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Real-Time PCR Improves Detection of Active Infection in Human Feces and Treatment Failure Following Multiple Chemotherapeutic Rounds in Schistosoma mansoni Low-Endemic Area

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- Schistosomiasis
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Abstract

The diagnosis of active schistosomiasis and the subsequent therapeutic regimen based on Kato-Katz test (KK) are highly compromised in the low-endemic area (LEA). Recognition of individuals with decreased or no egg-excretion and assessment of drug response became impaired. Therefore, the appropriate assessment of surveillance strategies like chemotherapy use is inaccurate. To overcome these limitations, we describe results of the diagnosis of active schistosomiasis by using, in addition to microscopy and serology, DNA-detection assay pre and post-chemotherapy. In 108 individuals, KK, serology, and real-time PCR were performed for diagnosis of schistosomiasis. In 32 out of 108 individuals, had schistosomiasis based on laboratorial diagnosis, and 14 individuals who accepted treatment with praziquantel were followed up to 2 years post-chemotherapy. Positive egg excretion and DNA fragments were demonstrated in 8/108 (7.4%) individuals. IgE levels were detectable in 8/8 egg-excretors, although IgG1 was reactive in 7/8 egg-excretors. DNA amplification was able to demonstrate active infection in 24 individuals even without egg-excretion and/or IgG1 reactivity. Post-chemotherapy, lack of amplified DNA correlated with a therapeutic response in 7 out of 14 individuals after 6 months. Follow up showed that 2 years later all but one presented DNA amplification. Furthermore, a significant decrease in IgG1 and IgE reactivity also correlated with drug responses at 2 years post-therapy. This proof-of-concept study suggests that DNA detection might become an essential tool to diagnosis schistosomiasis in LEA where light infection with low parasite burden predominates. Real-time PCR is a robust approach for revealing schistosomiasis active infection despite no detection of egg excretion and /or immunoreactivity. Also, results highly suggested that real-time PCR can be reliably used to assess therapeutic responses and permit monitoring strategies for schistosomiasis surveillance in LEAs.

ABBREVIATIONS

KK: Kato-Katz Test; LEA: Low-Endemic Area; NPSC: National Programs for Schistosomiasis Control; ELISA: Enzyme-Linked Immunosorbent Assays; PCR: Polymerase Chain Reaction; A.U.: Arbitrary Units; COX: Cytochrome C Oxidase Subunit 1; PZQ: Praziquantel

INTRODUCTION

Schistosomiasis presents with a broad array of clinical manifestations during both acute and chronic phases of infection. In general, its manifestations are unspecific, and asymptomatic infections are far more common. Additionally, when based on clinical findings, the outcomes and response to chemotherapy are quite imprecise, thereby undermining its clinical management. Thus, the diagnosis of an active Schistosoma infection in individuals with a history of exposure (living in endemic areas, travelers, migrants) with or without clinical signs or symptoms is primarily dependent on diagnostic tests. The laboratory investigation of Schistosoma infection consists of different techniques including parasitology, immunology and molecular biology [1-3].

The conventional diagnosis of Schistosoma infection relies

on egg detection by parasitological methods such as Kato-Katz test (KK). KK is an affordable quantitative method for diagnosis of other soil-transmitted helminthes as well as Schistosoma spp. Albeit low cost and easy-to-do, KK sensitivity reduces drastically with low parasite loads which results in misdiagnosis of light infections characterized by nil or low egg counts. Also, KK method uses a few fecal samples that might contribute to KK lower diagnostic accuracy in light infection [4]. Other parasitological methods such as formol-ether sedimentation, salt flotation and centrifugation, and Helmintex, improve egg detection in stool samples and they have been valuable for diagnosis in low endemic areas. Although these methods improved the diagnostic sensitivity, the pitfalls of the direct visualization of eggs by microscopy remain. In addition, all modified methods are time-consuming and laborious being less suitable for routine or large-scale screening [2].

An infection with *Schistosoma mansoni* is quite difficult to detect in individuals living in non-endemic and low endemic areas when based solely on parasitological tests. The lack of egg shedding interferes directly with the sensitivity of microscopic examination of stool samples, thus compromising the detection of low-intensity infections in low endemic areas (LEAs). Furthermore, coproscopy is an unreliable test to monitor response to therapy. "True" cured status cannot be assessed because egg excretion is erratic and may be absent despite the presence of an active infection [5]. Because establishment of Schistosoma infection based on egg-count became limited, other methods were investigated. In contrast to parasitological methods, immunological tests demonstrated to be a suitable choice in LEAs as a complementary test [6]. Also, immunodiagnosis have become useful for showing active infections in recently exposed individuals, such as travelers or chronically infected immigrants residing in non-endemic areas [7,8]. Despite its infrequent use in National Programs for Schistosomiasis Control (NPSC), serology is a potent auxiliary diagnostic approach in LEA that permits the diagnosis of non-egg excretors. However, the presence of active infection may be undermined by persistent reactivity despite successful treatment [1,6].

