COMPARISON OF PERFOMANCE AND FACTORS THAT AFFECT CIRCULATING CATHODIC ANTIGEN, KATO-KATZ AND ELISA TESTS FOR *SCHISTOSOMA MANSONI* DIAGNOSIS IN SCHOOL CHILDREN IN WESTERN KENYA

BY

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THE SCHOOL OF PUBLIC HEALTH AND COMMUNITY DEVELOPMENT

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DECLARATION

This thesis is my original work and has not been presented to any other university or institution for a degree or any other award.

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DEDICATION

To my father Pius Ochola, mother Florence Ochola, and siblings Fredrick, Stella, Joyce, Helen, Ambrose and James for their love and encouragement.

ABSTRACT

Schistosomiasis infects approximately 207 million people worldwide with 90% of the cases occurring in Africa. Kato-Katz, is the standard method of *S.mansoni* diagnosis, however it is characterized by low sensitivity. Another method of diagnosis is the antibody ELISA, which is highly sensitive but lacks specificity. The PCR test is 98% sensitive and 100% specific but it is costly for use in limited resource settings. The high sensitivity and specificity of the PCR makes it a useful gold standard for evaluation of new diagnostic tests. Children below 14 years are mostly affected by schistosomiasis and Asembo region in western Kenya is where most of these infections are found. This study which was cross-sectional in nature aimed to compare the sensitivities and the specificities of POC/CCA, ELISA, and Kato-Katz against PCR in the detection of *S.mansoni* in school children in Asembo, Rarieda district. It further looked at factors that may affect the performance of the POC/CCA, ELISA and Kato-Katz tests. A sample of school children (n=950) provided stool, urine, and blood samples. Stool samples were used for Kato-Katz and PCR testing, urine samples for POC/CCA testing and finally the blood samples for antibody ELISA. The sensitivity of a test was defined as the proportion of the participants with the disease who had a positive PCR result. Similarly, specificity was defined as the proportion of the participants without the disease who had a negative PCR result. McNemar's test was used to determine whether there were row and column marginal frequencies between the PCR positive and negative groups in respect to soil-transmitted helminths (STHs), hematuria, pyuria and malaria. Results showed that POC/CCA had a sensitivity of 78.30% while Kato-Katz and ELISA had sensitivities of 70.44% and 50.16%, respectively. Kato-Katz had a specificity of 92.70% while POC/CCA and ELISA had specificities of 61.20% and 85.11%, respectively. In order to assess if infection with STHs (*Trichuris trichiura*, *Ascaris lumbricoides*, and hookworm) had any effect on the performance of the POC/CCA tests, responses were compared between individuals who were PCR positive and infected with the helminths and those who were PCR positive but not infected with the helminthes, respectively. The results showed no significant differences in percentages of positive POC/CCA with *Trichuris trichiura* at 76.3% vs. 78.6 % (P=0.8338), POC/CCA positive with hookworm at 81.8% vs. 78.2% (P=1.000) and POC/CCA with *Ascaris lumbricoides* at 66.7% vs. 78.9 % (P=0.3315). These results showed that STHs infections did not affect the performance of POC/CCA tests. Similar comparisons were also made to determine the effect of hematuria, pyuria and malaria on POC/CCA performance. The results showed no significant difference in percentages of positive POC/CCA with hematuria cases at 100% vs. 88.8 % (P=1.0000), POC/CCA with pyuria at 100% vs. 88.3 % (P=1.0000) and POC/CCA with malaria at 72.7% vs. 83.4 % (P=0.0862). Findings presented here showed that POC/CCA was the most sensitive test when screening for *S.mansoni* in high prevalence areas. Co-infections did not affect the sensitivities of the tests. However, there is need to further improve on the specificity of the POC-CCA.

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ABBREVIATIONS

Abbreviation	Meaning
CAA	Circulating Anodic Antigen
CCA	Circulating Cathodic Antigen
CDC	Centers for Disease Control
CSEA	Circulating soluble egg antigen
DALY	Disability-adjusted life years
ELISA	Enzyme Linked Immunosorbent Assay
IL	Interleukin
KEMRI	Kenya Medical Research Institute
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
POC	Point of Contact
STH	Soil Transmitted Helminthes
TT	*Trichiura trichiura*
SWAP	Schistosome Worm Antigen Preparation
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1. Background

Schistosomiasis and soil-transmitted helminths (STHs) are attracting an increasing interest among health researchers, policy makers and donor agencies due to their impact on subtle and severe morbidity in endemic regions. Approximately 207 million individuals worldwide are living with schistosomiasis (Steinmann *et al.*, 2006), with 90% of the world-wide schistosomiasis cases occurring in sub-Saharan Africa (Hotez and Kamath, 2009; Steinmann *et al.*, 2006). Out of the approximated 150 million schistosomiasis-infected Africans, an estimated 5 million live in Kenya; moreover 12 million of the Kenyan population is at risk of acquiring the infection (Foo *et al*., 2015). In a previous survey of 1,246 covering 10–12 years old children in 32 primary schools in Kenya near Lake Victoria, it was found that the mean school prevalence of *Schistosoma mansoni* infection was 16.3% (range = 0–80%), with 63% of students infected with one or more geohelminths (Handzel *et al.*, 2003). Accurate diagnosis of infection is essential for the coordination of control programs to high risk individuals such as school children who are prone to re-infection with schistosomiasis.

In 2001, the World Health Assembly (WHA) passed resolution 54.19, which endorsed regular treatment of high-risk groups, particularly school age children, as the best way of reducing morbidity and mortality (WHO, 2001). Under the World Health Organization guidelines, the decision to treat all persons (community-based treatment) or only school children and other high-risk groups (selective treatment) depends on the prevalence of infection in a particular region (WHO, 2001). Affordable diagnostic tools that can identify high-risk populations and prioritize

such groups for mass treatment are required. This need has raised a great interest in the health research community (Hotez and Fenwick, 2009), which is responding by evaluating currently available diagnostic tools.

Diagnosis of schistosomiasis is usually performed by Kato-Katz technique which involves microscopic detection of eggs (Katz *et al*., 1972). This technique continues to be the standard method for assessing schistosomiasis prevalence and intensity (Kongs *et al*., 2001). However, due to the day to day fluctuations in egg counts, low infection levels can easily be missed and this in turn reduces the sensitivity of the test (Engels *et al.*, 1996). This methodology is time consuming, its accuracy is dependent on the microscopists' skill, and it can expose laboratory workers to potentially harmful fresh stools which can contain infectious agents. Therefore, there is need for an evaluation of a more sensitive test (Barreto, 1978).

