

Detecting Schistosoma Haematobium Infection by Microscopy and Polymerase Chain Reaction (Pcr) In School Children In The Three Senatorial Districts of Cross River State, Nigeria

Rosemary Kaiso Esiere

University of Calabar Teaching Hospital

Emmanuel onyekachukwu Ibeneme

University of Calabar

Emmanuel Offiong Effanga

University of Calabar

Edema Enogiomwan Imalele (✉ edemaeddy@gmail.com)

University of Calabar <https://orcid.org/0000-0003-3774-5121>

Miracle Kaiso Esiere

University of Calabar

Paul Columba Inyang-Etoh

University of Calabar

Ambrose Anyanwu Alaribe

University of Calabar

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Abstract

As a result of the poor sensitivity and specificity of the standard parasitological diagnostic methods currently being used, this study was conducted to compare the standard parasitological diagnostic methods and Polymerase Chain Reaction (PCR) in determining the prevalence of urinary schistosomiasis in Cross River State (CRS). The study was conducted between April 2015 and March 2016. Seven hundred and seventy seven (777) urine samples were randomly collected from selected school-age children. The urine samples were subjected to standard parasitological and molecular examinations. Chi-square test was used to test the differences between the data on subgroups and the results from specimen examinations. An overall prevalence of 1.7% was recorded using microscopy and 34.7% recorded using PCR. The highest prevalence of infection by microscopy occurred in the Southern Senatorial District (2.3%), while the Northern Senatorial District recorded the highest prevalence of infection by PCR (53.2%) ($p < 0.05$). Males were more infected (2.4%) than females (0.6%) using microscopy. With PCR, males were also more infected (35.7%) compared to females (33.3%) ($p < 0.05$). The highest prevalence of infection using microscopy and PCR both occurred in school-age children aged 5–8 years (3.6% and 47.8% respectively), while the lowest prevalence for both methods occurred in participants aged 17–20 years (0% for both methods) ($p < 0.05$). This study has shown PCR to be effective in detecting schistosomiasis infection and also re-affirms the endemicity of urinary schistosomiasis in the three Senatorial Districts of CRS.

Introduction

Schistosomiasis is a worldwide problem afflicting about 249 million persons and 97% of the infected persons reside in Africa (Anazaku et al. 2017). Approximately 779 million people are at risk of being infected in 76 endemic countries globally, 85% of these reside on the African continent (Utzinger et al. 2009). Every year schistosomiasis is responsible for about 1.7 million disability adjusted life years (DALYs) (Chitsulo et al. 2000). *Schistosoma mansoni* and *S. haematobium* cause intestinal and urinary schistosomiasis respectively in many African countries where they may occur concurrently.

Schistosomiasis causes health, labour loss, hypertension and significant reduction in socioeconomic benefits, particularly, late complications such as irreversible urinary tract obstruction and risk of renal failure are often associated with urinary schistosomiasis (King, 2009).

Control programmes for schistosomiasis are mainly founded on treating of infected people, hence adequate-case finding is essential. Hitherto, the existing protocols for the diagnosis of schistosomiasis are search for ova of the schistosomes in urine or stool samples and detection of ova or adult worm antigens in sera and urine of infected persons. Detecting antigens of ova or adult worm in sera/urine of diseased persons can distinguish current infections from past ones with almost 100% specificity. Egg counts and haematuria are the only parameters presently employed in surveillance. However, Poggensee et al. (1998) reported the presence of morbidity of the urinary tract in some women who were schistosomiasis-infected in Tanzania who had scanty or no egg output and no haematuria. This implies that there is need for methods of detection with more specificity and sensitivity of the disease in humans.