DNA detection has proven to be a feasible strategy for diagnosing *S. mansoni* infection in both experimental settings and in individuals living in high endemic areas whether carrying high or low parasitic loads. In experimentally induced schistosomiasis, an absence of DNA amplification correlates highly with the response to chemotherapy. However, the role of DNA detection in monitoring treatment response in individuals living in LEA has not been well established.

In Brazil, schistosomiasis morbidity has declined significantly since the introduction by the NPSC in the 1970s of mass chemotherapy in highly prevalent areas [1]. However, the successful reduction in severe forms paralleled a decrease in parasite burden, and assays based on the direct detection of eggs by parasitological methods, such as KK test, became less sensitive. Also, the lack of egg-shedding during the pre-patent phase in acute infections is easily missed. In travelers, recent infection is often missed after exposure in transmission areas [8]. Both situations pose an important obstacle to the diagnosis of schistosomiasis inside and outside endemic areas. For diagnostic purposes, as an alternative when stool specimens are negative, biopsy specimens from the rectum, liver, and other sites may be obtained in tertiary health care facilities. For instance, rectal snips may be examined for the presence and viability of trapped eggs. However, the sensitivity of tissue examination may also decline as the infection evolves to a chronic form. Persistent ova passage leads to submucosal fibrosis, itself leading to a lack of egg elimination and/or tissue deposition [10]. Additionally, disease severity does not always correlate with high egg-shedding rates, for example, ectopic clinical presentations including gynecological, and neuroschistosomiasis may show negative results. Other drawbacks related to biopsies may include tissue access, which may require a highly invasive sampling, such as in central nervous system [10]. In contrast, the indirect detection of Schistosoma infections based on immunological assays is non-invasive and cost-effective. Immunodiagnostic tests have already been proven to be reliable in confirming infection in naïve populations, such as travelers [11]. In contrast, a lack of reactivity can almost rule out schistosomiasis in suspected cases of acute or chronic infection. However, these tests are unable to differentiate between active and past infection. Even in the presence of progressive moderate-to-severe forms of the disease, such as hepatosplenomegaly with ongoing portal hypertension, these assays are not used routinely in laboratory tests or for clinical management.

Currently, DNA detection assays have been drawing attention due to their robustness, making them applicable to both community and individual diagnoses. PCR-based methods are specific and sensitive in detecting Schistosoma DNA in the stool, urine, blood, plasma, serum and genital specimens obtained from Schistosoma-infected individuals. DNA detection approach is a promising strategy. Most clinical laboratories have now started to standardize PCR-based assays [5,12-14]. Also, DNA detection assays accurately diagnose both egg-positive and egg-negative individuals pre-treatment. Also, DNA assays have a potential role as a marker of treatment responses as shown in some studies [15]. Nevertheless, large-scale study series and long-term follow-ups are still lacking. Thus, DNA detection assays have not been used extensively in either routine diagnoses or clinical management strategies [8,12,13]. In this proof-of- concept study we evaluated a real-time PCR combined with coproscopy and immunodiagnostic assay as a diagnostic tool for *S. mansoni* active infection in LEA both pre and post chemotherapy. The use of combined strategies showed potential applicability in surveillance by monitoring programs for the control and/ or eradication of Schistosoma infection post-chemotherapeutic intervention in a low endemic area.

MATERIALS AND METHODS

Ethical statement

The study population was informed of the protocol and written informed consent was obtained from each participant and in the case of minors from legal guardians. Individuals enrolled in the study approved by the Hospital Clementino Fraga Filho, Universidade Federal do Rio de Janeiro (HUCFF/UFRJ) Ethics Committee (n°058/09) subsequently provided fecal and blood samples prior to and after chemotherapy.