Antibody detection tests such as ELISA usually require more advanced laboratory settings but may yield a higher sensitivity. Specificity may also be a problem with ELISA since specific antibodies continue to be present long after the worms have been eliminated from the body (Ayele *et al.*, 2008).

Antigen detection of *Schistosoma* using circulating anodic antigen and circulating cathodic antigens (CAA and CCA) in blood and urine have many advantages, such as the demonstration of active infections, since the circulating antigens are produced by a live adult worm inside the host making the antigens not to persist after treatment compared to antibodies (Ayele *et al.*, 2008). A field-based point of contact circulating cathodic antigen (POC/CCA) assay is currently commercially available from Rapid Medical Diagnostics (Pretoria, South Africa). This test is a highly sensitive assay (van Dam *et al.*, 2004).

The POC/CCA tests detects one of the major antigens regurgitated by the adult worm inside the hosts (Legesse and Erko, 2008). This test is more specific to *S.mansoni* diagnosis but can be used in diagnosis of moderate to high level infections of *S.hematobium* and *S.japonicum* (Stothard *et al.,* 2006).

A previous evaluation of the POC/CCA test done in Usoma village in western Kenya showed that the test was more sensitive compared to Kato-Katz in detecting *S.manson*i infections in children, however its specificity was questionable (Verani *et al*., 2011). However, the evaluation of the sensitivity and specificity of the test in high *S.manson*i prevalence settings remains unknown.

A real-time PCR assay has been evaluated as an alternative diagnostic method for schistosomiasis, and has further been shown to be highly sensitive and 100% specific (Oliveira *et al*., 2010). Real-time PCR testing can detect as few as 2.4 eggs per gram of feces, making it 10 times more sensitive than the Kato-Katz examination (ten Hove et al., 2008). However, the procedure is expensive and requires more elaborate laboratory set-up (Pontes *et al*., 2002). In order to compare the different diagnostic approaches for *S.mansoni*, a gold standard test which is highly sensitive and specific was required to be the standard of comparison for the evaluation.

Cross-reactivity with other parasitic helminths is also a concern with regard to specificity of the POC/CCA since the test is under evaluation (Stothard *et al*., 2009). Very few studies have looked at the effects of cross-reactivity on the POC/CCA. Pyuria and hematuria which are symptoms of urinary tract infections have been found to be associated with false positive results in urine-based CCA and CAA assays (Zamanzad, 2009). So far Pyuria and hematuria are the only two factors that have been identified to affect the performance of the POC/CCA. However

there was need to confirm if there were additional factors that could affect the performance of the POC/CCA. Western Kenya is an area endemic for malaria and soil transmitted helminths, which presents together with schistosomiasis therefore it was worthwhile to investigate if such factors could affect the performance of the POC/CCA.

Generally, there was need to compare how these different tests performed using samples collected from school children living in areas of high schistosomiasis prevalence in western Kenya. Although various studies had previously compared a number of different diagnostic methods, this study included the comparison of a newly developed antigen detection test (POC/CCA) against a very highly sensitive and specific real-time PCR. The different assays were evaluated in a group of school children living in areas of >25 % schistosomiasis prevalence around Lake Victoria, western Kenya.

1.2. Problem Statement

Currently, there is a great need for affordable and easy to use point of contact diagnostic tools for schistosomiasis testing. Lack of such tools in turn compromises the ability to accurately assess the impact of control programs, which makes it hard to monitor and evaluate such activities (Shane *et al*., 2011). Asembo region of Rarieda District is in close proximity to Lake Victoria which is the major source of schistosomiasis infection (Handzel *et al*., 2003), and has the highest prevalence of the disease. The age group of 8-12 years is most vulnerable to schistosomiasis related morbidity (Verani *et al*., 2011). Therefore, there is need to evaluate tests which are more sensitive than the current Kato-Katz method. Studies have shown that infection with STHs, blood in urine (hematuria), and white blood cells in urine (pyuria) may affect the performance of diagnostic tests (Shane *et al*., 2011). However, it remains unknown whether these parameters

have any influence on the performance of the tests. As such, the current study compared the sensitivities and the specificities of the different methods (POC/CCA, ELISA, Kato-Katz and PCR) methods used in the detection of *S.mansoni* in school children in areas of high schistosomiasis prevalence in western Kenya and tested whether co-infections had any influence on the tests in an *S.mansoni* high prevalence area in western Kenya.

1.3. Objectives of study

1.3.1. Broad objective

To compare the sensitivities and the specificities of circulating cathodic antigen, Kato-Katz and ELISA tests against PCR for *Schistosoma mansoni* diagnosis and identify factors that may affect performance of the tests in school children living in areas of high schistosomiasis prevalence in western Kenya.

1.3.2. Specific objectives

1) To evaluate the performance of the POC/CCA, ELISA and Kato-Katz assay against PCR in school children living in areas of high prevalence settings.
2) To identify factors that may affect the performance of the POC/CCA, ELISA and Kato-Katz tests.

1.3.3. Research questions

1. What are the performance of the POC/CCA, ELISA and Kato-Katz assay against PCR in school children living in areas of high prevalence settings?
2. What are the factors that may affect the performance of the urine POC/CCA, ELISA and Kato-Katz tests in school children living in areas of high prevalence settings?

1.4. Significance of Study

Diagnostics are important for rapid identification of high risk communities who are in need of regular treatment and follow up patient management. Proper diagnostics are essential for monitoring progress of control interventions and drug efficacy during mass drug administration. The results from this study have the potential to inform on the most sensitive and easy to use diagnostic tool that can be applied to screen high-risk populations and prioritize such communities for schistosomiasis interventions. Findings of this study also provide a clear understanding on whether co-infection with other diseases such as malaria and soil-transmitted helminths has any effect on sensitivities and specificities of the various tests. Both findings are important for policy makers in *S.mansoni* endemic areas.

CHAPTER TWO

LITERATURE REVIEW

2.1. Diagnosis of schistosomiasis

Despite the wide global distribution of schistosomiasis in Sub-Saharan Africa, there is still need for proper diagnosis of the disease (Pillay *et al*., 2014). As the control programs are boosted, highly sensitive tools need to be put in place to diagnose the very low intensity levels. This reduction in prevalence levels compromises on the sensitivity of parasitological tests such as Kato-Katz (Knopp *et al*., 2009). The design of an effective control program relies heavily on accurate estimate of infection(Hotez and Kamath, 2009). The efficiencies gained from focused drug administration, drug efficacy measurements, pharmacovigilance, impact monitoring, and integration with other infection control programs are all contingent upon accurate diagnostic assessment (Utzinger *et al*., 1998).

