Sandwich ELISA & Conventional PCR for Diagnosis of Schistosoma mansoni infection (A Field Study)

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Abstract

Schistosomiasis is a major public health problem. Accurate early diagnosis is strongly recommended to provide proper treatment before progressing to complications.

Objective and methods: the aim of this study was to detect *S. mansoni* infected cases in two villages at Fayoum Governorate, Egypt, through application of different diagnostic methods. In field, subjects were primary screened by Kato Katz (KK) and Sandwich Enzyme Linked Immunosorbent Assays (ELISA). ELISA was performed for antigen detection in both serum and saliva samples. Then conventional Polymerase Chain Reaction (PCR) was used to examine 366 selected preserved serum samples, and results were compared with earlier tests.

Results: Among 1330 subjects completed the study and gave the required samples, KK revealed prevalence of *S. mansoni* infection among the study population (1.8%). In comparison with the standard KK technique, ELISA detecting antigen in serum and/or saliva samples gave sensitivity rates up to 100% and specificity rates (≥ 99%). PCR sensitivity and specificity were (95.8% & 96.2%) in comparison with KK.

Conclusion: *S. mansoni* is still prevalent in the study areas with low rate of transmission. Sandwich ELISA assay for *S. mansoni* circulating antigen detection is a sensitive and specific

diagnostic tool (either applied on serum or saliva samples) with results in the same range with PCR.

Key words

Schistosomiasis, circulating antigens, Saliva, serum.

Introduction

Schistosomiasis is a major public health problem in over 70 countries with substantial impacts on human health and economic development. People become infected when larval forms of the parasite – released by freshwater snails – penetrate the skin during contact with infested water. Transmission occurs when people suffering from schistosomiasis contaminate freshwater sources with their excreta containing parasite eggs, which hatch in water (Barakat 2013 and WHO, 2017).

The WHO strategy for schistosomiasis control focuses on reducing disease through periodic, targeted treatment with Praziquantel through the large-scale treatment (preventive chemotherapy) of affected populations (WHO, 2017). First of all, an accurate diagnosis of an active infection should be made in order to effectively treat and control the disease (CORSTJENS et al., 2014).

In Egypt, schistosomiasis is a traditional public health problem. Fayoum is an Egyptian governorate located south-west of Cairo with 3,340,564 inhabitants, working mainly in agriculture and related industries (CAPMAS, 2016). According to world schistosomiasis risk chart (2015), Fayoum was considered as endemic area for schistosomiasis (WHO, 2015). The prevalence rates of *S. mansoni* and *S. haematobium* in Fayoum were 4.3% and 9.95%, respectively (El-Khoby et al., 2000 and Barakat,2013) and it was reduced to (1.66 %) for *S. haematobium* as recorded by Shoeib et al. (2016). The detection of eggs in stool (*Schistosoma mansoni*) or in urine (*Schistosoma haematobium*), although laborious, is still the most common method to determine schistosome infections and the only tool formally recommended by the World Health Organization (WHO) in low-resource settings (Corstjens et al., 2014). However, it lacks sensitivity, particularly in low-endemic areas. Alternatively, several immunological techniques for detection of parasite-specific antibodies and/or circulating antigens are available including different formats of ELISA (Gomes et al., 2014). Immunodiagnosis of *S. mansoni* is still applied in different parts of the world. These methods are inexpensive, reproducible,

objective, rapid and potentially automatable (Carneiro et al., 2013). Antibody detection assays are more sensitive than parasitological examination, but can't reliably specify cure in the short-term. Detection of *Schistosoma* circulating antigens is more valuable. It is becoming an important tool for the diagnosis of active infections. For field applications, the preferred sample is blood, or less invasive samples such as urine or saliva (Ochodo et al., 2015).

Saliva can be used as a diagnostic fluid for many diseases comparable to serum samples, especially when used for antibody detection (Malamud & Rodriguez-Chavez, 2011).

Many techniques for identifying circulating *S. mansoni* DNA have been widely used. Such methods are able to detect infections as early as one day after parasite exposure. It is a very specific method particularly in low-intensity infections (Carneiro et al., 2013 and He et al., 2016).