Study sites and population

The study was performed in two rural areas of Sumidouro, a small municipality known as a low endemic area for schistosomiasis that is located in Rio de Janeiro State (22°02" 59'S, 42°40" 29'W), Brazil. In Sumidouro, rural areas occupy valleys with several small streams and irrigation channels that are tributaries of the Paquequer II river (Paraiba do Sul River tributary). These areas have been occupied by both permanent and fluctuant population involved in agriculture and livestock activities. The study population comprised 108 individuals including permanent residents and immigrants, newly arrived and returning individuals, with or without the history of previous treatment (2003 and 2006). Criteria used to diagnose active infections, treatment response, and follow-up are: *S. mansoni* DNA detected in at least one fecal sample in the presence and/or absence of KK and/or IgG1 and/or IgE positivity. If positive, individuals were treated with praziquantel (40 to 60 mg/kg, single dose). Additionally, individuals positive for other helminth infections were treated with albendazole (400 mg, single dose) as recommended by the Brazilian Ministry of Health and the World Health Organization [16]. The criteria for the absence of active infection after chemotherapy (response to therapy) were no excretion of eggs (KK in two independent samples) and/or no DNA amplification.

Parasitological and immunological diagnoses

S. mansoni infection was determined based on Kato-Katz tests on two independent samples/individual (two slides/stool sample, KK). Determination of the mean egg count resulted from the sum of eggs counted in each slide prepared by the parasitological method and divided by the total number of slides examined (4 slides/in 2 samples/individual). The result was multiplied by 24 [17]. Any value found was considered including zero. The mean egg count of the quadruplicate KK slides was calculated to determine infection intensity. Specific IgG1 and IgE anti-adult worm membrane soluble antigen (SMMA) was measured using an ELISA (SMMA-ELISA) as described previously [6]. The results are expressed in arbitrary units (A.U.) that were calculated by dividing the sample OD by the daily cut-off OD value. Values above 1.0 were considered positive.

DNA detection

Real-time PCR was used for DNA detection in fecal samples. *S. mansoni* probes and primers targeting the cytochrome c oxidase subunit 1 (COX) gene in the mitochondrial genome were designed as described previously [14]. DNA was extracted from fecal samples by using the Fast Prep DNA kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions and purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The reaction was prepared using 5 µL of eluted DNA as a template and mixed with 2.0 pmoles/µL of each *S. mansoni* primer (SMCYT748F and SMCY847R), 2.5 pmoles/µL of SMCY785T detection probe, 11.0 µL of Platinum qPCR SuperMix Rox-UDG (Invitrogen) and 2.3 µL of deionized water to make a final volume of 20 µL. The amplification conditions were 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification, detection, and data analyzes were performed using the 7500 Real-Time PCR System (Applied Biosystems, CA, USA). DNA amplification was positive when Ct values were < 38. All methods, molecular and parasitological, were performed on stool specimens collected in the same conditions, and immunological tests were performed on serum samples, obtained in the very same day pre and post-treatment.

Statistical analyses

Descriptive statistics (and normality tests) were performed by using Stata version 13 (College Station, Texas). Data analyses such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of the tests were done only when the individuals enrolled in the study provided serum for IgG1 and IgE levels determinations and fecal samples (two independent samples over different days) for KK and DNA detection. The sensitivity, specificity, PPV, and NPV were calculated with Kato-Katz test used as a reference test. The correlation between coproscopy and ELISA-IgG1/IgE or real-time PCR prior to treatment was assessed using the kappa statistic (k). The k index was interpreted as following: excellent agreement defined as being between 1.00 and 0.81, good was between 0.80 and 0.61, moderate was between 0.60 and 0.41, weak was between 0.40 and 0.21 and negligible was between 0.20 and 0.00. Pre- and post-treatment IgG1 and IgE responses, respectively, were compared using Wilcoxon test. Samples from individuals who did not comply with study requirements were excluded from the final analysis.