Accurate assessment of the prevalence of *S. mansoni* is important for the design and evaluation of control programs. The most widely used tools for diagnosis have been found to be limited by suboptimal sensitivity, slow turn-around-time, or inability to distinguish current from former infections (Sandoval *et al*., 2006; Shane *et al*., 2011).

The Kato-Katz technique, which is a (semi) quantitative stool examination technique, has been generally recommended for diagnosis and evaluation of *S. mansoni* infection due to its relatively simple technology and low material cost (Katz *et al*., 1972). However, the cost of labor is high as the process is time-consuming and must be performed by trained microscopists', who in addition may be exposed to infectious agents when handling fresh stools (Kongs *et al*., 2001).

Egg detection using this method is largely dependent on the skill of the technician. Moreover, results are subject to intra-specimen variations in egg distribution and daily fluctuations in egg excretion (Gower *et al*., 2012). Therefore, there was a need to evaluate a test that is highly sensitive compared to Kato-Katz, relatively easy to use (doesn't require skilled labour), and has minimal intra-specimen variation.

Antibody detection techniques such as enzyme-linked immunosorbent assays (ELISA) offer improved sensitivity over direct microscopic techniques but they require well equipped laboratories and highly trained technicians (Kongs *et al*., 2001). In addition, antibody levels vary across individuals. Antibodies do not always correspond with worm burden, and typically persist in the bloodstream for years after an infection has cleared (Doenhoff *et al*., 1985). As a result, current antibody detection methods are generally unable to distinguish between light and heavy infection or current and past infection, severely limiting their utility as operational tools ((Stothard *et al*., 2009). Cross-reactivity with other parasitic helminths is also a concern with regard to specificity of these techniques and this needs to be further investigated (Shane *et al*., 2011).

Circulating cathodic antigen (CCA) is one of the major antigens regurgitated by the adult worm inside the host (Ayele *et al.*, 2008). The major portion of CCA released by the live adult parasite is secreted in the urine. A positive CCA test indicates an active schistosomiasis infection (Erko *et al*., 2013).

The POC/CCA cassette works on the principle that the CCA antigen present in the urine sample binds to the labeled monoclonal antibody immobilized on the sample membrane. The urine sample is left to run over the strip where the antigen-antibody complex attaches to another

monoclonal antibody immobilized at the test line. A pink colored test line usually develops if there is binding. The second line which is a procedural control always shows up to make sure that the test works correctly (van Dam *et al.*, 2004). The intensity of the line is semi-quantitatively related to the intensity of infection (Lieshout, 1994). The POC/CCA test is easy to use in field setups and the cassettes do not require special storage conditions thus can be stored at room temperature for up to one year (van Dam *et al.*, 2004).

A study carried out in Egypt to detect CCA in serum of infected individuals and compare it to the ELISA test of people infected with both *S. mansoni* and *S. haematobium*, showed that ELISA and CCA tests had a sensitivity range of 91-98% (Corachan, 2002). This study suggested that ELISA and CCA tests were better methods of diagnosing schistosomiasis but recommended that future studies need to be carried out to determine if the specificity of the two tests are adequate (Noya *et al*., 2002).

Recently, an internal transcribed spacer (ITS) - based *Schistosoma*- specific real-time polymerase chain reaction (PCR) technique was developed. This PCR technique amplifies and detects a highly repeated *S. mansoni* DNA sequence present in human fecal samples (Obeng *et al.*, 2008). This test has a sensitivity of 98% and a specificity of 100% (Pillay *et al*., 2014). However, this procedure is not appropriate for regular use in control programs as it is expensive and requires a more sophisticated laboratory than what is available in most *S. mansoni*-endemic field settings (Pillay *et al*., 2014). The real-time PCR makes a better gold standard when evaluating new tests for schistosomiasis control (ten Hove *et al*., 2008). The high sensitivity of this approach makes it possible to detect parasite DNA in fecal samples containing as few as 3 eggs per gram of feces

and this makes it 10 times more sensitive than the Kato-Katz examination (Aryeetey *et al*., 2013) .

A study carried out in an endemic region of Brazil, comparing Kato Katz and PCR showed that PCR gave highly sensitive results compared to 3 consecutive stool samples diagnosed by Kato-Katz. PCR was able to detect 41.6% of the positive cases that were misdiagnosed as negative results by Kato-Katz (Pontes *et al*., 2003). There is need to further study if PCR can be used for diagnosing of schistosomiasis in discordant samples (Pontes *et al*., 2003). Despite the above literature, the sensitivities and specificities of POC/CCA, ELISA, and Kato-Katz against PCR in school children living in areas of high prevalence settings remain unknown. As such, the current study evaluated the performance of POC/CCA, ELISA, and Kato-Katz against PCR in school children living in areas of high prevalence settings.

2.1.1. Factors affecting performance of the POC/CCA tests

Pyuria and hematuria which are symptoms of urinary tract infections have been found to be associated with false positive results in urine-based CCA and CAA assays (Zamanzad, 2009). Previous studies have evaluated only pyuria and hematuria and found them to be associated with false positives for POC/CCA assays (Keiser *et al*., 2002). In contrast to this, a study carried out in Ivory Coast found that POC/CCA had no cross-reactivity to *S. haematobium* and hematuria (Coulibaly *et al*., 2011) but recommended that future studies be done to confirm this. Therefore, there was need to test whether hematuria, pyuria and factors such as malaria and STH had any effect on the POC/CCA tests. As such, the current study evaluated factors that may influence the performance of the urine POC/CCA, ELISA and Kato-Katz tests.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study site

The study was conducted in Asembo division (latitude-.0.2000000; longitude 34.3333300) of Rarieda district along the shores of Lake Victoria in Nyanza province of western Kenya (Figure 1) from October 2012 to February 2013. Asembo Division covers an area of approximately 200km^2 and consists of 79 villages with an estimated population of 57,000km. The region has 58 primary schools. Approximately 96% of the populations belong to the Luo ethnic group. The majority of the residents of Asembo are subsistence farmers and fishermen.

Previous schistosomiasis studies conducted in the region associated high *S .mansoni* infection levels with proximity to Lake Victoria (Handzel *et al*., 2003). Asembo region has not had any history of mass drug administration.