Several studies have looked at the prevalence and intensity of *S. haematobium* in communities in Nigeria using methods like the detection of haematuria and ova in urine (Okon et al. 2009, 2010; Adie et al. 2013). Though they are useful in the detection of heavy infections, their low sensitivity in detecting light/early infections is a drawback that cannot be overlooked. As a result of the poor specificity and sensitivity of the standard parasitological diagnostic methods currently being used, the real estimate of the prevalence of schistosomiasis in sub-Saharan endemic areas of Africa is not clear (Hotez and Fenwick, 2009). Earlier studies have shown that detection of schistosomal parasite-specific DNA can be done in urine of persons who are infected (Lodh et al. 2013). Adult schistosome worms are the source of DNA because they shed teguments at regular intervals. Therefore if parasite-specific DNA is detected in urine it confirms the parasites' presence (Ibironke et al. 2012). In recent years, multiple authors have demonstrated Schistosome parasite-specific DNA in urine (Lodh et al. 2013). This study, therefore, sought to compare microscopy and PCR in the characterization of *S. haematobium* in Cross River State (CRS), Nigeria.

Materials And Methods

Study area and population

This study was carried out in Cross River State with Calabar as the capital. The state is divided into three Senatorial Districts (North, South and Central) with 18 Local Government Areas (LGAs). Samples were collected from Central, Southern and Northern Senatorial Districts and were analysed at the University of Calabar Teaching Hospital (UCTH), Calabar and Molecular Biology Laboratory, Niger Delta University, Bayelsa State. The study population consisted of children/adolescents aged 5–20 years attending primary and secondary schools in the study area.

Ethical consideration

Subjects for this study were enrolled after approval was sought and obtained from Ministry of Health Ethical Committee, Cross River State (REC No.: RP/REC/2016/423). The village heads were informed of the study through advocacy visits to their palaces and local school authorities. Written informed consent was obtained from parents/guardians of participants.

Sampling and sample size determination

Seven hundred and seventy seven (777) samples were randomly obtained from children/adolescents in secondary/primary schools in the study area. Selection of children from schools in the study area was done randomly by picking one out of every four pupil within the range of the study.

Sample collection

The study was conducted between April 2015 and March 2016. Seven hundred and seventy seven (777) clean appropriately labelled universal containers were issued to the randomly selected children for collection of urine samples. The urine samples from these individuals were used for parasitological/molecular examinations. Subjects were instructed to include the last drops of urine

(Cheesbrough, 2005). The samples were collected between 10.00am and 2.00pm, on each collection day to ensure maximum yield of schistosome eggs (Cheesbrough, 2005). All samples collected were preserved with 2 drops of 10% formalin in 25mL capacity (WHO, 2003) and transported to the parasitology laboratory, University of Calabar Teaching Hospital.

Parasitological examination

A modified filtration system adopted by Useh and Ejezie, (1999) was used. Briefly a funnel holding a What-man No. 1 filter paper was suspended on a conical flask to act as the filtration system. 10mL of the urine sample was filtered for the eggs of *Schistosoma*. The thoroughly agitated sample was allowed to pass through the filtration system. Using a blunt-ended forceps, the filter paper was removed with great care and placed on a slide. The filter paper was placed upwards (eggs on surface) on the slide using ×10 objective, with the condenser iris closed adequately to give a good contrast. A systematic examination of the entire filter paper was conducted for *S. haematobium* ova. Any urine sample with ova was recorded as positive. The number of eggs counted was recorded per 10ml of urine samples collected.

Molecular examination for schistosome infection

Urine cell pellet preparation

The urine sample was spun at 5,000 rpm for 10 min, the supernatant decanted and cell pellets washed thrice with 25mL phosphate buffer saline (0.8% NaCl, 2.7mM KCl, 1.8mM KH₂P0₄, 8.mM Na₂HP0₄ pH 7.4). These were immediately stored at -80°C until used.

Genomic DNA extraction from urine cell pellet

The urine cell-pellets were re-suspended using 150µL of DNA elution buffer, then transferred into individual Eppendorf tubes, followed by 200µL bio-fluid - cell buffer solution (red) and 20µL proteinase K enzyme to digest the sample. Vortexing was done to have a thorough mix, and then incubated at 55°C for 10 minutes on a heating block. Exactly 420 µL of genomic binding buffer was added to the digested sample and mixed thoroughly. The mixture was then transferred to a Zymo-Spin IIC – XL column in a collection tube and centrifuged at 14,000 rpm for one minute. The columns were then placed in new collection tubes, where 400µL DNA of pre-wash buffer was added and spun for one minute at 14,000 rpm and the collection tubes emptied. Seven hundred (700µL) g-DNA wash buffer was then added and spun at 14,000 rpm for one minute, and the collection tube emptied again. Furthermore, 200µL of g-DNA wash buffer was added and centrifuged again at 14,000 rpm for one minute and the collection tube discarded with the flow through. The columns were transferred to clean micro-centrifuge tubes and 50µL of DNA elution buffer was added, incubated at room temperature for 5 minutes, and then spun at 14,000 rpm for one minute to elute the DNA. Finally, the columns were discarded and the g-DNA, already extracted in the micro-centrifuge tubes, stored at -20°C until further use for PCR reactions.

