



# Progress towards Schistosomiasis Elimination: Application of Sensitive Diagnostic Techniques

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## Abstract

**Objective:** Pursuing successful control programs aiming at schistosomiasis elimination, the diagnostic performance of Kato-Katz, urine Circulating Cathodic Antigen (CCA) point of care rapid test and real-time PCR techniques were compared for the detection of *Schistosoma mansoni* among school children with different infection intensities.

**Methods:** Stool and urine samples were collected from 110 school children after obtaining the consent of their parents and school guardians. The study was conducted in Motobus village, Kafr El-Sheikh governorate, Egypt. The collected stool samples were examined after Kato Katz (two slides, 41.7 mg each). Urine specimens were examined for *S. haematobium* by filtration technique and were tested for *S. mansoni* by POC-CCA. For of real time PCR, part of each stool sample was kept at -20°C until used.

**Results:** All the examined children were negative for *S. haematobium*. The performance of the three tests revealed that examination of double Kato-Katz smears detected *S. mansoni* infection in 51% of school children, where most of them had low infection intensity (64%). However, higher *S. mansoni* prevalence of 83% and 60% were diagnosed by real-time PCR and CCA, respectively. It was also found that real-time PCR detected a higher proportion of *S. mansoni* negative cases after Kato-Katz- compared to CCA (70% and 22%) respectively. Kato-Katz, CCA and real time PCR showed similar results with moderate and heavy infection intensities. Increasing the number of cycles of real time PCR to 40 cycles could detect the low infection intensity.

**Conclusion:** To move towards schistosomiasis elimination, real time PCR due to its great sensitivity is suitable for established control programs to detect cases overlooked by Kato-Katz technique and CCA test. The adoption of this technique can enhance the monitoring of *Schistosomiasis mansoni* control measures in pre-elimination or elimination settings.

**Keywords:** *Schistosoma mansoni*; Kato-Katz; Circulating cathodic antigen point of care cassette test (CCA-POC); SYBR green; Real-time PCR

## Introduction

Schistosomiasis is a chronic disease caused by infection with parasitic worms of the genus *Schistosoma*. It is considered one of the neglected diseases responsible for disabilities each year around the globe [1,2]. Approximately 240 million human individuals are infected globally and about 700 million people are living at risk of this infection [3].

Schistosomiasis control has received support through global alliances and partnerships. A cardinal step was taken by the WHO in setting out a clear strategy for the scale up of preventive chemotherapy and assistance to countries to appraise their future praziquantel needs until 2020 and beyond. Its current strategy for the control of schistosomiasis is the reduction of morbidity through regular treatment with praziquantel of populations at risk [4,5]. Efforts to control morbidity of the disease are being stepped up worldwide. In some countries, there are existent prospects to turnover from schistosomiasis control into transmission control and eventually elimination [6]. What is more, elimination in selected areas by 2025 is now an aim [7,8].

In conditions where elimination of schistosomiasis is aimed at, case detection may pose a problem as the commonly used methods lack the necessary sensitivity to accurately determine the prevalence of schistosomiasis or low infection intensity [9,10]. Active surveillance should rely upon proper diagnosis, particularly in situations of low intensity [9-11].

Kato-Katz is the method recommended for diagnosis of *S. mansoni* infection by the WHO for its high specificity, handiness and cost effectiveness [12]. The concern that Kato-Katz method misses *S. mansoni* infection in a proportion of cases in regions of low prevalence and intensity has a very important implication, principally when it is used in monitoring the effectiveness of preventive chemotherapy [13].

Serodiagnostic assays designed for the detection of specific anti-schistosome antibodies and antigens have been developed over the years [14,15]. However, the drawback intrinsic to antibodies assays is the discrimination between active and old infections [15]. In addition, blood collection is not easily applicable for field studies in endemic areas [14,15].

The commercially available point of care urine Circulating Cathodic Antigen (CCA-POC) has been shown to be a promising approach to

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the identification of active *S. mansoni* infection and to the assessment of treatment efficacy [16,17]. POC diagnosis is beneficial not only for individual patients but it allows as well for rapid mapping of disease prevalence and the acceleration of schistosomiasis control programs, factors which are imperative to meet the 2020 goals [18].