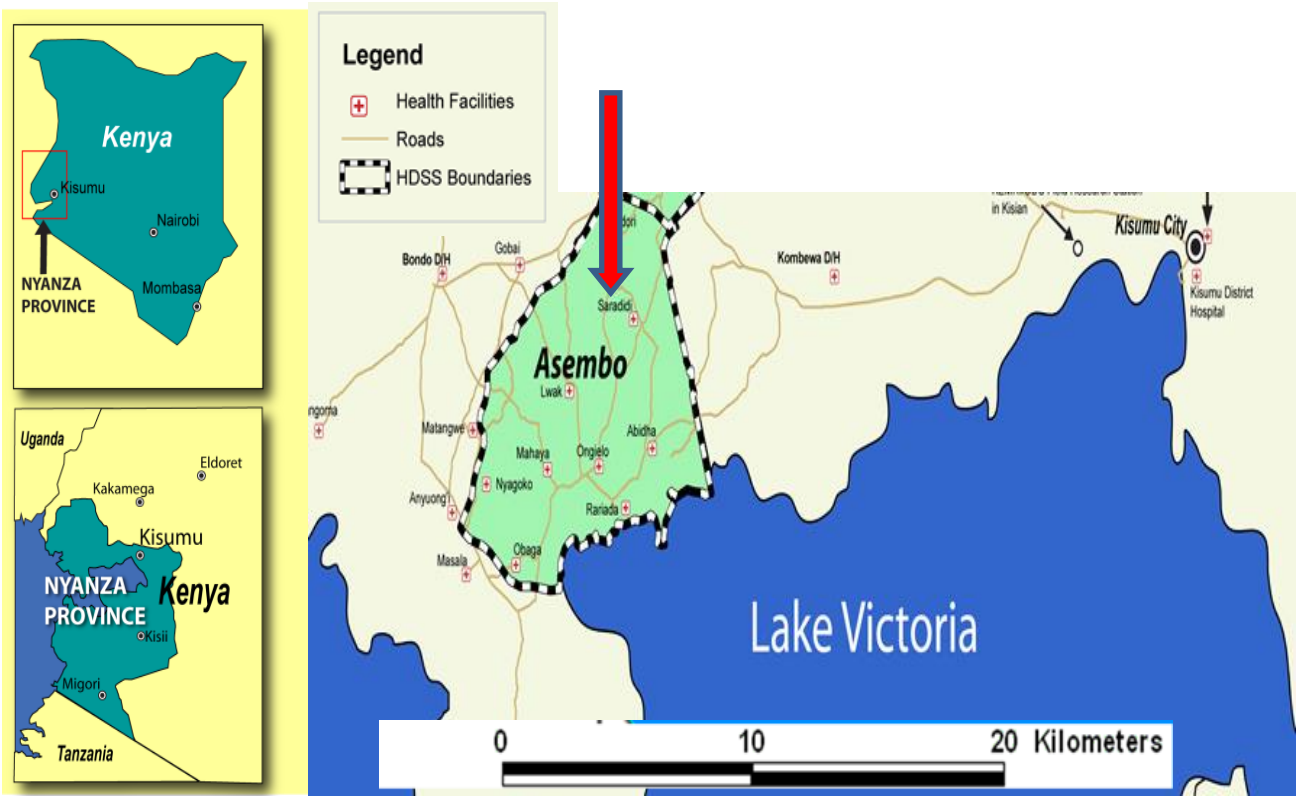

Figure 1: Map showing Asembo study site in Rarieda division (Odhiambo *et al.*, 2012)

3.2. Study population

The study population was 950 school going children living in Rarieda District at the shores of Lake Victoria.

3.2.1 Inclusion criteria

Children enrolled in the study were both male and female aged between 8 to 12 years of age. The children at the time of enrollment were required to be attending primary schools located in Asembo region. They were also expected to provide their assent to participate in the study; in addition to their parents/guardians giving informed consent.

3.2.2 Exclusion criteria

Children were excluded from the study if they did not reside in Asembo region and their ages were found to be outside the 8 to 12 year age bracket. Children who were clinically sick and their hemoglobin levels were below 8g/dL were also excluded from the study. Finally; children who were unwilling to give assent and whose parents did not give consent were also excluded from the study.

3.3. Sample size calculation

The current study recruited 950 school going children (aged 8-12 years) from primary schools in Asembo region of Rarieda District. Sample size calculations were based on previous observations in the same study population (Handzel *et al*., 2003), which demonstrated that 25% of schools in Asembo region lie on high prevalence zones of 25%. As such, the prevalence of 25% was used to calculate sample size using the (Magnani, 1997).

The sample size was then determined based on the 25% prevalence levels using the statistical formulae

$$\frac{N = Z^2 P(1-P)}{D^2}$$

Where:

N= minimum sample size required

Z= 1.96 standard error

P= postulated prevalence rate of 25% (0.25)

D= 0.05= the inverse of 95% confidence limit

Substituting,

$$N = \frac{1.96^2 \times 0.25(1- 0.25)}{0.05^2}$$

=288.12

A minimum sample size of 289 children was required to take part in the study. As such, the sample size of 950 children was sufficient to evaluate the diagnostic tools in the study. The advantage of such a sample size was that it limited the influence of outliers and extreme observations in the study population.

3.3. Study design

The following is a diagrammatic presentation of the study design:

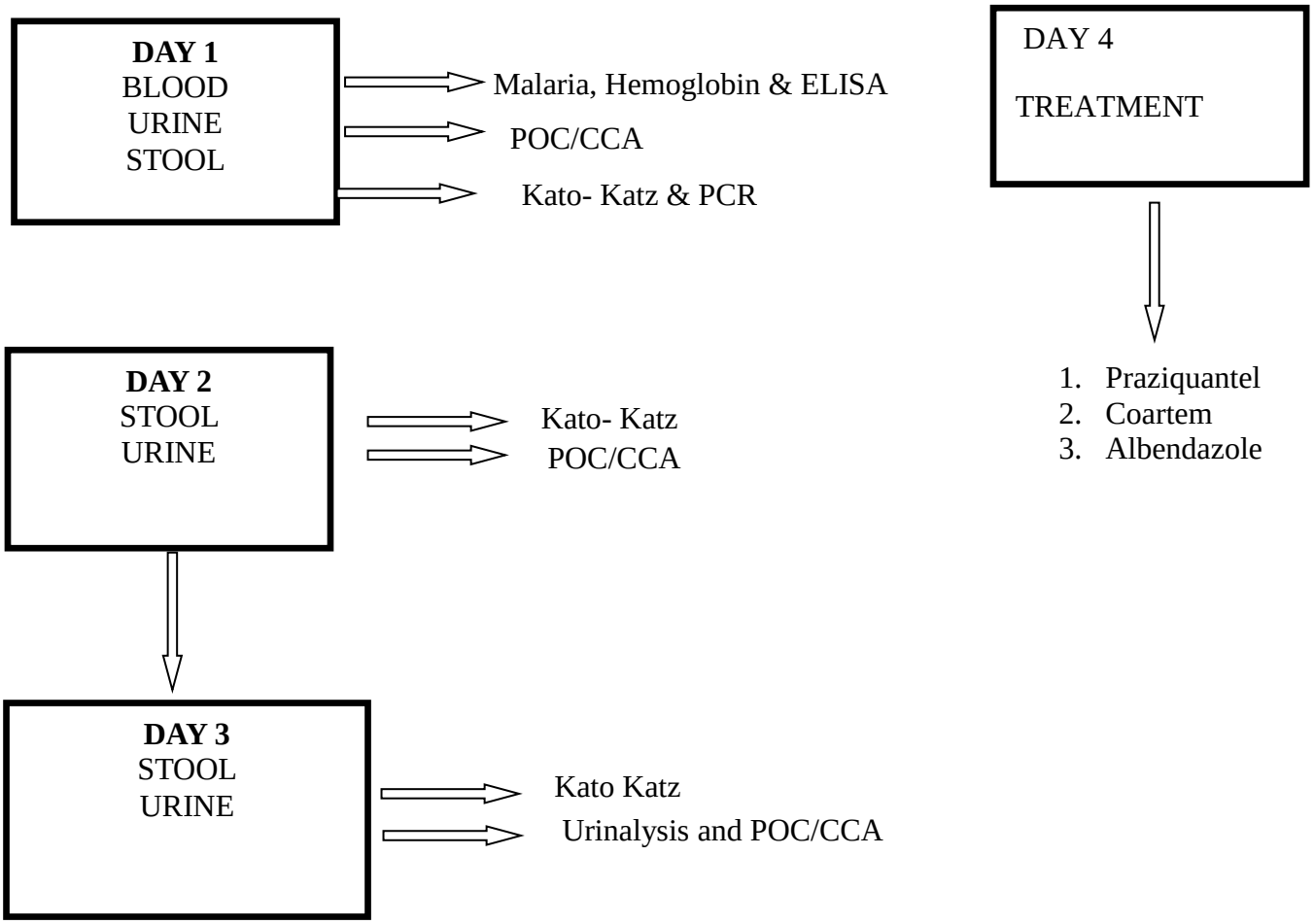

Figure 2: A diagram showing the study design

3.4. Sample collection

On the first day of sample collections, children were asked to provide a stool sample and a midstream urine sample. A finger-prick sample of blood was also obtained. The blood sample was used to determine hemoglobin concentrations using a portable battery operated hemoglobinometer (HemoCue, Angelholm, Sweden), Thick and thin blood smears for malaria testing was also collected from the same Finger-prick blood. The remaining blood was collected in a Microvette CB 300 tube (Sarstedt Inc., Newton, NC) for serum isolation in the lab.

On arrival into the lab, the blood smears were stained with Giemsa and examined for the number of malaria parasites per 300 leucocytes. The blood collected in the microvette tubes was centrifuged in order to collect the sera which was then stored at -20^{o}C and assayed by ELISA.

On the second and third day, stool and urine samples were collected. The reason for this was due to the variability in schistosome egg counts in both the urine and stool samples. Treatment for schistosomiasis, malaria, and STHs was done on the fourth day at the school premises for those children who were infected.

3.5. POC/CCA testing

Urine samples were tested at the school site for positivity and intensity of infection by the POC/CCA assay. One drop of urine was added to the well of the testing cassette and allowed to absorb. Once it was absorbed, then a drop of buffer (enclosed within the kit) was added into the same well and the assay allowed to develop for twenty minutes and later the results were read. The tests were considered invalid if the control band did not appear /develop or if the tests were left to develop for more than 25 minutes after addition of the buffer. For any invalid tests, the samples were re-run with a new test kit and later scored appropriately. To score the intensity of the POC/CCA assay results, the intensity of the test band was compared to that of the control band (a control line is a procedural line that shows up to confirm that the test has worked correctly). Positive results were given a score of 1^{+} if the test band was less visible than the control band, 2^{+} if the test band was of equal visibility with the control band, and 3^{+} if the test band was more visible than the control band. All the results were read by one person for consistency. Urine samples were then tested by dipstick urinalysis for hematuria and pyuria. All POC/CCA intensity results, dipstick results, hemoglobin level, were recorded.

3.6. Laboratory Testing

3.6.1 Stool sample testing

Stool samples collected were transported to the laboratory for preparation of Kato-Katz slides. Stool samples were processed within 24 hours of collection using Kato-Katz (Katz *et al.*, 1972; WHO, 1994) thick smear technique in duplicate for the quantitative determination of *S.mansoni* and soil-transmitted helminths. A template size of 1.5mm thick was used to deliver 41.7mg of stool sample. Each slide was read within 1 hour of preparation to diagnose presence of hookworm, *Ascaris lumbricoides*, and *Trichuris trichuria* by two well-trained microscopists. The *S.mansoni* slide readings were recorded as eggs per gram (EPG). The results of all the six slides collected on three consecutive days were averaged and later classified according to the WHO intensity guidelines (WHO, 2002). As per the WHO guidelines, intensity levels of 1-99, 100-399 and greater than 400 EPG were classified as light, moderate and heavy infections, respectively. Soil-transmitted infections were categorized as either positive or negative.

3.6.2. Real-time PCR for parasitic detection

A portion of the first day stool that remained after Kato-Katz processing was preserved in 70% ethanol which is a field friendly preservation method that does not require freezing (approximately 0.7grams of stool was placed in 2mL of absolute ethanol and stored at 4^{o}C for a maximum of 3 months). 950 of such samples were tested by PCR. The samples were transported under frozen conditions to Leiden University Medical Center (Department of Epidemiology and Parasitology), The Netherlands for testing using *Schistosoma* real-time PCR (Applied Biosystems, Netherlands).

On arrival, the samples were washed to remove all traces of ethanol. This was performed by a custom-made automated liquid handling station (Hamilton®, Switzerland).

