Circulating *Schistosoma* DNA in seropositive patients: A two-step diagnostic approach to rule-out acute and active chronic schistosomiasis in low transmission settings

Original Article

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ABSTRACT

Background: Since its discovery in 1850 by Theodor Bilharz at Kasr Al-Ainy hospital, Cairo, Egypt, schistosomiasis continues to be a significant health challenge with a wide spectrum of chronic sequelae. There is no reliable single gold standard conventional method (microscopy or serology) for communities with low intensity and low transmission settings.
Objectives: To evaluate a two-step immunomolecular diagnostic approach to rule-out recent *Schistosoma* re-infection in patients with characteristic pathological radiological features of chronic schistosomiasis.
Subjects and Methods: Single serum samples were collected from patients from Kafr El Sheikh, Egypt. Selection of patients was based on microscopic absence of *Schistosoma* spp. eggs in either stool or urine samples; abstinence from treatment by Praziquantel in the last 2 months; and clinical and radiological diagnosis of pathological features of chronic schistosomiasis. All serum samples were serologically examined using indirect hemagglutination test (IHAT) for identification of anti-*Schistosoma* antibodies. Serum samples from 100 seropositive patients using IHAT were tested by multiplex PCR (mPCR) for detection of species-specific cell-free circulating *Schistosoma* DNA.
Results: None of the seropositive serum samples were positive by PCR targeting *Schistosoma* species-specific gene. Patients who were farmers and/or who had received Praziquantel treatment were associated with *Schistosoma* seropositivity with statistical significance.
Conclusion: A two-step immunomolecular diagnostic approach, using a single serum sample, can rule-out acute or active chronic schistosomiasis in patients that have characteristic pathological and radiological features of chronic schistosomiasis. Also, the two-step approach avoids over-treatment and development of drug resistance to Praziquantel, the only effective broad anti-*Schistosoma* medicine.

Keywords: circulating *Schistosoma*-DNA; Egypt; IHAT; PCR; praziquantel; schistosomiasis; seropositive.

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INTRODUCTION

Schistosomiasis is a neglected tropical and subtropical helminthic parasitic disease caused by the *Schistosoma* spp. This neglected disease is multi-constituent including parasite, host, and vector factors. A successful massive drug administration (MDA) *Schistosoma* control program was implemented for the last 40 years in many countries including Egypt. However, due to re-infection schistosomiasis continues to be a significant health challenge, posing as one of the most disability-adjusted life years diseases and the cause of mortality in over 70 endemic countries in Africa, Middle East, South America and Asia[1-4].

Humans can be infected by two forms of *Schistosoma* infection, urogenital and intestinal, caused by five *Schistosoma* species: *S. haematobium*, *S. mansoni*, *S. japonicum, S. intercalatum* and *S. mekongi*. *Schistosoma haematobium, mansoni,* and *japonicum* are the main three species infecting humans worldwide. In Egypt, the two prevailing species are *S. mansoni* and *haematobium* including mixed infection by both species[3]. The resulting schistosomiasis is an acute parasitic infection that tends to persist as a life-long chronic debilitating disease. There is a wide spectrum of chronic sequelae of schistosomiasis, some of which are fatal, including chronic cystitis, carcinoma of the bladder, portal hypertension, pulmonary hypertension, male and female genital schistosomiasis, and cerebral schistosomiasis[5,6].

The majority of the currently used diagnostic methods are conventional, including microscopic identification of *Schistosoma* ova in stool, urine and/or tissue smear, or immunological detection of parasite-specific antigen/antibodies[1,7,8].

The MDA control program for schistosomiasis is based on the application of a treatment strategy for all populations living in *Schistosoma* endemic regions,

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whether they are infected or not. In Egypt, since 1996, a widespread MDA schistosomiasis control program has been applied. This control program maintains low *Schistosoma* transmission and low intensity of infection, engendering the challenge of missed cases of acute schistosomiasis based on parasitological microscopy. In addition to misdiagnosis, microscopy is of limited value in diagnosis of schistosomiasis during chronic infections with a wide spectrum of chronic disease sequelae[3,9,10].

Diagnosing chronic schistosomiasis, in many countries including Egypt, is mainly dependent on serological diagnostic tests, mainly IHAT, in the presence of characteristic radiological image features and in the absence of detectable *Schistosoma* ova in urine, stool samples or tissue biopsy. Serological tests that depend upon antibody detection cannot differentiate between recent and old infection, persistent and re-infection, or co-occurrence of the two or more *Schistosoma* species. They are also limited by the variability in sensitivity and specificity of different serological test formats. In addition, antibodies persist for months or even years following clearance of infection[7,8].

Cell-free circulating *Schistosoma*-DNA are fragments detached from *Schistosoma* cells of a live-parasite and released in blood, saliva, urine, and other body fluids. Molecular detection of cell-free circulating *Schistosoma*-DNA is used to detect early acute schistosomiasis and active chronic schistosomiasis in absence of *Schistosoma* eggs in stool and urine. These DNA fragments rapidly disappear within weeks following treatment making molecular methods a useful follow up tool[11-13].