PCR method displayed high sensitivity and specificity for the detection of *S. mansoni* DNA in stool samples, yet it gave negative results among few cases positive by microscopy [19]. Lodh et al. [20] showed that the diagnosis of *S. mansoni* from urine samples by PCR is an effective way to diagnose light infections and thus enhance the effectiveness of surveillance and control programs of schistosomiasis.

Real time PCR assay using SYBR Green (fluorescent based technology) and Taq Man probe for the detection and quantification of *S. mansoni* DNA in fecal samples were developed and evaluated as a diagnostic method to study the epidemiology of schistosomiasis [21].

The present study aimed at comparing the performance of urine Circulating Cathodic Antigen (CCA) cassette test and real time PCR as well as PCR Ct values in cases of *S. mansoni* with different intensities as diagnosed by Kato-Katz. Focus for adequate management for schistosomiasis transmission interruption and elimination was considered.

## Methods

### Study Area

The study was carried out in Motobus village, El-Riyad District, Kafr El-Sheikh governorate, Egypt. Motobus is 100 km away from Alexandria. Farming is the major source of economic activity and agriculture employs most of the work force.

### Administrative design and ethical considerations

For the conduction of the present work, ethical clearance was permitted by the ethical committee of the Medical Research Institute and parasitology department. Approval of the study was obtained from the Egyptian Ministry of Health and Population and the Administration of Health Affairs in Kafr El-Sheikh governorate. Preliminary meetings were held with the directors and teachers of the school to explain the outline of the study. Informed consents were obtained from school guardians and parents before participation of their children in the study. Ethically, all cases with *S. mansoni* and other parasites (diagnosed after Kato-Katz) were treated with conventional drugs.

**Study subjects and sample size:** Based on a 35% infection rate of *S. mansoni* diagnosed previously in this area [22,23] and on the hypothesis that the sensitivity of the tests used in the present study is around 90% and accepting a margin of error  $\pm 7$  at level of confidence 95%, a sample size of 110 pupils randomly selected were enrolled in the study. Children aged 6-12 years in Arab El-Mahder primary school were asked to provide stool and urine samples.

**Collection of urine and stool samples:** The school was visited on two consecutive days every week for collection of stool and urine samples. On the first day, tightly closed plastic containers labelled with student's identification number, name and class, were distributed. On the next day, the containers were collected from all students enrolled in the study and returned to the parasitology laboratory in the Medical Research Institute. All the collected stool samples were examined microscopically after Kato-Katz technique (41.7 mg/slide, 2 slides for each sample) [24]. A portion of each fecal specimen was stored at -20°C for further processing by real time PCR. A single midstream urine

sample was collected from each child and was examined by filtration technique for the presence of *S. haematobium*. Part of each urine sample was stored at -20°C until the CCA assay was performed.

### Laboratory techniques:

**CCA cassette test:** CCA assay was obtained from Rapid Medical Diagnostics (Pretoria, South Africa). In brief, one drop of each urine sample was added to the circular well; the CCA if present in the urine sample binds to the labelled monoclonal antibody immobilized on the membrane of the well. The buffer solution then runs over the strip where the antigen-antibody complex attaches to another monoclonal antibody immobilized at the test line developing a pink color. The other line is a control, which should always give a pink color to make sure the test works correctly [16,17].

**DNA extraction and real time PCR:** Extraction of egg DNA from all stool specimens was done by using QIAGEN amp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For detection of *S. mansoni* specific DNA, primers targeting the 28 S rDNA region from *S. mansoni* (SmF/ SmR) were used by Sandoval et al. [25] to diagnose *S. mansoni* from urine samples. These primers were used in the present study to amplify the extracted *S. mansoni* DNA from stool samples. Primers SmF GAGATCAAGTGTGACAGTTTTGC and SmRCAGTGC GCGCGTTCGTAAGC, rDNA (ribosomal DNA) region localization 28S were selected to amplify a fragment of 350 bp, which was detected by the SYBR Green [21]. Positive control sample for *S. mansoni* DNA was used. In addition, DNA controls of other parasites were extracted from routine stool samples submitted to department of parasitology, Medical Research Institute, Alexandria University. A DNA negative control was obtained from the stool specimen of parasite free individual.