The aim of this study was to detect *S. mansoni* infected cases in two villages at Fayoum Governorate, Egypt, through application of different diagnostic methods. The primary screening techniques were KK and Sandwich ELISA. ELISA was performed for antigen detection in both serum and saliva samples. In the 2nd part of study, 366 preserved serum samples were examined by conventional PCR and results compared with the earlier test.

Materials and Methods

Study area and population: This study was conducted in two villages (Abo–Elsoud and Abo Ish) in Fayoum Governorate. They have a fresh water supply from River Nile. They constitute about 29,000 inhabitants; most of them were farmers, with a higher chance for exposure to infection. Written informed consents were obtained from participants. The study received approval by local health authorities and the Faculty of Medicine, Fayoum University Ethics Committee and performed in accordance with the Declaration of Helsinki.

Study design:

This is a descriptive cross sectional study for detection of *S. mansoni* infected cases in the study areas through application of different diagnostic methods. A systematic sample of the houses (every tenth house) was identified and numbered in the study areas and then samples were collected. Participants who gave a history of current intake of anti-parasitic drugs were excluded from the study. The study was conducted from December 2011 to April 2014.

The study consisted of two parts: (i) parasitological and immunological baseline screening for *S. mansoni* included 1330 participants, (ii) detection of *S. mansoni* DNA in 366 serum samples. Positive cases for *S. mansoni* were treated with Praziquantel (PZQ). Cases with other helminthic infections were provided suitable drugs as recommended by the Egyptian Ministry of Health.

Collection of samples:

For each participant, the name, age, sex, history of schistosomiasis and previous anti-*Schistosoma* treatment were recorded. Then they were asked to provide only one of the following samples in suitable sterile labeled containers**.** Preparation of samples and direct parasitological examinations were performed within 24 hours of collection in the Laboratory of Parasitology, Faculty of Medicine at Fayoum University. Body fluid samples were transferred within 24 hours to polypropylene tubes and stored at - 20°C until tested by ELISA and PCR in the laboratory of the Department of Parasitology, Faculty of Veterinary Medicine at Cairo University.

A venous blood sample, used to prepare sera.

A saliva sample collected according to Nagler and Hershkovich (2005). Samples were transferred to test tubes containing phosphate buffer saline (PBS), centrifuged to remove debris.

A urine sample, at least 10 ml of early morning, midstream urine then centrifugation sedimentation technique and direct microscopic detection for *S. haematobium* egg was done.

A stool sample; of over 50 grams, not mixed with urine then two slides were prepared from each sample within 24 hours of collection using the KK method (Katz et al., 1972), examined for *S. mansoni* eggs and/or other helminthic infections. For *S. mansoni* eggs only, the average egg count from the examined slides was recorded as egg per gram (EPG).

S. mansoni excretory -secretory antigen (ESA) preparation:

ESA was extracted from living *S. mansoni* worms obtained from Theodor Bilharz Research Institute, Egypt, according to Marrero et al. (1988). The protein content was measured (Lowry et al., 1951), and ESA was used for preparation of Rabbits Hyper-immune serum (RHIS) according to Osorio et al. (1998). Rabbits' sera were collected prior immunization (negative control sera). Sera were collected one week after the last injection (positive control sera), stored at -20°C until used.

Two-antibody Sandwich ELISA (Antigen capture assay):

Two antibodies sandwich ELISA was used to detect *S. mansoni* circulating antigen in serum and saliva, according to Osori et al. (1998). The test conditions were adjusted after checkerboard titrations; the optimal dilutions of the sera and enzyme conjugate were determined. ELISA micro-titer plates were coated with 100µL/ well of 1:100 RHIS diluted in 0.1mL carbonate buffer (pH 9.6). Plates were incubated overnight at 4°C. Plates were then washed thoroughly with PBS (50 mM, pH 7.2) then blocked with 0.2% bovine serum albumin fraction V (Sigma) in PBST (pH 7.2), 100 µl/well. After washing two replicate of each serum (1:50) and saliva (1:2 dilution) samples were added and incubated for 3 hours at 37^{o}C. After washing 100µL/well of the previously prepared RHIS (1:100 diluted in PBS) were added then incubated at room temperature for 2 hours. After washing, 100µl of Horseradish peroxidase conjugated goat anti-rabbit IgG, diluted 1:1000 within PBS was added to each well and incubated for 1 hour. After plate washing, 100µl of the Ortho-phenylene diamidine (OPD) substrate was added to each well. The reaction was allowed to proceed for 15 minutes at room temperature in the dark. The reaction was terminated with 0.2 M H_2SO_4. The absorbance was read at 450 nm with an automated Titerteck multiskan plate reader. The cutoff value was established based on the mean optical density of negative control sera plus 2 standard deviations.