DNA quantification

NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, California, USA) was used to determine the quantity and purity of DNA. The equipment was initialized and blanked before readings. DNA concentrations of the samples were recorded, in ng/ μ L while the absorbance 260/280 was used to determine the purity. Samples with absorbance ranging from 1.70 to 2.10 were selected for amplification by PCR.

Amplification of *Schistosoma haematobium* DNA repeat fragment from urine samples

Polymerase chain reaction was carried out on all the 13 positive samples and 108 negative samples, respectively, by microscopy, using 0.4 μ L of species-specific primers *ShDra1* F (forward: 5'-GATCTCACCTATCAGACGAAAC-3' and *ShDra1* R (reverse: 5'-T CACAACGATACGACCAAC-3'), which were previously designed by Hamburger et al., (2001) for specific amplification of 121 bp *Dra1* tandem repeats of *S. haematobium*. Amplification reactions were carried out in 20 μ L volume. Two microlitre (2 μ L) of DNA (concentration: 4-6ng/ μ L) served as PCR template in the reaction volume, 10 μ L of 2X Quick-Load Master-mix containing 200 μ M of dNTPs, 1.5 μ M of Taq polymerase (Inqaba Biotech, Johannesburg, South Africa) and 1.5 μ M of MgCl. The PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for one minute, annealing at 55°C for 90 seconds and extension at one minute at 72°C for 5 minutes.

Agarose gel electrophoreses

One gram and half (1.5gm) of agarose powder was measured and poured in 100ml of 1X TBE buffer (Tris Boric Ethylene diamine Tetra-acetic acid.) then heated in a microwave for 5 minutes to allow the agarose powder dissolve and make a clear solution. It was allowed to cool and 4 μ L of ethidium bromide was added. The solution was poured in an electrophoretic casting tray, the comb was placed and then the solution was allowed to cool and solidified for about 30 minutes then placed in an electrophoresis tank. Exactly 10 μ L of PCR product was pipetted, already mixed with DNA loading dye, and then loaded in the agarose gel lane after removing the comb. DNA ladder and negative control were also loaded and the gel was subjected to an electric field at 120V for 25 minutes. The gel was visualized on a UV trans-illuminator for band detection.

Data analysis

All analyses were performed using the SPSS package (version 22). Descriptive statistical analysis was used to calculate the prevalence and intensity of infections. Chi-square test was used to test the differences between the data on subgroups and the results from specimen examinations. Differences were considered significant at a p -value less than 0.05 ($p < 0.05$).

Results

Seven hundred and seventy seven (777) school children/adolescents randomly selected from eight schools in the three Senatorial Districts of Cross River State were screened to determine the current status

of urinary schistosomiasis in these districts. The Northern Senatorial District had the highest number of participants (348/777; 44.8%) followed by the Southern Senatorial District (220/777; 28.6%) and the Central Senatorial District (209/777; 26.9%). The school children/adolescents also comprised of 450 (57.9%) males and 327 (42.1%) females. The age distribution of the participants in the study was 5–8 years (248/777; 31.9%), 9–12 years (339/777; 43.6%), 13–16 years (116/777; 14.9%) and 17–20 years (74/777; 9.5%).

Table 1 shows the prevalence of *S. haematobium* infection by microscopy and PCR in the Senatorial Districts. The highest prevalence of infection by microscopy occurred in the Southern Senatorial Districts (2.3%), while the Northern Senatorial District recorded the highest prevalence of infection by PCR (53.2%); there was a statistically significant difference ($p < 0.05$) in the prevalence of infection in the Senatorial Districts using microscopy and PCR.