PCR included 10  $\mu$ L of extracted DNA from each stool sample, 0.1  $\mu$ L of each *S. mansoni* primer (forward and reverse), 12.5  $\mu$ L SYBR Green universal PCR master mix as well as water free RNAase added in the reaction tube to a final volume of 25  $\mu$ L. The real time PCR was then performed under the thermal profile as follows: An initial denaturation step at 95°C for 15 min was firstly carried out. 40 cycles of amplification were performed (94°C for 20 s, 61°C for 20s and 72°C for 30 s). Well defined DNA extracted from *S. mansoni* positive stool samples was used as positive control and included in each PCR run. Melting curve analysis was done to determine the purity and specificity of the amplified product. It was subsequently performed after PCR run, by increasing temperature slowly from 72°C to 95°C for one min, then at 55°C for 30 s and finally at 95°C for 30s [21].

**Intensity of infection:** Intensity was determined by microscopy after Kato-Katz and compared with the cycle threshold (Ct) value of the real-time PCR.

### Statistical analysis

Data were analyzed using IBM SPSS for Windows, Version 20.0 (Armonk, NY: IBM Corp). The statistical program was utilized for both data presentation and statistical analysis of the results. For descriptive analysis, the prevalence by different methods was articulated in percentages, while the median was used to present the real-time PCR Ct values. Geometric mean was used to express the *S. mansoni* egg counts (GMEC) [26]. Confidence Interval (CI) of 95% was applied as measures of central tendency and dispersion respectively for normally distributed quantitative data. The final judgment of the results for each sample was based on the three tests (KK, PCR and CCA), the positive and negative assessment was based

on the next predictions according to Lodh et al. [20]. If Kato-Katz, CCA and real time PCR are positives the results are True Positives (TP). If the three methods gave negative results, then True Negative (TN). If KK positive and CCA or PCR negative then the sample considered False Negative (FN). The samples are False Positive (FP) when PCR or KK are negative and CCA positive. Built on these assumptions, the results of all specimens collected were classified and all diagnostic parameters were calculated at 95% CI using Med Calc 12.4.0 (Med Calc Software, Belgium). The diagnostic parameters of the three methods were evaluated by establishing a reference standard incorporating all the positive cases by KK, CCA and real time PCR. Kappa agreement (K) test was applied for measuring the agreement between the results of the diagnostic tests (at  $k < 0.2$  poor agreement,  $k > 0.2-0.4$  fair agreement,  $k > 0.4-0.6$  moderate agreement,  $k > 0.6$  high

agreement). Spearman's non-parametric coefficient ( $r$ ) was used to detect the correlation between the Ct values and intensity of infection. In all statistical tests, significance was accepted as  $p < 0.05$  [27].