RESULTS

Demographic characteristics, as well as a laboratorial determination of *S. mansoni* infection in the study population, are depicted in Table (1). Based on Schistosoma egg-patent infection determined by Kato-Katz test (KK-positive), active infections were diagnosed in eight (8/108) individuals. The intensity of infection expressed by parasite loads varied from 6 to 72 epg / individual (Table 1). Levels of IgG and IgE determined by SMMA-ELISA revealed reactivity in 64 and 59 out of 108 individuals, respectively. Since eight individuals failed to provide sufficient fecal samples for DNA extraction, only 100 individuals were included for analysis. Results showed DNA amplification in 32 out of 100 individuals tested by real-time PCR (Table 1). When immunoreactivity assessed in the egg-excretor group, IgG1 and IgE confirmed active infection in 7 and 8 out of 8 egg- excretors, respectively. S. mansoni DNA amplification was also demonstrated in all egg-patent infections (8/8). Ct values ranged from 23 to 36 (mean 29.0). In 100 individuals without egg-patent infection (KK-negative), 41% (41/100) individuals presented both IgG1 and IgE reactive (IgG+IgE+) while 16% (16/100) were IgG positive IgE negative (IgG+IgE-), 10% (10/100) IgG negative IgE positive (IgG-IgE+) and 33% (33/100) IgG negative IgE negative (IgG-IgE-). Real-time PCR showed Schistosoma DNA amplification in 24 % (24/100) in the total egg-negative population, and Ct values varied from 24 to 37.5 (mean 30.1). In KK negative and immunoreactive population, 20 out of 67 (29.85%) individuals had *S. mansoni* DNA in fecal samples being 14 IgG+IgE+, 2 IgG+IgE- and 4 IgG-IgE+. Also, DNA was also detected in 4 individuals without egg and immunoreactivity (KK negative and IgG and IgE negative). When diagnostic parameters were calculated by using quadruplicate KK (four slides) as a reference, duplicate KK (two slides) sensitivity was 75% (95% CI = 34.91 – 96.81). A single

real-time PCR presented sensitivity of 100% (95% CI = 63.06 – 100). Moreover, real-time PCR combined with KK and/or ELISA-SMMA presented the same sensitivity as single. Duplicate KK had 100 % specificity (95%CI = 96.38 - 100). However, single real-time PCR presented 73.91% (95% CI = 63.71 - 82.52) specificity that was superior to serology or KK and /or immunodiagnosis and PCR all combined. Real-time PCR presented positive and negative predictive values of 25% (95% CI= 11.46 - 43.41) and 100% (95% CI = 94.72 - 100), respectively. As a single approach, real-time PCR positive predictive value was superior compared to immunodiagnosis alone or the combination of KK and/or IgG and/or IgE and real-time PCR. Further statistical analysis showed a k index of 0.312, indicating a fair agreement between real – time PCR and quadruplicate KK used as reference test.

Individuals with egg-patent infection (8) and /or PCR positive (24) were candidates for treatment. From 32 actively infected individuals, 14 accepted treatment and agreed with follow-up visits at 6 and 24 months post-treatment. Seven out of 14 individuals had the egg-patent infection. After a single dose of praziquantel, seven individuals (6 KK + PCR+ and 1 KK -PCR+) responded to chemotherapy. Both egg and DNA amplification were absent in stool samples after six months post-PZQ use (Table 2). However, DNA amplification was still detected in 7 individuals (1 KK+ and 6 KK-) after six months post-chemotherapy. Thus, those individuals were resubmitted to another round of PZQ. After re-treatment, all seven individuals presented with no DNA amplification. A 2-year post-treatment follow-up showed that two individuals presented active infection which one being KK positive PCR negative (KK+PCR-) and the other, KK negative PCR positive (KK-PCR+). Cure rate calculated by using Kato-Katz negative as criteria of cure was 100 % and 92.3 % after six months and two years post-treatment, respectively (Table 2). When an absence of DNA amplification was used as sole criteria of cure, the rate was 50% in 6 months and 92.3 % in 24 months post therapy. The non-response to therapy rate was 7.14% and 14.29% for KK and PCR, respectively, in the two year follow-up period (Table 2).

Treatment with PZQ can be followed by changes in reactivity (less than 6 months post-treatment) which can be used as an indirect marker of drug response. Also, PZQ seems to affect susceptibility to new infection after re-exposure [25]. So, post treatment response based on IgG1 and IgE reactivity was also evaluated (Table 2). After 6 months post treatment, IgG1 levels pre and post treatment presented no significant difference ($p > 0.05$; mean 1.92 ± 0.80; 95% CI= 1.511- 2.433 and 1.72 ± 0.84; 95% CI= 1.238 – 2.203, respectively (Figure 1A). In contrast, IgE pre-treatment levels had significant increase when compared to post treatment levels ($p < 0.01$; mean 1.63 ± 0.54, 95%CI= 1.309 – 1.958 versus 2.34 ± 0.61, 95% CI= 1.98 – 2.69) (Figure 2 A). A 2 year follow-up showed that pre-treatment IgG1 levels (mean 1.72 ± 0.84; 95% CI= 1.238-2.203) compared with post treatment levels (1.34 ± 0.61, 95% CI= 0.973 - 1.706) had a significant decay ($p < 0.05$; Figure 1 B). IgG1 reactivity was absent after PZQ use in 21.43% (3/14). In the same period, IgE levels pre-treatment also had a significant decrease when compared to post-treatment levels ($p < 0.01$; mean: 2.34 ± 0.61, 95% CI=1.984 to 2.686 versus 1.33 ± 0.39, 95% CI=1.098 to 1.564; Figure 2B). Lack of IgE reactivity was observed two years post-treatment in 3 out 14 individuals.