This study's intention was to evaluate a two-step immunomolecular diagnostic approach to rule-out *Schistosoma* recent re-infection in patients who had radiological characteristic pathological lesions of chronic schistosomiasis with a history of schistosomiasis or anti-*Schistosoma* treatment.

SUBJECTS AND METHODS

This laboratory-based cross-sectional study was carried out on serum samples from patients residing in Kafr El Sheikh from May, 2016 to February, 2017. Immuno- and PCR assays were carried out in the Diagnostic and Research Unit of Parasitic Diseases (DRUP), the Molecular Medical Parasitology Lab (LMMP), respectively, Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt.

Patients: They were clinically and radiologically diagnosed with pathological features of chronic schistosomiasis and referred to the lab for serological testing for schistosomiasis. The exclusion criteria included patients less than 6 years old, patients with other underlying illnesses, patients for whom *Schistosoma* eggs were microscopically detected in their stool or urine samples and those who had taken Praziquantel in the last 2 months. A single blood sample was obtained from each patient, separated by centrifugation, then kept at -20°C for immunoassays and DNA extraction, followed by PCR assay. Related patients' data were obtained with each sample and analyzed as predictors for *Schistosoma* seropositivity and molecular prevalence.

Immunoassay: Part of the serum from all study individuals was examined for detection of anti-*Schistosoma* antibodies using IHAT (Fumouze Diagnostics, France) following the manufacturer's instructions.

Molecular assay[14,15]: Serum samples from 100 seropositive patients using IHAT were tested by mPCR assay. DNA extraction of all serum samples was done using QIAamp DNA Blood Mini Kit (Qiagen- Germany) following instructions by the manufacturer. The mPCR was used to amplify the extracted genomic-DNA targeting *Schistosoma*-species specific DNA mitochondrial cytochrome oxidase I (mtDNA) gene. It can differentiate between *S. haematobium* and *S. mansoni* based on length of mPCR products. The mPCR was performed following the reaction condition, reaction mix and a set of 3 primers[14,15], including common forward for *Schistosoma* (5`-TTT TTT GGT CAT CCT GAG GTG TAT-3), and 2 reverse primers for *S. haematobium* (5`-TGA TAA TCA ATG ACC CTG CAA TAA-3′) and *S. mansoni* (5′-TGC AGA TAA AGC CAC CCC TGT G-3′). The mPCR products (543 bp for *S. haematobium* and 375 bp for *S. mansoni*) were electrophoresed on agarose gel (1.5%) after staining the gel by ethidium bromide and viewed by a UV transilluminator.

Statistical analysis: Collected data was statistically analyzed using IBM SPSS V23 software. Patients associated quantitative variables were presented as mean and standard deviation; and the associated qualitative variables were tabulated as numbers and percentages. Variables were compared using the Chi-square test and/or Fisher exact test when appropriate. *P* value equal to or less than 0.5 was considered significant.

Ethical Consideration: The study was started after approval by the ethical committee of the Faculty of Medicine, Al-Azhar University. Patients or their guardians were orally informed about the study goals; serum samples collection was done after acquiring their consent. Joining the study was voluntary.

RESULTS

A total of 100 patient serum samples were sero-positive for schistosomiasis by IHAT. All seropositive

patients met the inclusion and exclusion criteria of this study. Urine and stool specimens from included patients were microscopically negative for *Schistosoma* eggs. All sero-positive serum samples were PCR negative using PCR targeting *Schistosoma*-species specific DNA.

The patient's characteristics were presented in table (1). Among seropositive patients, 75 (75%) were males and 25 (25%) were females. Those > 40-50 years old were the most affected age group (42%). The majority of seropositive patients were farmers (65%), lived in rural areas (81%) and had received Praziquantel treatment (68%).

In our study, being a farmer (P=0.01) and/or having received Praziquantel treatment (P=0.02) were associated with *Schistosoma* seropositivity, with statistical significance (Table 1).

Table 1. Distribution of patients' characteristics in 100 seropositive *Schistosoma* patients.

Patients' characteristics		Seropositive (No. 100)		*P* value
Age (Year) (Mean±SD)		46.5 ± 12.1		**0.2**
Age groups	**20-30**	21	21.0%	**0.6**
	>30-40	25	25.0%	
	>40-50	42	42.0%	
	>50	12	12.0%	
Sex	**Males**	75	75.0%	**0.07**
	Females	25	25.0%	
Occupation	**Farmer**	65	65.0%	**0.01***
	Housewife	21	21.0%	
	Worker	11	11.0%	
	Unemployed	3	3.0%	
Residence	**Rural**	81	81.0%	**0.12**
	Urban	19	19.0%	
Praziquantel treatment	**Yes**	68	68.0%	**0.02***
	No	32	32.0%	
Total		100	100.0%	

***:** Significant (P<0.05)

DISCUSSION

Since its discovery in 1850 by Theodor Bilharz at Kasr Al-Ainy hospital, Cairo, chronic schistosomiasis continues as a significant health challenge worldwide, including Egypt[3]. In the current study, circulating *Schistosoma*-DNA was not detected using PCR in all sera of seropositive patients with characteristic radiological imaging features of chronic schistosomiasis, supporting the idea that seropositivity of *Schistosoma* antibody among the studied population with chronic infection may be related to old *Schistosoma* infection. Serology alone is not a reliable diagnostic method for these patients, because it cannot rule out the possibility of reinfection or active chronic *Schistosoma* infection[1,8,16].