## Results

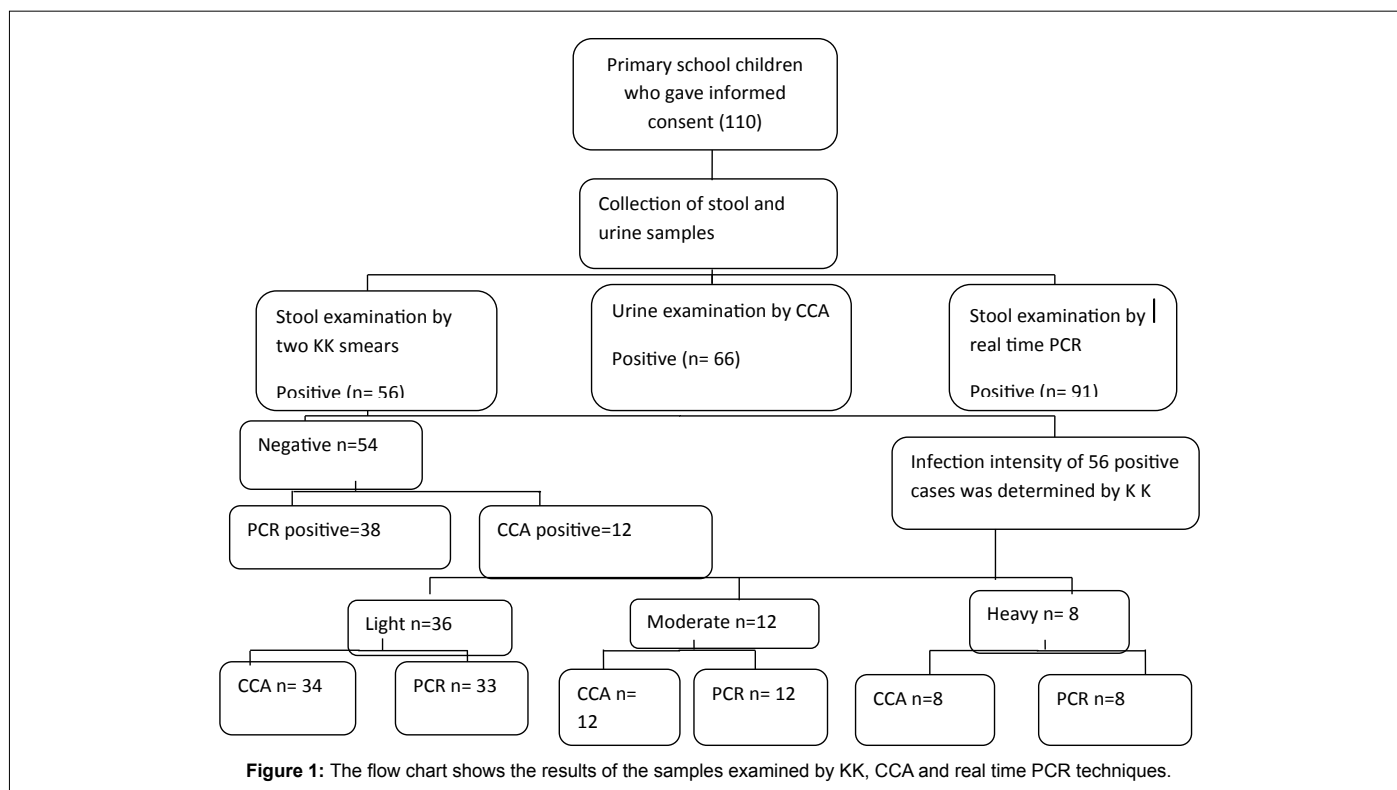
By using the urine filtration technique, all samples were negative for *S. haematobium*.

The performance of the three diagnostic methods to detect *S. mansoni* infection among the examined school children is presented in Table 1 and Figure 1. Out of 110 tested samples, 94 cases were true positive (by KK+ CCA+PCR). Real time PCR gave the highest positive (91 true positives) results compared to the three tests. The number of false negatives was highest with KK (38 cases).

Table 2 showed the calculated diagnostic parameters including sensitivity and specificity of KK (60%, 100%), CCA (67%, 81%) and PCR (97%, 100%). The Positive Predictive Values (PPVs) were relatively similar, ranging from 96% to 100% for the three used methods. The Negative Predictive Values (NPVs) were inconsistent. Moreover, the positive and negative likelihood ratios were reflective of PPVs and NPVs for the used diagnostic methods.

Test	True positive	False positive	True negative	False negative	Total
KK	56	0	16	38	110
CCA	63	3	13	31	110
PCR	91	0	16	3	110

**Table 1:** Evaluation of the performance of Kato Katz (K-K), circulating cathodic antigen (CCA-POC) and real time PCR for detection of *S. mansoni* among the examined school children.



**Figure 1:** The flow chart shows the results of the samples examined by KK, CCA and real time PCR techniques.

Diagnostic test	Positive No (%)	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (PPV)	Negative predictive value (NPV)	Positive likelihood ratio	Negative likelihood ratio
Kato-Katz	56 (51)	60% (49%-70%)	100% (79-100%)	100%	70% (60-81%)	Gold +ve	0.4 0.32-0.52
CCA	66 (60)	67% (73 % -91%)	81% (76%-98%)	96% (88%-98%)	71% (59%-80%)	3.5	0.4 0.32 to 0.52
PCR	91 (83)	97% (91-99%)	100% (79-100%)	100%	84% (64-94%)	Gold +ve	0.03 0.01-0.1

CI=confidence interval.

**Table 2:** Prevalence, sensitivity, specificity, predictive values, and likelihood ratios of the three diagnostic tests for detection of *S. mansoni* infection among 110 examined children.