Table 2 shows the prevalence of *S. haematobium* infection by microscopy and PCR with respect to gender. Males were more infected than females using microscopy (2.4% for males and 0.6% for females) and PCR (35.7% for males and 33.3% for females); there was a statistically significant difference in prevalence of infection by gender, using microscopy and PCR ($p < 0.05$).

Table 3 shows the prevalence of *S. haematobium* infection by microscopy and PCR in participants with respect to age. The highest prevalence of infection using PCR occurred in participants aged 5–8 years (47.8%), while the lowest prevalence occurred in participants aged 17–20 years (0%). Using microscopy, the highest and lowest prevalence occurred in age groups 5–8 years (3.6%) and 17–20 years (0%). There was a statistically significant difference ($p < 0.05$) in the prevalence of infection among the different age-groups using microscopy and PCR.

Table 1
Prevalence of *Schistosoma haematobium* infection by microscopy and PCR by Senatorial Districts

Senatorial District	No. Examined by microscopy	No. (%) positive by microscopy	Number examined by PCR	Number(%) positive by PCR	χ^2	p -value
South	220	5 (2.3)	31	5 (16.1)	11.082	0.004
Central	209	1 (0.5)	46	14 (30.4)		
North	348	7 (2.0)	44	23 (52.3)		
Total	777	13 (1.7)	121	42 (34.7)		

Table 2
Prevalence of *Schistosoma haematobium* infection by microscopy and PCR by gender

Gender	No. examined by microscopy	No. (%) positive by microscopy	No. examined by PCR	No. (%) positive by PCR	χ^2	<i>p</i> -value
Males	450	11(2.4)	70	25(35.7)	0.257	0.012
Females	327	2(0.6)	51	17(33.3)		
Total	777	13(1.7)	121	42(34.7)		

Table 3
Prevalence of *S. haematobium* infection by microscopy and PCR of subjects examined by age

Age group (years)	No. Examined by microscopy	No. (%) positive by microscopy	No. Examined by PCR	No. (%) positive by PCR	χ^2	<i>p</i> -value
5–8	248	9 (3.6)	46	22 (47.8)	8.106	0.044
9–12	339	3 (0.9)	58	18 (31.0)		
13–16	116	1 (0.9)	13	2 (15.4)		
17–20	74	0 (0.0)	4	0 (0.0)		
Total	777	13 (1.7)	121	42 (34.7)		

Plate 1 shows an agarose gel electrophoresis of the amplified repeats of *Schistosoma haematobium* from positive samples by microscopy. Lane 1, 2, and 10 are negative samples, Lanes 3, 9, 11–13 represents positive samples with 1 to 3 repeats. M is a 100bp ladder. Lane 14 represents the negative control.

Plate 2 shows an agarose gel electrophoresis showing the amplified repeats of the *Schistosoma haematobium* from selected samples negative by microscopy. Lanes showing 1 band or more represent positive samples for *S. haematobium* while lanes with no bands represent negative samples. L is 100bp ladder.

Discussion

Studies have compared different diagnostic procedures used to detect schistosomiasis. These include tests for specific antibody, circulating antigens, haematuria, egg detection and ultrasound scans of the urinary tract. Nonetheless, it is difficult to find a “gold” standard because of the variability of the diagnostic performance of these techniques especially in areas with variable prevalence of *S.*

haematobium infection (Koukounari et al. 2009). However, assays based on polymerase chain reaction (PCR) have recently begun to show potentials as effective methods in detecting DNA of parasites in urine and saliva (Mharakurwa et al. 2006).

In this study, the 121bp *Dra1* tandem repeat PCR developed by Hamburger and colleagues (Hamburger et al. 2001) was adapted to detect *S. haematobium* DNA in urine. The *Dra1* fragments were detected in samples from both children and adolescents when ova of schistosome were detected, and also in specimens where no ova were detected. However, these fragments were not detected in some of the samples. Out of the 777 urine samples, 13 (1.7%) were positive by microscopy, but PCR showed that 42 (34.7%) out of 121 randomly selected samples showed parasite-specific DNA for *S. haematobium*. This speaks volume of the sensitivity of PCR over microscopy. This agrees with results reported by Gasmelseed et al. (2014).