Characteristic radiological imaging features are important for diagnosing chronic schistosomiasis in the affected target organs, as well as assessing the complications and the severity of the disease. The primary target organs in schistosomiasis include urinary bladder in schistosomiasis *haematobium*, rectum and distal colon in schistosomiasis *mansoni* and schistosomiasis *japonicum*. The secondary target organs include the urinary tract, particularly the upper part, liver, spleen and lungs. In addition, accidentally *Schistosoma* eggs may deposit in ectopic secondary target organs and affect them via their venous anastomoses with the inferior vena cava. This results in genital schistosomiasis, cerebral schistosomiasis or skin ectopic infection. In chronic schistosomiasis the diagnosis is essentially by radiological imaging and confirmed by serology in absence of etiological microscopic diagnosis[16,17]. Serological tests, including IHAT, have many limitations: they cannot differentiate between recent/active *Schistosoma* and chronic old infections, persistent and re-infection, or co-infection of more than one *Schistosoma* species. They are also limited by variance in sensitivity and specificity of different serological test formats. In addition, antibodies persist for months to years after clearance of infection, which renders serological tests targeting antibodies useless in these circumstances[7].

Rapid diagnostic test as a point of care test (POCT) is an easy applicable method that can detect specific circulating and secreted worm antigens. This test is easy, equipment-free, applicable, and rapid[18]. *Schistosoma* antigen-detection tests are limited by their false negative results due to low sensitivity and are not suitable for low infection intensity setting. However, they are not appropriate for epidemiological studies and monitoring MDA *Schistosoma* control programs[1,7,18]. This is especially true in underdiagnosed infections using low sensitive microscopy and antigen detection

tests, or over diagnosis when using serological tests. Implementation of molecular diagnosis of schistosomiasis should not be limited to reference labs and should be readily available in small local and rural hospitals as well[7].

Based on our results, in agreement with the results of different studies[1,7,8,16], serological diagnosis is a poor positive test to rule-in the possibility of re-infection or active chronic schistosomiasis, and for those who were prescribed anti-*Schistosoma* treatment for characteristic radiological features of chronic schistosomiasis in target organs. Thus, we recommend a two-step approach for diagnosis of chronic schistosomiasis with late manifestations. Patients with a positive serological test should be further examined molecularly for *Schistosoma*-DNA, in order to avoid unnecessary treatment and the development of drug resistance to Praziquantel. Praziquantel is, to date, the only safe and effective anti-*Schistosoma* medicine, used for all types of human schistosomiasis as well as for control programs[4,10].

This accurate, cost effective two-step active schistosomiasis diagnostic approach requires the implementation of molecular diagnosis in endemic areas, especially those in which DNA-based techniques, particularly PCR, is already available. Our study found a statistical significance association between *Schistosoma* seropositivity and being a farmer and/or having received Praziquantel. No association was found between *Schistosoma* seropositivity using IAHT and the clinical picture, age group, sex and living in rural/urban setting. In endemic countries, both female and male gender predominance or non-predominance with *Schistosoma* infection was reported[19-22]. Children aged 5–17 years-old have been identified as the highest *Schistosoma* infected group in developing countries[3,22,23]. Additionally, farmers in contact with contaminated water when irrigating fields are in the highest risk occupation group for acquiring *Schistosoma* infection[3,4,24].

In conclusion, recent *Schistosoma* re-infection needs to be excluded in patients who have characteristic pathological and radiological features of chronic schistosomiasis and are living in low endemic regions with low transmission settings. For these patients, a two-step immunomolecular diagnostic approach (detection of cell-free circulating *Schistosoma*-DNA in sero-positive patients using a single serum sample) can rule-out acute or active chronic schistosomiasis. Also, it avoids unnecessary treatment and development of drug resistance to the only available effective broad anti-*Schistosoma* medicine, Praziquantel.

Author contribution: All authors made substantial contributions to the study design, analyzing, and interpreting data, revising the manuscript and approved the final version submitted. Gawish FG, Bayoumy AMS, Abd El Raheem MA, and Abo Hashim AH contributed to the collection of samples and to parasitological and immunological processing of samples. El-Badry AA conducted the molecular work for the study. Gawish FG drafted the manuscript and El-Badry AA wrote the final manuscript.

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