### The agreement between the different diagnostic techniques in the detection of *S. mansoni*

Statistical analysis showed a kappa index of 0.75 indicating high agreement between Kato-Katz and CCA. Fourteen cases showed discordant results. By comparing the results of the real time PCR and Kato-Katz, 53 patients gave concordant positive results and 41 showed discordant results. Statistically, a fair agreement was detected between both techniques (K=0.25). Also, CCA and real time PCR showed fair agreement with discordant results among 33 cases and the kappa index was 0.31 (Table 3).

### Comparison of CCA, real time PCR performance and Ct values with Kato-Katz according to *S. mansoni* infection intensity

Table 4 and Figure 2 illustrate the distribution of the positive cases by the Kato-Katz according to the intensity of infection. 64.3% of positive cases had light infection intensity, 21.4% and 14.2% showed moderate and heavy intensity. Results of the CCA and PCR are presented in the different egg count categories. All cases with moderate and heavy infections were positive by both techniques. Among the light intensity category, both CCA and real time PCR missed cases with equal or less than 12 eggs. The table shows as well the range and the median PCR Ct values in the different categories. Statistically, there was a negative correlation between the Ct values (Figure 3) and *S. mansoni* egg counts

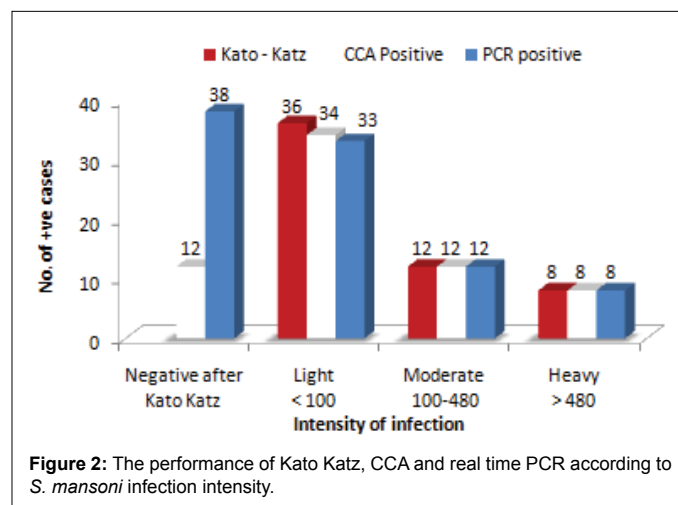


Figure 2: The performance of Kato Katz, CCA and real time PCR according to *S. mansoni* infection intensity.

but this correlation was not significant (r=0.208; n=56; p=0.124). The melting curve analysis determined the specificity of the sample product (Figure 4).

### Discussion

Egypt has been overwhelmed by schistosomiasis for many years. Fortunately, due to the immense and sustained adoption of different control programs, the prevalence fell down to less than 2% [13]. Despite of this control effort, noted foci of endemicity still exist, possibly due to a lack of improvement in rural sanitary conditions [6].

In the present study it was found that, despite the high prevalence in Motobus village (51% as diagnosed by Kato-Katz), the majority of children (64%) had light infection while few had moderate and heavy infections. An explanation of the low GMEC may be a lower transmission potential due to low snail infection together with the availability of passive chemotherapy as rural health units and local physicians are present throughout the governorate. In addition, self-referral for examination and treatment is common.

As we have moved towards low intensity and elimination of schistosomiasis, reconsideration of the present results in the light of intensity is needed. The wide transmission potential is existent even when small numbers of miracidia are released in water. In this study, the performance of Kato-Katz, CCA test and real time PCR were compared for the diagnosis of *S. mansoni* with different infection intensities. This study revealed that the highest prevalence (83%) was detected by real time PCR, followed by CCA (60%) while the lowest prevalence was diagnosed by Kato-Katz (51%). Tchuente et al. and Coulibaly et al. [16,18] reported that single urine CCA cassette test produced similar prevalence as multiple Kato-Katz thick smears. Regarding sensitivity, PCR showed a sensitivity of 97%, while CCA currently in use as a point of care rapid diagnostic test has low sensitivity problems as KK (67%, 60% respectively). Similar results were reported by Lodh et al. [20].