Table 1: Demographic, Proportion of Positive, Immunoparasitological and Molecular Baseline Characteristics of the Study Population.

Characteristics		Absolute numbers and Percentages
Number and Proportion	Male	59 (54.63%)
	Female	49 (45.37%)
	Sex Ratio	1:2
Age (years)	Mean	36.12
	Range	8 – 76
Coproscopy (K-K)	Number of positive individuals	8
	Proportion of positive*	7.4%
	Arithmetic mean of epg	32.3
	Range of number of epg	6-72
ELISA-IgG1	Number of positive individuals	64
	Proportion of positive*	59.26%
	number of excretors + non excretors	7 KK pos+57 KK neg
	Mean ± S.D. A.U.*	1.97 ± 0.68 A.U.
	Range of A.U.*	1.00 - 3.52 A.U.
ELISA-IgE Proportion of positive*	Number of positive individuals	59
	Proportion of positive*	54.63%
	number of excretors + non excretors	8 KK pos + 51 KK neg
	Mean ± S.D. A.U.*	1.76 ± 0.73 A.U.
	Range of A.U.*	1.00 – 2.59 A.U.
Real-time PCR	Number of positive individuals	32
	Proportion of positive*	32%
	number of excretors + non excretors	8 KK pos+24 KK neg
	Mean of Ct values*	29.6
	Range of Ct values*	23 to 37.5

Abbreviations: (%): Percentages are presented in parenthesis. KK: Kato-Katz test; K-K po= Kato-Katz positive; K-K neg: Kato-Katz negative; A.U.: arbitrary units.ELISA-IgG1: ELISA for detection of specific isotype response to *S. mansoni* antigen SMMA; ELISA-IgE: ELISA for detection of specific isotype response to *S. mansoni* antigen SMMA.ELISA-IgG1 and ELISA-IgE were considered positive ≥ 1.00 A.U. Real-time PCR POS: detection of amplified products (Ct ≤ 38) and NEG, no amplification (Ct > 38). *Inclusion of both egg-excretors and non-excretors.

DISCUSSION

Coproscopy is still considered central to the diagnosis of *S. mansoni* infection diagnosis.

Active Schistosoma infection in LEA has been minimized when based solely on a single microscopy test. Usually, individuals infected in LEA present low parasite loads that may remain undetectable. Despite KK high specificity and sensitivity improvement by increasing the number of slides, microscopy results in underestimation of the number of actively infected individuals by excluding non-egg patent infections. Alternative methods have been applied to diagnosis in light infections such as immunodiagnosis and DNA-based assays [6,19]. Our findings demonstrated that all individuals with egg-patent infection presented with IgG and/or IgE reactivity and positive DNA

Table 2: Therapy response and long-term (2 years) follow up of individuals living in low endemic area for schistosomiasis.

ID	Pre-Treatment				Post-Treatment (6 months)				Follow up (24 months)			
	KK	PCR (Ct)	IgG1 (A.U.)	IgE (A.U.)	KK	PCR (Ct)	IgG1 (A.U.))	IgE (A.U.)	KK	PCR (Ct)	IgG1 (A.U.)	IgE (A.U.)
Sm48	POS	28.0	2.09	1.12	NEG	NA	2.60	3.10	NEG	NA	1.30	1.60
Sm52	POS	27.0	2.29	1.03	NEG	NA	1.35	1.35	NEG	35	1.00	1.00
Sm53	POS	35.0	2.04	2.59	NEG	NA	0.94	2.50	NEG	NA	0.51	1.50
Sm56	POS	23.0	1.77	1.19	NEG	NA	1.20	2.60	NEG	NA	1.00	0.90
Sm77	POS	24.2	1.72	1.15	NEG	NA	1.46	1.53	ND	ND	ND	ND
Sm83	POS	30.0	0.40	1.02	NEG	37.3	1.64	1.60	NEG	NA	1.90	1.70
Sm102	POS	31.0	2.80	1.70	NEG	NA	2.41	2.70	NEG	NA	2.40	1.20
Sm01	NEG	30.5	2.41	1.76	NEG	24.8	2.28	2.11	NEG	NA	1.30	0.90
Sm23	NEG	31.0	1.54	ND	NEG	29.3	0.97	1.60	NEG	NA	0.60	0.70
Sm41	NEG	28.0	2.22	2.04	NEG	31.6	1.40	2.20	NEG	NA	1.20	1.50
Sm61	NEG	29.0	3.06	2.46	NEG	26.0	1.90	3.00	POS	NA	1.40	1.10
Sm70	NEG	26.0	3.11	1.90	NEG	NA	3.7	2.60	NEG	NA	2.40	1.50
Sm80	NEG	30.2	0.63	1.93	NEG	34.3	0.38	3.10	NEG	NA	0.80	1.80
Sm93	NEG	30.2	1.53	1.35	NEG	30.8	1.86	2.70	NEG	NA	1.60	1.90