DNA extraction from sera and *S. mansoni* adult worm crude extract by using QIAamp DNA Blood and Body Fluid Mini Kit (Qiagen GmbH, Hilden, Germany) was according to manufacturer's instructions.

DNA amplification from serum samples: using primers designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni* that composes approximately 10% of the parasite genome (600,000 copies per cell) described by Hamburger et al. (1991). The forward primer sequence is (5′ GATCTGAATCCGACCAACCG-3′) and the reverse primer has the following sequence (5′ ATATTAACGCCCACGCTCTC-3′).

PCR conditions: twoµL of undiluted extracted DNA was used as initial template. All reactions were carried out in PTC-100 Thermal Cycler in a 10µL mixture containing: 20mM Tris–HCl (pH 8.4), 50mM KCl buffer; 1.5mM MgCl2; 0.5µM of each primer; 200µM dNTPsand 0.75U of Taq DNA polymerase. A total of 35 cycles were performed each of them consisted of three steps: 45 sec denaturation at 95°C, 30 sec annealing at 63°C and 30 sec extension at 72°C.

The denaturation step was prolonged for 5 min in the first cycle, and the last cycle included an extension step of 2 min. To prevent contamination, extraction, amplification of DNA and the preparation of the PCR mixture were conducted in separate environments using sterile and disposable laboratory supplies. Contamination was monitored using negative controls containing water instead of extracted DNA in the amplification reaction. As PCR positive controls, 1ng of *S. mansoni* adult worm DNA was used as a template. PCR assays were conducted 3 times for each sample. The PCR products were subjected to electrophoresis on 6% polyacrylamide gels and analyzed after silver staining.

Statistical analysis

SPSS software package (version 16.0 for Windows; SPSS Inc, Chicago, Ill) was used for statistical analysis. Data were presented as mean ± standard deviation (X ± SD). Evaluation of diagnostic performance was done by calculation of sensitivity (SN), specificity (SP), diagnostic accuracy (ACC), Positive and negative predictive values (PPV & NPV).

Concordance between results of various tests was assessed using Cohen's Kappa index at 95% confidence intervals. The Kappa index values range interpretation was as follows: poor agreement (<0.20); low agreement (0.20-0.40); moderate agreement (0.41-0.60); good agreement (0.61-0.80); and excellent agreement (0.8-1.00) (Altman, 1990). Chi-squared test was applied to detect levels of association between the results of various tests. All P values ≤ 0.05 were considered statistically significant.

Results

Out of the 1758 participants, 127 didn't supply stool samples, and 326 refused to supply blood samples so they were excluded from the study owing to incomplete data. Finally, 1330 participants remained with complete samples. They were 685 (51.5%) subjects from Abo-Elsoud village and 645 (48.5%) from Abo-Ish village. They were 693 males (52.1%) and 637 females (47.9%) with age ranged from (7- 70) years old.

Stool examination revealed prevalence of *S. mansoni* infection in 24 cases among the total population (1.8%), with egg count ranged from 2-49, and a mean of 25.9±18.9 EPG for the infected cases. Kato Katz test also revealed that a total of 268 cases (20.2%) were found to be

infected with at least one parasite other than *S. mansoni*. Eggs of *Ascaris lumbricoides, Hymenolepis* spp. and hookworms were the most commonly encountered (Table 1).

Harboring more than one parasite (multiple helminthic infections), other than *Schistosoma* worms was reported for 232 subjects (17.5 %). Examination of urine samples revealed a total of 22 (1.65%) positive samples for *S. haematobium*.