By studying the agreement between the three techniques, statistical analysis showed high agreement between urine CCA cassette test and Kato-Katz and a fair agreement between Kato-Katz and real time PCR as well as between CCA and real time PCR. These results confirm the low sensitivity of Kato-Katz as compared to both techniques and the highest efficacy of real time PCR in the detection of *S. mansoni*. The fair agreement between Kato-Katz and PCR as well as between CCA and

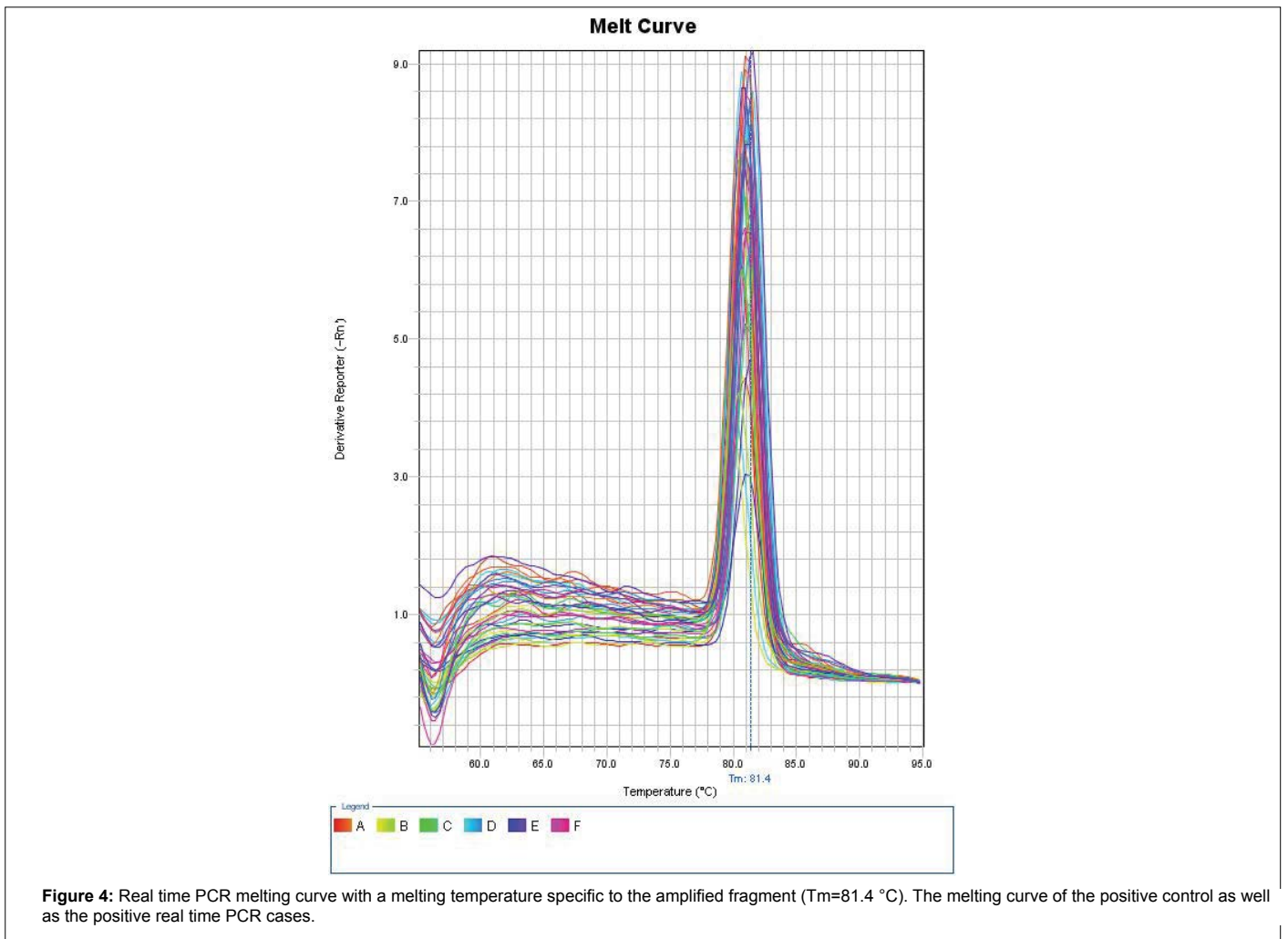
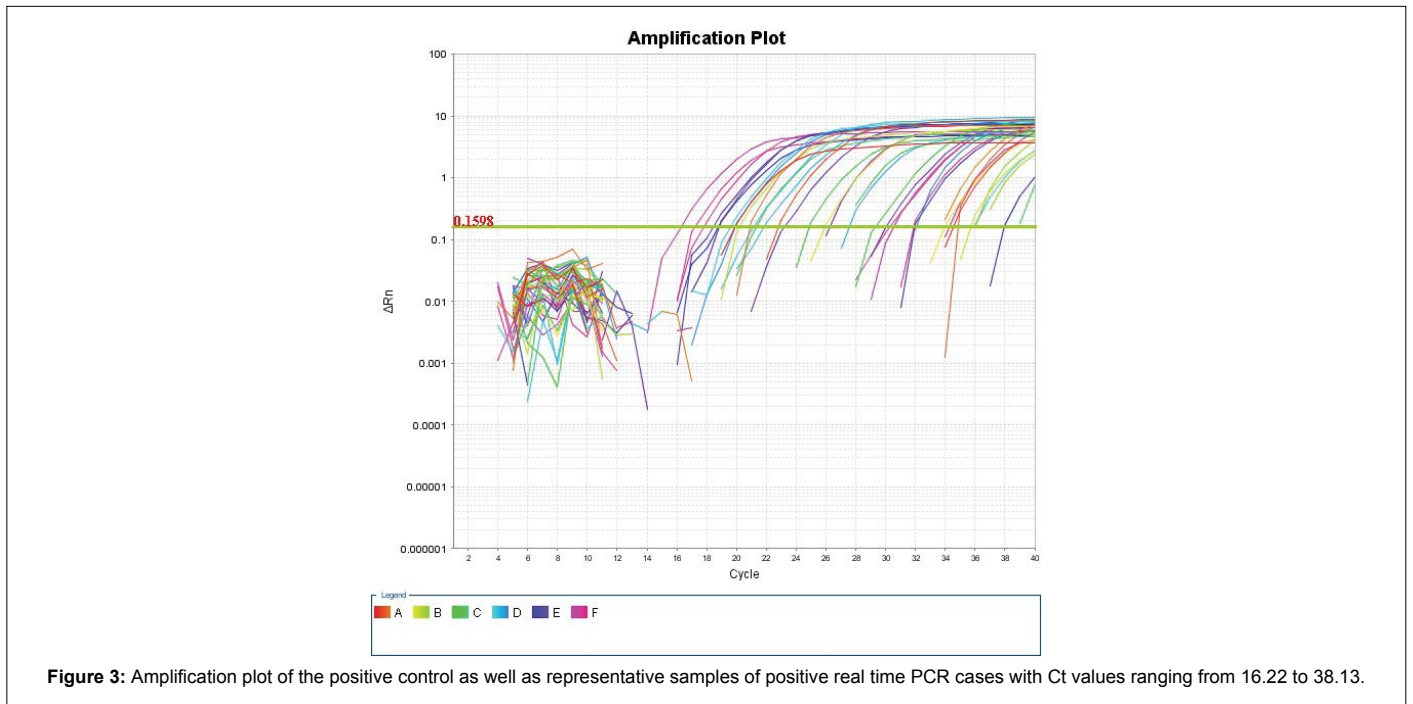
CCA	Kato-Katz		Total
	Positive	Negative	
Positive	54	12	66
Negative	2	42	44
Total	56	54	110
k=0.75			
PCR	Kato-Katz		Total
	Positive	Negative	
Positive	53	38	91
Negative	3	16	19
Total	56	54	110
k=0.25			
PCR	CCA		Total
	Positive	Negative	
Positive	62	29	91
Negative	4	15	19
Total	66	44	110
k=0.31			