For the DNA isolation, 200 mL of feces suspension was centrifuged and the pellet washed twice with 1mL of phosphate buffered saline (PBS). After centrifugation the pellet was resuspended in 200 mL of 2% polyvinylpolypyrolidone (Sigma) suspension and heated for 10 min at 100°C. Sodium dodecyl sulfate–proteinase K was added to the suspension and gently heated at 55°C in a water bath for 2 hours, DNA was then isolated using QIAamp DNA-easy 96-well plates (QIAgen). In each sample, 103 PFU/mL Phocin Herpes Virus 1 (PhHV-1) was included within the isolation lysis buffer (Niesters, 2002; Verweij *et al.,* 2001).

A *Schistosoma* multiplex real-time PCR was later performed as described previously (Obeng et al., 2008). This PCR targeted the *Schistosoma*-specific internal transcribed spacer-2 (ITS2) sequence and PhHV-1 was an internal control. *S.mansoni* (AF503487) the Schistosoma-specific primers, Ssp48F (59-GGT CTA GAT GAC TTGATY GAG ATG CT-39) and Ssp124R (59-TCC CGA GCG YGT ATA ATG TCATTA-39) amplified a 77-bp fragment. The double labeled Ssp78T [FAM-59-TGGGTT GTG CTC GAG TCG TGGC-39-Black Hole Quencher (Biolegio)] was used as a probe, to detect any Schistosoma-specific amplification. Amplification, detection and data analysis was performed using the Bio-Rad CFX platform and the CFX96 Real-Time System by CFX software version 1.1 (Bio-Rad, Hercules, CA). The PCR output from the system consisted of a cycle-threshold (Ct) value, which represented the amplification cycle on the parasite specific DNA load present in the sample when the level of fluorescent signal exceeded the background fluorescence.

3.7. IgG Detection using SWAP (Schistosome worm antigen preparation) ELISA

ELISA plates were coated with 100uL of 0.01mg/ml SWAP in 0.5 M sodium carbonate buffer, pH 9.6, for four hours at room temperature. After four hours the plates were washed five times and then blocked with 100uL of PBS containing 0.3% Tween 20 and 5% nonfat dried milk and later incubated overnight at 4^{o}C. Diluted sera (1:100 in PBS/Tween20/.01% milk) were added to the plates in duplicate wells and incubated at room temperature for one hour, then washed five times in PBS containing 0.05% Tween 20. Affinity purified (HRP), peroxidase labeled mouse anti-human IgG_1, IgG2, IgG_3, IgG_4 (Southern Biotech) diluted 1:1000 in PBS/Tween/0.01% milk was added for one hour at room temperature followed by five washes. The plates were then developed using 100uL per well of Tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and stopped with 18% sulfuric acid. The plates were read on molecular Diagnostics Emax microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 450 nm and analyzed with Softmax software (Molecular Devices). To ensure consistency between the plates a standard curve was developed from each plate. A 1:3 serial dilution curve was made from a highly positive serum pool of adult male car washers from the area and assigned arbitrary units. A five-parameter curve fitting model was used to assign units to each unknown sera. The positive cutoff value was set at two standard deviations above the average anti-SWAP IgG value of the negative controls. The ELISA was coded as 0 to indicate no infection, and 1 for a positive infection.

3.8. Data Management and Analysis

The sensitivities and the specificities of the different tests were estimated using PCR as the reference test. The sensitivity of a test was defined as the proportion of the participants with the disease who had a positive test. Similarly, specificity was defined as the proportion of the

participants without the disease who had a negative test. This analysis was done by SAS version 9.2. All field and laboratory data were entered into Microsoft Excel (Microsoft Corporation, Redmond, WA), and analyzed by SAS (vers. 9.2; SAS institute Inc., Cary, North Carolina, USA). Comparison of proportions between and within groups of the PCR positive and negative individuals was computed using McNemar's test with *P*-values <0.05 regarded as significant.

3.9. Ethical considerations

The scientific approval of this study was obtained from the School of Graduate Studies (SGS) Maseno University while the ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (Appendix 1). Written informed consent was obtained from the participants prior to their recruitment into the study. Confidentiality was maintained throughout the study. All children who were positive for schistosomiasis were treated with praziquantel (40mg/kg), children positive for STHs were given Albendazole 400mg as a single dose, and children positive for malaria were treated with Coartem (Artemether-Lumefantrine) as per Ministry of Health, Kenya guidelines.

CHAPTER FOUR

RESULTS

4.1. Demographic and laboratory characteristics of the study participants

A total of 494 boys were enrolled (52%) against 456 (48%) girls in the study. The proportion of the males vs. females was comparable (*P*=0.801). The mean age was 10 years. The mean hemoglobin level of the children was 10.5g/dL.

4.2. Diagnostic test results

The POC/CCA tests results for an individual was scored as 0, 1, 2 or 3. POC/CCA score was based on the average intensities of results across the three days. The cut-off was set at 0.67 with a score below that being considered a negative, and a score above 0.67 being regarded as a positive. Table 1 summarizes the results of the Kato-Katz and POC/CCA results in actual number (N=950) and proportions.

Table 1: Diagnostic test results

Category	N	%
POC/CCA^{+} KATO-KATZ^{-}	276	29.1
POC/CCA^{+}KATO-KATZ^{+}	219	23.0
POC/CCA^{-}KATO-KATZ^{+}	51	5.4
POC/CCA $^{-}$KATOKATZ^{-}	404	42.5
TOTAL	950	100.0

Table 2: Sensitivity and specificity analyses against PCR

TEST	SENSITIVITY%(CI)	SPECIFICITY%(CI)
PCR	N/A	N/A
POC/CCA	78.30(73.85,83.18)	61.20 (56.16,66.04)
ELISA	50.16(44.50,55.81)	85.11 (82.06,87.33)
KATO KATZ	70.44(65.42,75.46)	92.70 (89.98,94.19)

The highest sensitivity was shown by the POC/CCA test at 78.30%, followed by Kato-Katz at 70.44% and finally ELISA at 50.16%. Kato-Katz had the highest specificity at 92.70%. ELISA and POC/CCA had a specificity of 85.11% and 61.20% respectively. The figures in brackets signify the upper and lower confidence limits at 95%.

4.4. Factors that may affect performance of the tests

4.2.1. STH infection on POC/CCA performance

Factors that could influence the performance of the POC/CCA tests such as STH coinfection, hematuria, pyuria and malaria were evaluated and results presented on Table 3 and 4. The overall prevalence of soil transmitted helminths on the children in the study was 6.5%, with *Trichuris trichiura*, *Ascaris lumbricoides* and hookworm having a prevalence of 9.1%, 4.2% and 4%, respectively.

