1] Durban University of Technology, South Africa, [2] University of KwaZulu Natal, South Africa, [3] University of Agder, Norway [4] Oslo University Hospital, Norway, [5] Leiden University Medical Center, The Netherlands

8th European Congress on Tropical Medicine and International Health, Copenhagen, Denmark, September 10-13, 2013. Oral Presentation title: Real-time *Schistosoma* PCR in vaginal lavage and urine of high school girls in South Africa as an indicator of Female Genital Schistosomiasis (FGS)

P Pillay[1,2], M Taylor[2], SG Zulu[2], SG Gundersen[3], E Kleppa [4], K Lillebo[4], EF Kjetland[2,4] , EAT Brienen[5] , L van Lieshout[5]

VIBE Scientific Day on the 10TH January 2013 at UKZN. Oral Presentation title: PCR for Schistosomiasis of vaginal lavage in young women. Feasibility and usefulness. **P Pillay**[1,3], M Taylor[3], J Verweij [5], G van Dam [5], SG Gundersen[4], EF Kjetland[2,3], E Brienen[5], SG Zulu [3], E Kleppa [2], L van Lieshout[5]

VIBE Scientific Day on the 9th of March 2012 at UKZN. Oral Presentation title: "Bilharzia in the urinary and genital tracts detected by PCR" **P Pillay**, M Taylor, J Verweij, G van Dam,SG Gundersen, EF Kjetland, E Brienen,S Zulu, E Kleppa, K Lillebo, L van Lieshout

American Society of Tropical Medicine and Hygiene, 61ST Annual Meeting Oral Presentation, -11-15 /11/12: Presented a paper entitled: Comparing high-throughput quantitative detection of *Schistosoma*-DNA using real-time PCR and extensive microscopy in urine samples from primary school girls in coastal KwaZulu Natal. **P Pillay**[1,3], M Taylor[3], J Verweij [5], G van Dam [5], SG Gundersen[4], EFKjetland[2,3], E Brienen[5], S Zulu [3], E Kleppa [2], L van Lieshout[5]

American Society of Tropical Medicine and Hygiene, 59th Annual Meeting Oral Presentation, 3-7 /11/10: Presented a paper entitled: The role of pap smears in young females from a Schistosomaisis endemic area. **P Pillay**[1,3], M Taylor[3], SG Gundersen[4], EF Kjetland[2,3], SG Zulu [3], E Kleppa [2]

Public Health Association of South Africa, 6th Conference Poster Presentation, 29/11/10: Presented a poster entitled: Female genital schistosomiasis (FGS) as an epidemiological risk factor for squamous cell atypia and genital disease in a longitudinal cohort of young women in KZN. [1,3] **P Pillay,** [2]E Kleppa, [3] CC Jinabhai , [4,5]SG Gundersen,[3] M Taylor, [2,6] EF Kjetland.

Society for Medical Laboratory Technologists South Africa. Oral Presentation, 7/09/09, Cervical screening in urban clinics in the eThekwini Municipal Area, **Pillay P**, Knight S, Rmiah WNS.