The overall prevalence (1.7%) recorded from parasitological examination of urine sample is lower than that reported for Cross River State (CRS) in earlier studies. Ejezie et al. (1991) established a schistosomiasis prevalence value of 43.5% in Adim, CRS, while Useh and Ejezie (1996) recorded a prevalence of 53.8% in the same study area. Ekanem et al. (1995) recorded a prevalence of 44% in Ijiman, Yakurr Local Government Area (LGA), a community close to Adim. Recent studies have shown that the urinary schistosomiasis prevalence in Adim community increased from 53.8% reported by Useh and Ejezie (1996) to 57.4% (Okon et al. 2009). In Adim, Etim et al. (2012) reported a 42.2% prevalence of urinary schistosomiasis, while Okon et al. (2007, 2009) reported 35% and 45.3% prevalence rates respectively from parasitological examination of urine samples. The present study has re-affirmed the endemicity of *S. haematobium* in the three Senatorial Districts; Southern (Biase LGA), Central (Yakurr LGA) and Northern (Bekwarra LGA). According to Useh (2013), the extensive, unrestrained and unchecked increase in irrigation, water impoundment schemes, and widespread ignorance of schistosomiasis in the majority of the endemic areas have elevated the disease to a very serious health predicament in the sub-Saharan part of the continent, including Nigeria. This disease is also considered as one of the most frequently occurring parasitic infections globally (Okon et al. 2007). In CRS, the agrarian population and suitable conditions that enable the snail intermediate host to thrive has added to the prevalence of schistosomiasis (Ejezie et al. 1991).

It is likely that the reduction in prevalence and intensity of urinary schistosomiasis recorded in these communities, is as a result of treatments carried out by previous researchers; Okon et al. (2010), evaluated the status of urinary schistosomiasis in Ogoja LGA, where he went ahead to treat infected persons with Artesunate and found 64% cure rate while Inyang-Etoh et al. (2004) had a 70% cure rate in their work in Adim, CRS, using Artesunate. Another work, in Adim, recorded a cure rate of 88% after utilizing Artesunate and Praziquantel combination (Inyang-Etoh et al. 2009). This reduction in morbidity of urinary schistosomiasis could also be as a result of the integrated health interventions introduced about eight years ago by the Neglected Tropical Diseases Control Unit in collaboration with the schistosomiasis/soil transmitted helminths unit of the Federal Ministry of Health, CRS, in November 2012 (Adie et al. 2015). Integrated control measures put in place included chemotherapy of infected individuals

with Praziquantel and health education on the predisposing factors responsible for the transmission of urinary schistosomiasis (Adie et al. 2015).

Infection pattern varied according to gender with more male participants (11; 2.4%) being infected than females (2; 0.6%). This difference varied significantly at ($p < 0.05$). Similar findings were reported in other studies (Inyang-Etoh et al. 2004; Ugbomoiko et al. 2010). This difference may be explained by the fact that males are more involved in water contact activities than females due to social inhibitions like being restricted from activities such as fishing, public bathing and swimming (Etim et al. 2012). However, this deviates from other studies that reported no significant difference in gender-related prevalence. Okon et al. (2007), Bala et al. (2012) and Morenikeji et al. (2014) all reported both genders to be equally at risk.

Prevalence of *S. haematobium* varied significantly across age groups with the least (0% for both microscopy and PCR methods) and highest (3.6% for microscopy and 47.8% for PCR) recorded in age group 17–20 and 5–8 years, respectively ($p \leq 0.05$). This is similar to previous result reported in Biase and Yakurr LGAs by Adie et al. (2015), but disagrees with other findings which recorded highest infection rates occurring in age brackets 12–15 (Morenikeji et al. 2014) and 10–14 (Ugbomoiko et al. 2010 and Deribe et al. 2011). The prevalence of urinary schistosomiasis varied with age in this study which is in contrast to findings reported by Ekwunife et al. (2009), while it was supported by Sam-Wobo et al. (2009) and Uwazuoke et al. (2009).