In order to assess if soil transmitted helminths (*Trichuris trichiura*, *Ascaris lumbricoides*, and hookworm) had any influence on the performance of the POC/CCA tests, responses were compared between individuals who were PCR positive and infected with the helminths and those who were PCR positive but not infected with the helminthes, respectively. The results showed no significant difference in percentages of POC/CCA positive with *Trichuris trichiura* at 76.3% vs. 78.6 %, (*P*=0.8338) POC/CCA positive with hookworm at 81.8% vs. 78.2% (*P*=1.000) and POC/CCA positive with *Ascaris lumbricoides* at 66.7% vs. 78.9 % (*P*=0.3315) (Table 3). These results show that STH infections did not affect the performance of the POC/CCA tests.

Table 3: STH infection on POC/CCA performance

TEST	**#**	**#(%) POC/CCA POS**	**#(%) POC/CCA NEG**	***P*-value**
PCR $^{+}$TT^{-}	280	220(78.6)	60(21.4)	
PCR $^{+}$TT^{+}	38	29(76.3)	9(23.7)	0.8338

PCR $^{+}$H/W^{-}	307	240(78.2)	67(21.8)	
PCR $^{+}$H/W^{+}	11	9(81.8)	2(18.2)	1.000
PCR^{+}AL^{-}	303	239(78.9)	64(21.1)	
PCR $^{+}$AL^{+}	15	10(66.7)	5(33.3)	0.3315

Legend: Table 3 shows the effects of STH on POC/CCA performance. The three soil transmitted helminths used for comparison were *Trichuris trichiura*, hookworm and *Ascaris lumbricoides.* The figures in brackets show the percentages. McNemar's test was used for comparisons between the PCR positive and PCR negative groups.

4.4.2. Hematuria, pyuria and malaria on POC/CCA performance

To further assess the influence of hematuria, pyuria and malaria on POC/CCA performance, responses were compared between individuals who were PCR positive and had hematuria, pyuria and malaria and those who were PCR positive but did not have the above conditions, respectively, using McNemar's test.

The results showed (table 4) no significant difference in percentages of POC/CCA positive with hematuria cases at 100% vs. 88.8 % (P=1.0000), POC/CCA positive with pyuria at 100% vs. 88.3 % (P=1.0000) and POC/CCA positive with malaria at 72.7% vs. 83.4 % (P=0.0862). These results show that hematuria, pyuria and malaria infections did not affect the performance of the POC/CCA tests.

Table 4: Hematuria, pyuria and malaria on POC/CCA performance

TEST	#	#(%) POS	POC/CCA	#(%) NEG	POC/CCA	*P* value
PCR $^{+}$Hematuria^{-}	98	87(88.8)		11(11.2)		
PCR $^{+}$Hematuria^{+}	2	2(100.0)		0(0.0)		1.0000
PCR $^{+}$ Pyuria^{-}	94	83(88.3)		11(11.7)		
PCR $^{+}$ Pyuria^{+}	6	6(100.0)		0(0.0)		1.0000
PCR^{+} Malaria^{-}	151	126(83.4)		25(16.6)		
PCR $^{+}$ Malaria^{+}	161	117(72.7)		44(27.3)		0.0862

Table legend. This table shows the effects of hematuria, pyuria and malaria on the performance of the POC/CCA. Comparison was made between the PCR positive and PCR negative individuals. The percentages are shown in brackets.

CHAPTER FIVE

DISCUSSION

The results obtained from this study revealed that POC/CCA had the highest sensitivity at 78.30%. ELISA and Kato-Katz tests had a sensitivity of 50.16% and 70.44%, respectively. Kato-Katz had the highest specificity at 92.70% followed by ELISA and POC/CCA at 85.11% and 61.20% respectively. The sensitivity of the POC/CCA was quite low. These findings are similar to the POC/CCA multi-country studies that showed the sensitivity of the POC/CCA to be at a range of 76-86% and suggested that the sensitivity of the commercial POC/CCA needs to be improved (Colley *et al.*, 2013). POC/CCA tests performance was evaluated with respect to co-infections with malaria, soil transmitted helminthes (STHs), hematuria and pyuria. The comparison was performed between the PCR positive and PCR negative groups. The results demonstrated that STHs, hematuria, pyuria and malaria infection did not in any way affect the performance of POC/CCA tests and this was also observed in an earlier study conducted in western Kenya (Shane *et al*., 2011).

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The crude antigens from the adult worms and eggs have been found to have high sensitivity for the diagnosis of *S.mansoni* infection (Noya *et al.*, 2002). However, they present a specificity lower than 80% giving false positives for infections with intestinal nematodes especially with hookworm (Noya *et al.*, 1995) this was in agreement with what was observed in our study setting for the POC/CCA test however for the ELISA tests the sensitivity and specificity was lowered in our setting due to the fact that antibodies were continually being produced long after treatment.

POC-CCA tests in this study allowed for the detection of circulating cathodic antigens which is an indication of active schistosomiasis infections (Ayele *et al.*, 2008). The advantage of the

POC-CCA test compared to the other tests is that it uses urine which is easier to produce and handle compared to blood or stool samples (van Dam *et al.*, 2004).

Studies have shown that circulating antigens have been clinically correlated with the intensity of infection and morbidity caused by schistosomiasis (Ashton *et al*., 2011; Coulibaly *et al*., 2011), POC-CCA tests have proven to be at least as sensitive as a single Kato-Katz thick smear for the diagnosis of *S.mansoni* but not quite as sensitive when it comes to diagnosis of *S.hematobium* infection. The cassettes don't work well in areas which are endemic for both schistosome species (Stothard *et al*., 2006). In this scenario, we didn't test for *S.hematobium* infection, but the sensitivity of the tests was compared to three Kato-Katz samples.

Molecular diagnosis by PCR has demonstrated its utility in the diagnosis of infection with *S.mansoni* (Pontes *et al.*, 2002) due to its high sensitivity and specificity. Real-time PCR done on stool samples is able to reveal the presence of DNA from a broad range of parasites (Verweij *et al*., 2007). The real time PCR has been found to be a potential diagnostic tool for both urinary and intestinal schistosomiasis (Verweij *et al*., 2007) therefore in this study it was used for *S.mansoni* diagnosis. The advantage of having this method in this study was that, the stool samples were collected in remote field areas and were stored in ethanol without refrigeration for at least 3 months without affecting the results of the PCR testing. Co-infection with soil-transmitted helminths, hematuria, pyuria and malaria did not in any way affect the performance of the POC/CCA as showed between the infected and non-infected groups. This also agrees with studies carried out previously in Kenya and Ivory Coast which showed that the percentages of POC/ CCA positive individuals did not significantly change based on STH infection status of the individuals, suggesting that STH infections did not influence the CCA assay results (Coulibaly *et al*., 2011; Shane *et al*., 2011).