Table 3: Agreement between Kato-Katz, CCA and real time PCR for the detection of *S. mansoni* infection.

Egg count category (Kato-Katz)	CCA positive No (%)	PCR positive No (%)	Ct value of positive PCR per egg count category Range (Median)
Negative (n=54)	12 (22.2)	38 (70.37)	8.83-37.92 31.92
Light<100 (n=36, 64.3%)	34 (94.4)	33 (91.66)	18.78-38.13 26.44
Moderate (100-480) (n=12, 21.4%)	12 (100)	12(100)	17.29-34.25 21.17
Heavy>480 (n=8, 14.2%)	8 (100)	8 (100)	16.22-24.85 19.84

\*GMEC=73 eggs/gm

Table 4: Comparison between results of CCA, real time PCR and Ct values with Kato-Katz categorized by the number of *S. mansoni* eggs.



PCR means that the combination of Kato-Katz and or CCA tests with PCR will improve the diagnostic results more than combining Kato-Katz with CCA test.

Concerning the discordant results, two cases were diagnosed only by Kato-Katz, three cases were diagnosed only by CCA while one case was detected by both Kato-Katz and CCA but missed by real time PCR and this may be attributed to specific inhibitors in stool or to DNA degradation during transportation and/or manipulation of the stool samples [21]. The two cases positive by Kato-Katz and negative by CCA could be explained by low intensity and small amount of antigen released. The three cases detected only by CCA, may be false positives or due to the presence of immature worms during the migration period to mesenteric veins before starting egg laying (egg negative/worm positive). Further studies in the laboratory and follow up of these cases may be required to address these issues.

Depending on the intensity of infection as estimated by Kato-Katz, the three tests detected almost similar prevalence in moderate and heavy infections. Parallel observations were reported by several authors, they documented that the current commercially available CCA urine cassette test was appropriate for diagnosis of *S. mansoni* in moderate and heavy transmission areas [16-18]. Inconsistency between microscopy, CCA and PCR was observed in samples with low egg counts or low parasite DNA concentration. Real time PCR diagnosed *S. mansoni* positive cases approximately three folds (70.4%) higher than CCA test (22.2%) among those missed by Kato-Katz. Referring to real time PCR, the present results were similar to those of Hove et al. [21] who reported that the sensitivity of real time PCR was 100% for subjects with *S. mansoni* intensity more than 100 eggs. They also stated that discrepancies between real time PCR and microscopy were observed in samples with low intensity of infection.

The present study illustrated that real time PCR showed the best performance as it detected the highest positive cases in comparison to CCA and Kato-Katz. The high positive and negative predictive values were also indicative of PCR robustness. The use of real time PCR had the advantage of quantitative analysis, as shown by the negative correlation between Ct values and number of target copies [28-30]. Increasing the number of real time PCR cycles to 40 cycles could detect the low intensity of infection. The specificity of the real time PCR based on SYBR Green product was verified by the performance of melting curve analysis. A characteristic melting temperature of the amplicon distinguishes it from amplification artefacts that melt at lower temperatures with broader peaks [29,30]. When comparing the real time PCR with microscopy-based assays, the PCR is less labor intensive when many samples are to be examined. The real time PCR method also has the advantage of being less influenced by observer variation/bias as compared to microscopy [31]. CCA is rapid, easy to use, less time consuming than Kato-Katz, field applicable and the easiness to collect urine than stool samples are significant characteristics for this test [16,32].

## Limitations

The higher prevalence obtained with KK together with CCA and real time PCR techniques argue for the continuation of Mass Drug Administration (MDA) in endemic areas. The costs to apply these tests and control interventions may constitute a major constraint. It may be proper to deviate from mass drug administration to a test re-ally and selective treatment development.

## Conclusion

In conclusion, Kato-Katz, CCA and real time PCR showed similar

results with moderate and heavy infection intensities. Real time PCR was the strongest detector of *S. mansoni* positive cases missed by Kato-Katz and CCA. It has also the advantage of quantitative analysis. To detect the low intensity of infection, it is recommended to increase the number of its cycles to 40 cycles.

The main strength of this study is the inclusion of the real time PCR and CCA results to provide more information on individual's infection status. The study also provides accurate diagnosis of *S. mansoni* and baseline for prospective studies. To proceed towards a goal of elimination, it is advised to begin the survey with Kato-Katz and then apply POC-CCA on the negatives to diagnose more cases and to detect egg-negative/worm-positive schistosomiasis. PCR should enact on the negative cases after Kato Katz and CCA. Efforts to reduce CCA and real time PCR costs are necessary to facilitate their large scale use.

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