Abbreviations: KK: Kato-Katz Test; Post-Treatment Corresponds to a Period of 6 Months Post PZQ Intake; Follow up Relates to 24 Month Period Post Treatment; KK POS: Positive; KK NEG: Negative; IgG1: A.U.: Arbitrary Units, ELISA for Detection of Specific Isotype Response to *S. mansoni* Antigen SMMA (Reactive > 1.00 A.U.); PCR: Real-Time PCR; PCR positive: (Ct ≤ 38); PCR Negative or NA: No Amplification (Ct > 38); ND: Not Done.

amplification that supports the high sensitivity of ELISA-IgG and IgE and real-time PCR.

Both serology and DNA-based assays have been used in some areas as an auxiliary tool for diagnosis of schistosomiasis in non-egg patent infections [6,19]. Among the non-egg excretors, 33% had no IgG and/or IgE reactivity. The result suggested an absence of active infection in the third part of the study population. In contrast, over 60% had IgG and/or IgE positive which might include both active and past infection. In populations living in LEAs submitted to rounds of successive treatments, some immunoreactive individuals may not be presently infected. Despite its high sensitivity (100%), immunodiagnosis showed very low specificity (33%) which might be the result of persistent serum reactivity in individuals previously treated. At least 30% of the study population had a history of praziquantel intake, 3 to 7 years before the enrollment in the present study.

The results showed that it is essential to discriminate between active and past infection, when immunoreactivity is present in non-egg excrector individuals. In a previous work, the authors demonstrated that conventional PCR successfully detected active infection in both egg-positive and egg-negative cases with serology positive in LEA [19]. In agreement with others, data suggest that real-time PCR could be used as a sensitive method to detect active infection in LEA [20]. Furthermore, use of DNA-based assays might avoid overestimation of Schistosoma infection based on serum reactivity in low endemic areas. In the present study, results showed that almost 30 % of non-egg patent infections with IgG and/or IgE positive had DNA fragments detectable in fecal samples and confirmed active Schistosoma infection. Also, since the negative predictive value of real-time

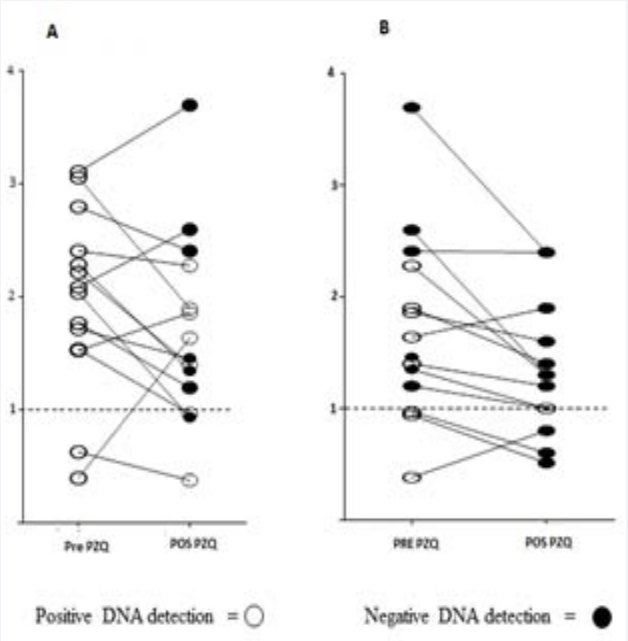

Figure 1 Determination of IgG1 levels before and after chemotherapy use. In A and B, the response to therapy was measured by IgG1 after six and 24 months, respectively. Statistical analysis was performed by the Wilcoxon rank test (two-tailed in A and B, one-tailed in C; see text for results) (Stata 13, Texas). The difference was considered significant when $p < 0.05$. The interrupted line indicates the level of IgG1 that is considered reactive (> 1.00). AU: arbitrary units. Individuals positive for DNA amplification are identified by open circles. Individuals without DNA amplification are represented by closed circles.