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

1. Results from this study indicate that the POC/CCA assay was the most sensitive tests compared to ELISA and Kato-Katz when screening for *S.mansoni* in high prevalence settings.
2. The POC/CCA test is easy to use and field-friendly making it an attractive tool when screening for *S. mansoni* infections in high prevalence settings.
3. Co-infection with soil-transmitted helminths, malaria, hematuria and pyuria did not affect the performance of the POC/CCA tests.

6.2. Conclusions

1. The POC/CCA assay is the most sensitive tests for schistosomiasis diagnosis in high prevalence areas.
2. Factors such as malaria, pyuria, hematuria, and soil-transmitted helminthes did not affect the performance of the POC/CCA tests.

6.4. Recommendations for future research

1. There is need for future adjustments to the POC/CCA to improve on its specificity.
2. There is need for future evaluation of the POC/CCA tests in low, moderate and high schistosomiasis prevalence areas.

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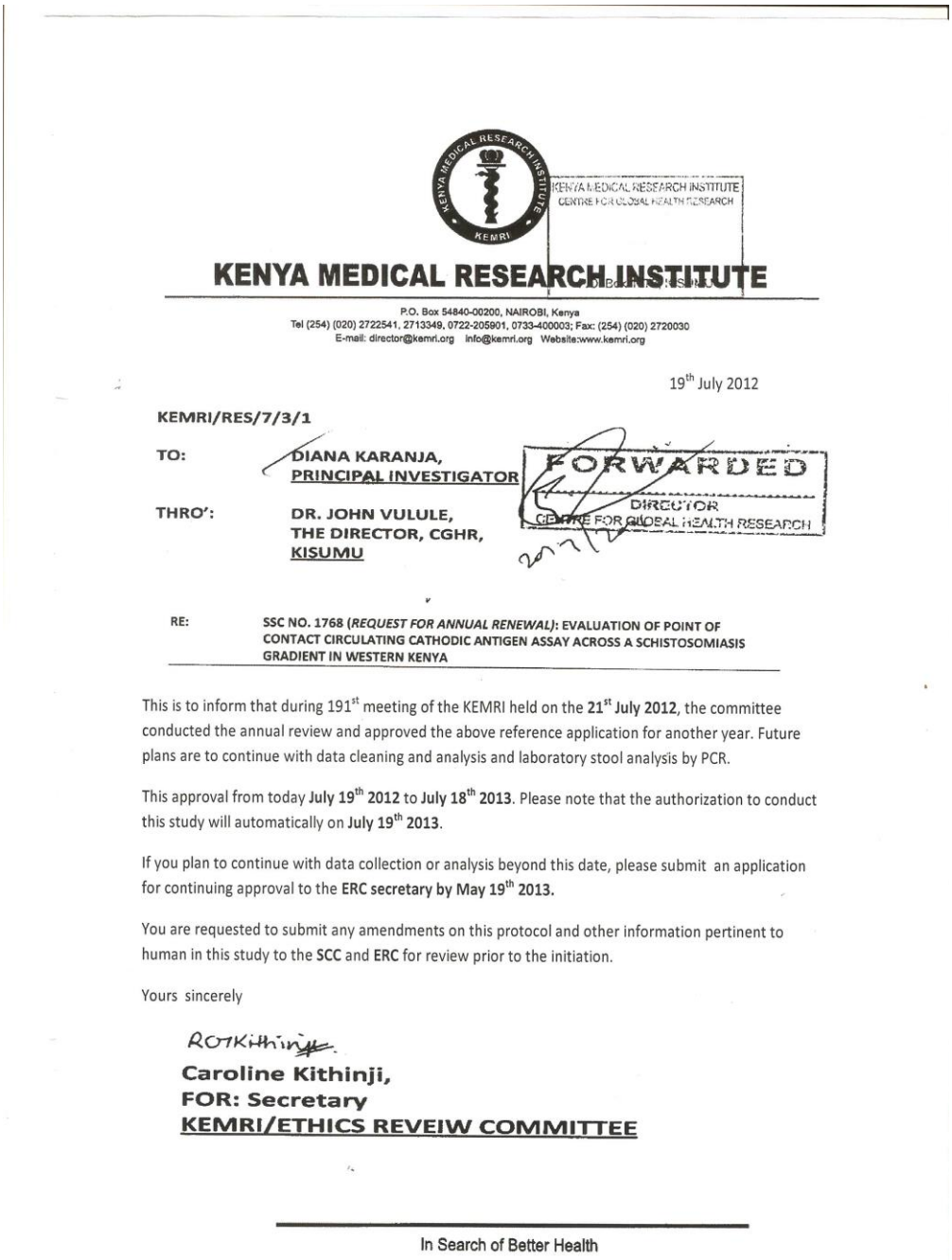
APPENDICES

APPENDIX 1: Research Approval



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19th July 2012

KEMRI/RES/7/3/1

TO: DIANA KARANJA,
PRINCIPAL INVESTIGATOR

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

THRO': DR. JOHN VULULE,
THE DIRECTOR, CGHR,
KISUMU

RE: SSC NO. 1768 (*REQUEST FOR ANNUAL RENEWAL*): EVALUATION OF POINT OF CONTACT CIRCULATING CATHODIC ANTIGEN ASSAY ACROSS A SCHISTOSOMIASIS GRADIENT IN WESTERN KENYA

This is to inform that during 191st meeting of the KEMRI held on the **21st July 2012**, the committee conducted the annual review and approved the above reference application for another year. Future plans are to continue with data cleaning and analysis and laboratory stool analysis by PCR.

This approval from today **July 19th 2012** to **July 18th 2013**. Please note that the authorization to conduct this study will automatically on **July 19th 2013**.

If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the **ERC secretary by May 19th 2013.**

You are requested to submit any amendments on this protocol and other information pertinent to human in this study to the **SCC** and **ERC** for review prior to the initiation.

Yours sincerely

RO Kithinji

Caroline Kithinji,
FOR: Secretary
KEMRI/ETHICS REVEIW COMMITTEE

In Search of Better Health