The distribution of cases between the two villages is shown in (Table 1). There were no statistical differences between the two villages regarding the number of cases with schistosomiasis or other helminthic infections ($p > 0.05$).

Prevalence of *S. mansoni* infection varied according to different techniques applied on 1330 samples **(**Table 2). Detection rate of *S. mansoni* circulating antigen in the examined sera was 37 cases (2.8%), while the detection rate using saliva samples was 28 cases (2.1%). PCR technique was performed for the detection of *S. mansoni* DNA in 366 preserved serum samples (having; *S. mansoni*, any parasitic infection, Hepatitis C virus infection, or history of treatment of schistosomiasis). The test was positive in 36 cases (9.8%) of the tested samples (Table 2).

The distribution of cases in relation to the KK test and PCR was presented in (Tables 3 and 4).

In comparison with the standard KK technique, the validity of ELISA for detection of *S. mansoni* antigen in the examined 1330 serum and saliva samples are presented (Table 3). Detection of *S. mansoni* circulating antigen in both serum and saliva samples revealed 100% sensitivity and specificity rates up to (99.7% and 99%) respectively.

The overall accuracy of *S. mansoni* circulating antigen detection assays in serum and saliva samples was high ≥ 99%. The sensitivity and specificity of PCR in comparison with KK were (95.8% and 96.2%) respectively (Table 3). The decreased sensitivity as PCR failed to detect 1 case diagnosed positive by KK test. Chi squared test showed significant change of results after performing various ELISA tests in comparison with KK technique ($p<0.001$) (Table 3).

In comparison with KK technique, Kappa test showed better agreement of *S. mansoni* circulating antigen detection in saliva than in sera samples (Kappa indices = 0.922, 0.782 respectively)**.** Results of PCR showed good degree of agreement with KK (Kappa index=0.747) with significant change of results after performing the test ($p<0.001$) (Table 3).

In the second part of this study, PCR technique was considered as a reference test and the performance of various tests was compared to it (Table 4). Thirty two cases diagnosed by both PCR and *S. mansoni* circulating antigen detection in sera with five false positive cases added by

antigen in sera (Table 4). One case was a true case of *S. mansoni* (positive KK) and was missed by PCR. The other 4 false positive cases (3 had *S. haematobium,* and 1 case had *Fasciola gigantic*a infection) were considered as cross-reactivity with *S. mansoni* antigens. There were 4 negative cases by *S. mansoni* circulating antigen in serum and were positive by PCR (Table 4). They all were KK negative; three of them had history of treatment from *S. mansoni*. The 4th PCR false positive case belonged to a female patient with *Ascaris* and *Strongyloids* infections.

In comparison with PCR, *S. mansoni* circulating antigen detection was a sensitive test, it gave a sensitivity rates in sera and saliva up to 88.9% and 75% respectively, while KK sensitivity was only 63.9%. As regard specificity, *S. mansoni* circulating antigen detection in saliva and sera were specific tests (99.7% and 98.5%) respectively. In comparison with PCR, KK gave specificity up to 99.7%. The overall accuracy of *S. mansoni* circulating antigen detection in serum and saliva samples reached up to 97.5% and 97.3% respectively (Table 4). *S. mansoni* circulating antigen detection in sera and saliva showed the best degree of agreement with PCR test (Kappa indices =0.863, 0.829 respectively). ELISA and KK tests showed significant change of results after performing the PCR test ($p<0.001$) (Table 4).

Discussion

Although considered to be among the neglected tropical diseases (NTD) of the world, schistosomiasis remains one of the most common parasitic diseases throughout the tropics and subtropics and is a major public health problem in these areas (WHO, 2017).

Egypt has been plagued by many NTD since Pharaonic time. Schistosomiasis is still persisting as a public health problem in the country, despite the continuing improvement in the economic status, standard of living, sanitation and ecology of the Egyptian society (Abou-El-Naga, 2015). The results of this study revealed decreased total prevalence of schistosomiasis, with a tendency to increase rates of *S. mansoni* over *S. haematobium* as previously observed (Barakat 2013 and Shoeib et al ., 2016). This was explained by the hydrological changes following construction of dams leading to changes in the ecology of the snail intermediate hosts. However, the migratory flow of the infected rural population that has occurred in consequence with an occupation and urbanization led to expansion and the emergence of new foci of schistosomiasis transmission with lack of sanitation and basic infrastructure (Abou-El-Naga, 2015). Within this perspective, it is crucial to identify infected cases, especially in low transmission areas (Gomes et al., 2012).