Conclusion

This study has re-affirmed the endemicity of urinary schistosomiasis in the three Senatorial Districts of CRS. The prevalence of 34.7% recorded in this study is still within the WHO recommended threshold of $\geq 20\%$, for mass drug administration (MDA). This study has shown that PCR is more sensitive than standard parasitological methods for the diagnosis of *S. haematobium* infection.

Declarations

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Conflicts of interest

The authors declare no conflict of interest exist.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

Esiere, Rosemary Kaiso: Conceptualization, Data curation, Investigation, Methodology, Writing - original drafting. **Ibeneme, Emmanuel Onyekachukwu:** Investigation, Methodology, Writing – review and editing. **Imalele, Edema Enogiomwan:** Methodology, Writing – review and editing. **Effanga, Emmanuel Offiong:** Data curation, Methodology. **Esiere, Miracle Kaiso:** Investigation, Data curation. **Inyang-Etoh, Paul Columba:** Formal analysis, Project administration, Validation. **Alaribe, Ambrose Andrew Anyanwu:** Conceptualization, Validation, Supervision

Ethics approval

Subjects for this study were enrolled after approval was sought and obtained from Ministry of Health Ethical Committee, Cross River State.

Consent to participate

The village heads were informed of the study through advocacy visits to their palaces and local school authorities. Written informed consent was obtained from parents/guardians of participants.

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Figures

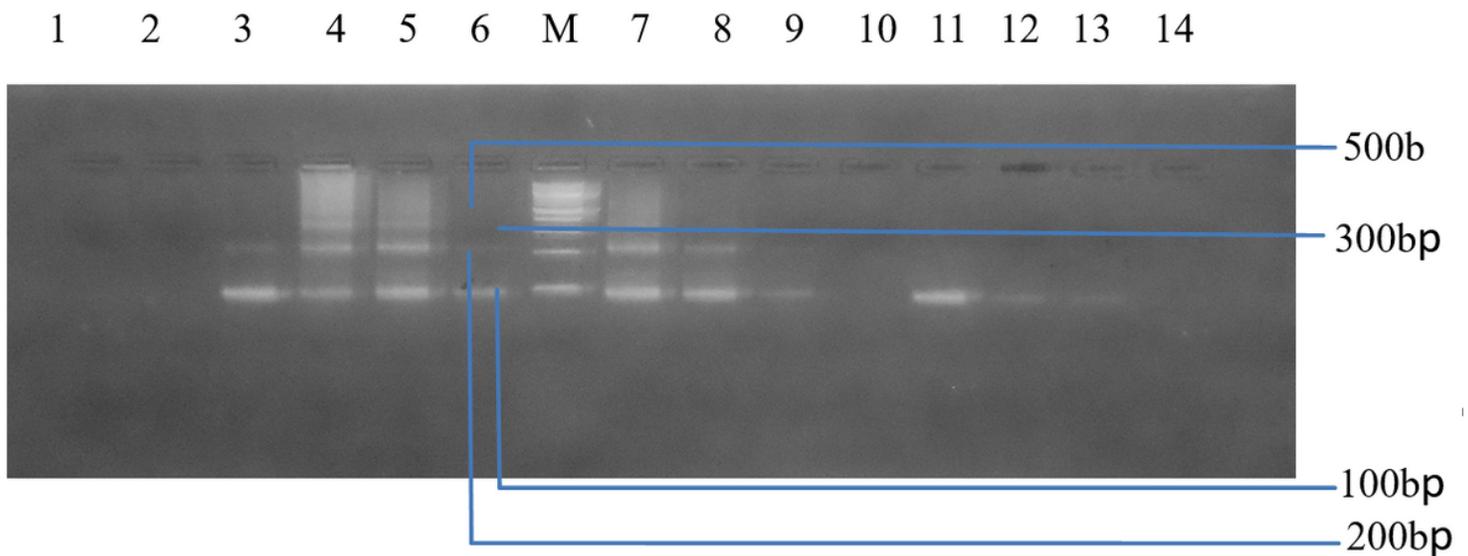
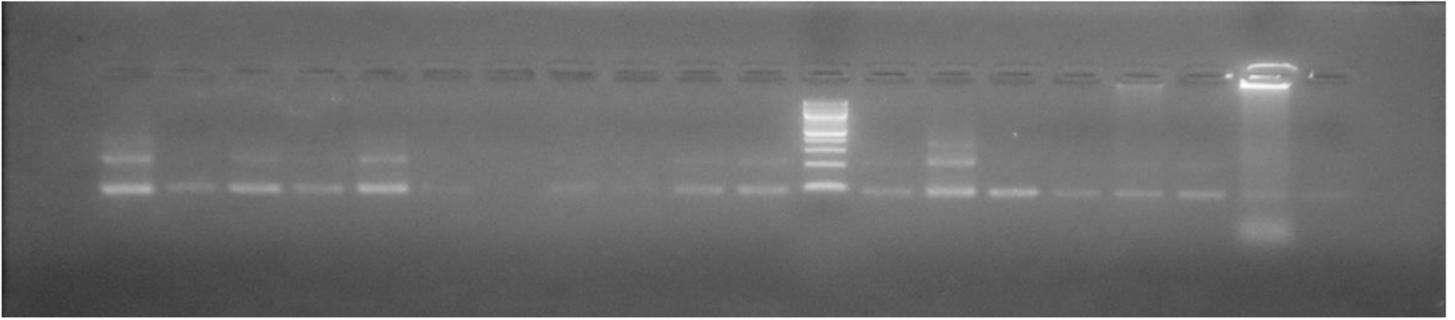