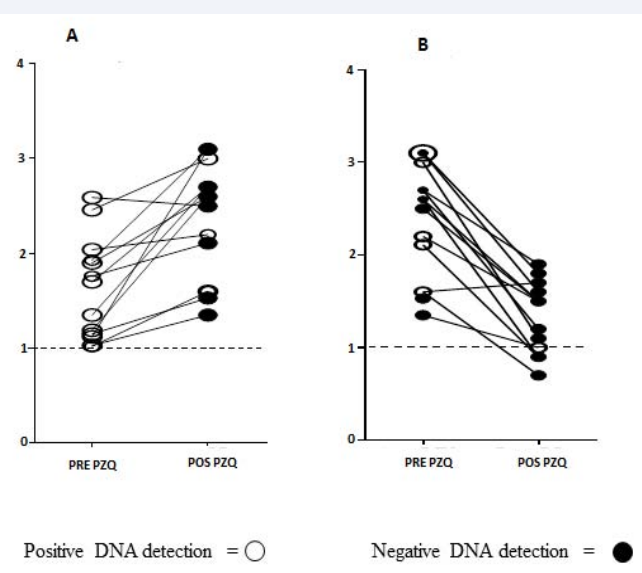

Figure 2 Detection of IgE levels pre- and post-chemotherapy. In A and B, the response to therapy was measured by IgE after six and 24 months, respectively. Statistical analysis was performed by the Wilcoxon rank test (two-tailed in A and B; one-tailed in C; see text for results) (Stata 13, Texas). The difference was considered significant when $p < 0.05$. The interrupted line indicates the level of IgE that is considered reactive (> 1.00). AU: arbitrary units. Individuals positive for DNA amplification are identified by open circles. Individuals without DNA amplification are represented by closed circles.

PCR is high (100%), the absence of DNA detection in non-egg excretors with or without immunoreactivity safely excludes *S. mansoni* infection.

In LEAs, not only low parasite load is present but also egg shed may not be detected. DNA – based assay was able to detect DNA fragments in both egg-excretors and non-egg excretors. Usually, Ct values are inversely correlated to parasite load. However, results showed no correlation with egg counts since low, medium and high Ct values ranged from 0 to < 50/epg. Lack of the correlation between egg counts and presence of DNA amplification might result from a low excretion of eggs viable and not viable as well as parasites at any stage [5]. Moreover, DNA degradation might also occur or the presence of Taq polymerase inhibitors that might interfere with the real amount of detectable DNA in the samples [21].

DNA-based detection assays proved to be a valuable tool for both diagnosing schistosomiasis and monitoring therapeutic response [15,22-24]. In the present study, real-time PCR targeting cox-1 gene sequence showed prevalence, sensitivity and specificity of 32%, 100 %, and 73.91%, respectively. Studies in LEAs, applying conventional PCR, PCR-ELISA targeting 121 bp sequence showed prevalence of 23.4 to 38.1%. In addition, sensitivity ranged from 83.3 % to 96.7% and specificity from 38.5% to 88% [24-26]. Moreover, gene target sequence used and other test intrinsic variables may play a role in different study results [14,27,28]. From 2 to 8 weeks after PZQ use, no signal amplification is detected by real-time PCR in different clinical samples suggesting response to therapy [15,23]. Data showed that 50% of the treated individuals had no detectable DNA in fecal samples after six months. The cure rate in a six month period after therapy was 50% in contrast to KK cure rate of 100%. In the present study, data showed a lack of correlation between drug response and no egg excretion. Microscopy failed to detect all non-drug responders that presented signal for DNA amplification. Discrepancies between the absence of egg excretion and persistence of DNA detection after treatment imply that the sensitivity of microscopy fairly differs from real-time PCR [23]. As a consequence, microscopy overestimates cure rates after PZQ use that characterizes inadequacy of the method to determine response to chemotherapy. In contrast, real-time PCR seems to be a reliable tool to estimate cure rates and detect failures in treatment response [15,23]. Nonetheless, differences in cure rates may also be seen among DNA assays. Data on studies using conventional and PCR-ELISA, targeting a 121 – bp repetitive sequence of *S. mansoni*, achieved cure rates of 87.8% and 96.5%, respectively [24]. Discrepancies in cure rates might result from differences in DNA assay methods and target sequences. Also, individual drug responses, age groups included in the study, infection intensity as well as reinfection rates might produce differences among studies [29]. Further studies must be done to clarify the differences found.