Accurate, reliable, and inexpensive diagnostic methods are keys for monitoring infection dynamics and treatment efficacy (WHO, 2015).

The low sensitivity of KK in the present study may be attributed to examination of only one stool sample per subject, despite preparing two slides /each fecal sample. Many authors recommend examination of multiple stool samples in order to estimate prevalence more accurately, since infection cannot be deduced from only one KK (Bergquist et al. 2009). This was, however, not a realistic option in this study and the procurement of two stool samples– together with urine, saliva and serum samples – was at the limit of acceptability by the population. Another explanation for low KK sensitivity, is due to low ova output in the early stages of infection (prepatent period) and in chronic cases or in areas of mass drug administration (Kato-Hayashi et al., 2014). In the present study, application of ELISA and PCR added new cases to parasitological prevalence and agreed with (Weerakoon et al., 2015) that in low transmission areas of *S. mansoni* infection, one diagnostic test is insufficient for diagnosis and can't give both 100% sensitivity and specificity (Weerakoon et al., 2015).

The results indicated that application of many tools can enhance the diagnosis *S. mansoni* infection especially in areas of low parasite load, this agrees with (Cavalcanti et al. 2013).

The present study considered KK as a reference technique in its 1st part (WHO, 1993). In the 2nd part PCR was used as reference test (Kato-Hayashi et al., 2014). *S. mansoni* circulating antigen detection in sera gave the best performance and concordance with both reference techniques ($k \geq 0.782$) in agreement with Gomes et al. (2014) and Wang et al. (2002). This was followed by antigen detection in saliva samples. Previously, (Gomes et al., 2014; Colley et al. 2014) declared that circulating antigens are highly specific and had positive correlation with worm burden. Furthermore, they can differentiate between active and past infections. In addition, they disappear rapidly after treatment. Therefore, they can be used for assessment of cure. Determination of *S. mansoni* circulating antigen is better than detection of antibodies which gave high false positive rates in chronic infection or after treatment of the parasite, since they could be present for more than 6 months post parasite eradication Gomes et al. (2014).

Using saliva samples for diagnosis of *S. mansoni* infection by estimation of circulating antigen was demonstrated in the present paper as a new diagnostic scope. Wang et al. (2002) diagnosed *S. japonica* infection by detection of circulating antibodies is saliva by indirect ELISA. Results of the present study agreed with Singh et al. (2005) that using saliva for diagnosis of infections is

equivalent to sera with respect to test efficacy, offering an alternative to the invasive blood sampling. Zhou et al. (2009) reported that the specificity of saliva (92.11%) was superior to serum samples (85.53%) in diagnosis of anti-*S.japonicum* antibody by ELISA. They concluded that salivary detection is a promising alternative to the serological test for schistosomiasis using serum. In the present study, the lower protein concentration in saliva than serum samples was reflected as a decrease in the mean ELISA OD values.

The good degree of agreement between KK and PCR results and significant change of the results subsequent to performing PCR technique was, on the contrary to a study by de Carvalho et al. (2012) as they obtained a Kappa coefficient = 0.234, showing weak agreement between the two diagnostic techniques. PCR sensitivity, specificity and total accuracy in comparison with KK were in agreement with Gomes et al. (2014). While Kato-Hayashi et al. (2014) reported that they were (100% & 59%) in serum samples.