Figure 1

Plate 1: Agarose gel electrophoresis showing the amplified repeats of the *Schistosoma haematobium* from the positive samples by microscopy. Lane 1, 2 and 10 are negative samples. Lanes 11-13 represents positive samples with 1 to 3 repeats. M is a 100bp ladder. Lane 14 represents the negative control

1 2 3 4 5 6 7 8 9 10 11 L 12 13 14 15 16 17 18 19



20 21 22 23 24 25 26 27 28 29 L 30 31 32 33 34 35 36 37 38

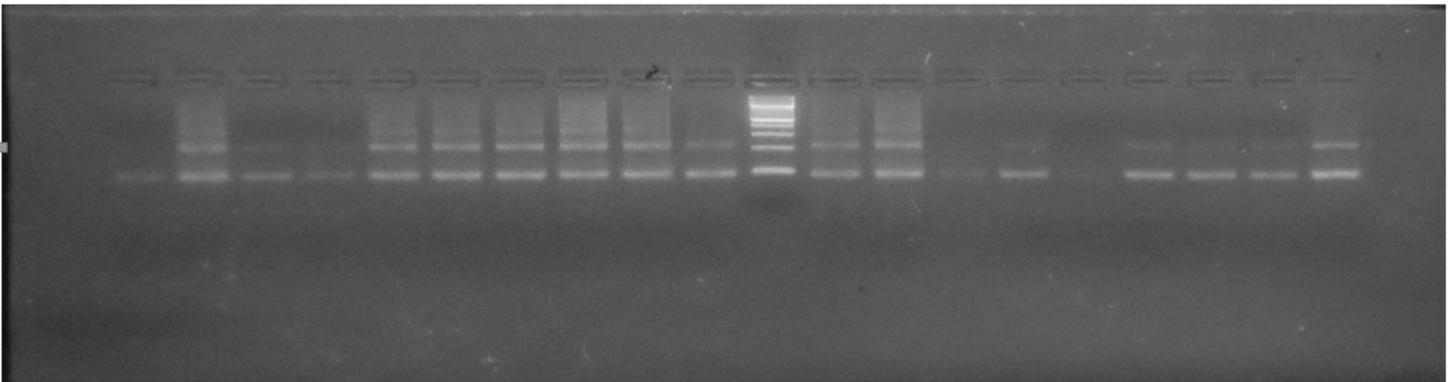


Figure 2

Plate 2: Agarose gel electrophoresis showing the amplified repeats of the *Schistosoma haematobium* from selected samples negative by microscopy. Lanes showing 1 band or more represent positive samples for *S. haematobium* while lanes with no bands represent negative samples. L is a 100bp ladder.