In response to anti-helminthic therapy, three outcomes are expected: absence of egg excretion, egg reduction or maintenance of egg detection. Nonetheless, the introduction of higher sensitive tests like DNA assays is changing the meaning of drug response. For instance, no DNA amplification seems to be associated with interruption of egg excretion in response to therapy. However, the persistence of DNA amplification appears to correlate with poor or non-response to chemotherapy [15,23]. DNA persistence post-therapy might be a result of an inability of PZQ to affect young, immature forms of Schistosoma. As a result, Schistosoma progress to more mature forms and sustain a residual infection that might contribute to the persistence of DNA amplification [30]. Also, delayed DNA shedding from dead parasites and/or inactive eggs might contribute to DNA amplification post-treatment. Individuals living in a transmission area are susceptible tore-infection. Continuous re-exposure may play an important role in persistent DNA detection [5,23,30]. After a second round of treatment, five individuals had no detectable eggs or DNA. Nonetheless, one individual presented eggs at the microscopy and another one, responsive to the previous therapy, had detectable DNA after two years post therapy. Results strongly suggest that continuous exposure and/or reinfection might be implicated in the persistence of detectable DNA. However, even during the long term follow up, residual infections might also contribute to the persistence of DNA fragment elimination in the stools. Nonetheless, individuals residing in areas of transmission are at risk of continuous re-exposure. Praziquantel use does not preclude reinfections despite previous rounds of effective treatment [30].

The introduction of a "new parameter" of drug response such as DNA detection may have an impact on the determination of drug effectiveness, response to therapy and disease progression. Evolvement to more severe schistosomiasis clinical presentations may occur in the absence of egg excretion after use of anti-helminthic therapy. Inflammatory responses seem mainly implicated in chronic progressive tissue damage. Moreover,

evolvement to severe schistosomiasis forms after "parasitic cure" occurs suggesting that reduction or elimination of egg excretion may not be sufficient to interrupt schistosomiasis pathological progression [31]. However, no data are available on persistent DNA shedding and risk of disease progression after therapy.

DNA assays have been showing promising results in schistosomiasis diagnosis and assessment of therapy response. However, DNA–based methods may have different performances. Conventional PCR could be used in schistosomiasis LEAs. However, low sensitivity, infection rates, and high numbers of misdiagnosed true positive cases may limit its use [26]. Future work must be done by analyzing different target sequences from Schistosoma genome in the same samples from LEAs. Amplification of distinct target gene may produce different sensitivities in samples from individuals with nil or very low egg counts [6,32]. Also, development of DNA based assays by using different biological samples such as urine may improve even further schistosomiasis diagnsosis. Ease sample collection and highly sensitive and specific DNA based assays optimized to detect Schistosoma DNA detection in urine samples show exciting results [33,34]. Larger studies must be done in LEAs.

Data showed the potential use of real-time PCR in schistosomiasis with nil or low egg counts as well as a monitoring marker of drug response. Despite the usefulness of real-time PCR in LEAs, there is an extensive discussion of its final cost. Final cost can range from US$ 4 to 10 in contrast to US$ 0.3 of Kato-Katz test [1,33]. Nonetheless, great numbers of low income countries have real-time PCR technology available for HIV viral load detection in reference laboratories [19,26]. The larger use of molecular technologies for multiple diagnostic purposes may rapidly decrease the costs of single tests. At last, due lack of information, the cost-effectiveness of different real-time PCR assays in developing countries must be assessed in future studies.

The use of PZQ also results in changes in immunoreactivity [35,36]. Findings here demonstrated that both IgG1 and IgE levels had increased levels until six months post-chemotherapy, followed by reduced levels after two years in most of the treated individuals. In community settings, specific isotypic immune response detection combined with real-time PCR might be a powerful instrument to monitor interventions like chemotherapy use in transmission areas. Also, combined strategy could be useful to detect emerging and re-emerging infections, resulting in more efficient support of national surveillance systems aimed at controlling and/or eliminating schistosomiasis [1].

CONCLUSION

The results strongly suggest that real-time PCR in LEAs is a reliable approach when diagnosing schistosomiasis in the presence or absence of egg excretion. Also, PCR-assay seems to overcome the coproscopy lack of sensitivity after PZQ use. Therefore, PCR-assay could become a new marker of therapy response. Overall, real-time PCR may redefine active infection in individuals with no or low parasite burden and become a potentially reliable tool to determine the effectiveness of chemotherapeutic interventions in surveillance settings when combined with immunodiagnostic assays.

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