In this study, PCR showed one false negative case, and 13 false positive over KK. False positive cases may be attributed to single worm infection or early stage of infection where no eggs had passed. According to de Carvalho et al. (2012), a positive PCR assay indicates the presence of the parasite without information regarding its life cycle phase or its viability, including the presence of mature male and female worms capable of egg deposition. PCR requires a well-equipped laboratory with highly trained technicians; which is more costly than using the usual techniques. Also, nucleic acid amplification is easy to be contaminated under unsuitable laboratory conditions, giving false positive results. Although experimental studies reported that the sensitivity and specificity of nucleic acid tests were significantly higher than those of the Kato-Katz method, yet they cannot completely replace it (Ochodo et al., 2015). Development of suitable detection techniques for large-scale application in endemic areas of schistosomiasis requires simplified procedures of the nucleic acid method and reduction of their cost (He et al., 2016).

Conclusion: *S. mansoni* is still prevalent in the study area despite control program with low transmission proved by simple parasitological examination. Application of different techniques improved detection methods. ELISA detecting circulating antigen assays is a sensitive and specific tool for diagnosis and gave results equivalent to PCR, either applied on serum or saliva

samples. This immunodiagnostic assay has the advantage of being simple, cheap, and field applicable with prevalent needed infrastructure than PCR.

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Table 1: Results of direct parasitological examination of stool (1330 subjects)

Parasite	Abo–Elsoud (685) N (%)	Abo-Ish (645) N (%)	Total (1330) N (%)
Eggs of *S. mansoni*	14 (2)	10 (1.6)	24 (1.8)
Eggs of *Fasciola gigantica*	9 (1.3)	12 (1.9)	21 (1.6)
Eggs of *Taenia* spp.	13 (1.9)	10 (1.6)	23 (1.7)
Eggs of *Hymenolepis* spp.	41 (6)	57 (8.8)	98 (7.4)
Eggs of *Ascaris lumbricoides*	66 (9.6)	61 (9.5)	127 (9.5)
Eggs of *Ancylostoma*	39 (5.7)	54 (8.4)	93 (7)
Eggs of *Entrobius vermicularis*	28 (4)	23 (3.6)	51 (3.8)
Larvae of *Strongyloides*	42 (6)	45 (7)	87 (6.5)
Double parasitic infections	120 (17.5)	112 (17.4)	232 (17.5)
Total infected	140 (20.4)	128 (19.8)	268 (20.2)

Table 2: Prevalence of *S. mansoni* infection according to different techniques

Technique	Abo–Elsoud (685) N (%)	Abo-Ish (645) N (%)	Total (1330) N (%)	OD Range	Mean ± SD
KK	14 (2)	10 (1.6)	24 (1.8)	-	-
Antigen in serum	19 (2.8)	18 (2.8)	37 (2.8)	0.325-0.768	0.669 ± 0.14
Antigen in saliva	16 (2.3)	12 (1.9)	28 (2.1)	0.312-0.542	0.504 ± 0.05
PCR performed for 366 cases	18/178 (10.1)	18/188 (9.6)	36 /366 (9.8)	-	-

Table 3: Efficacy and concordance degree of various tests in comparison with KK as a reference test

Test		KK		Total	SN	SP	PPV	NPV	ACC	Kappa index	p- value
		P	N								
S. mansoni circulating antigen in serum	P	24	13	37	100	99	64.9	99	99	0.782	<0.001
	N	0	1293	1293							
	Total	24	1306	1330							
S. mansoni circulating antigen in saliva	P	24	4	28	100	99.7	85.7	100	99.7	0.922	0.001
	N	0	1302	1302							
	Total	24	1306	1330							
PCR	P	23	13	36	95.8	96.2	63.9	99.7	96.2	0.747	<0.001
	N	1	329	330							
	Total	24	342	366							

P: positive N: negative

Table 4: Efficacy and concordance degree of various tests in comparison with PCR as a reference test

Test		PCR		Total	SN	SP	PPV	NPV	ACC	Kappa index	p-value
		P	N								
S. mansoni circulating antigen in serum	P	32	5	37	88.9	98.5	86.5	98.8	97.5	0.863	<0.001
	N	4	325	329							
	Total	36	330	366							
S. mansoni circulating antigen In saliva	P	27	1	28	75	99.7	96.4	97.3	97.3	0.829	<0.001
	N	9	329	338							
	Total	36	330	366							
KK	P	23	1	24	63.9	99.7	95.8	96.2	96.2	0.747	<0.001
	N	13	329	342							
	Total	36	330	366							