Diagnostic and prognostic value of cell free circulating *Schistosoma mansoni* DNA: an experimental study

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Received: 11 September 2014 / Accepted: 26 November 2014 / Published online: 20 December 2014
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Abstract Searching for a more sensitive and accurate marker for schistosomiasis diagnosis and treatment follow up is a potential necessity. Hereby, we evaluated usefulness of circulating free DNA as a marker for schistosomiasis diagnosis, assessing drug efficacy and monitoring the control interventions impact using SYBR green real-time PCR. A batch of mice were infected by 90 ± 10 *Schistosoma mansoni* cercariae. Starting from the 2nd day post infection (p.i.), groups of 2 or 3 mice were sacrificed every 3 days until 30 days p.i. The remaining animals were treated by a single dose of 400 mg/kg mefloquine and sacrificed in group at 5, 10, 21 days post treatment (35, 40, 51 days p.i.). Using SYBR green real time qPCR, pooled sera DNA were extracted and amplified. The results showed that, circulating free *S. mansoni* DNA was detected from the 2nd day post infection (p.i.) onwards with gradual decrease in the cycle threshold value C_t which indicates the gradual elevation of the DNA level (Log quantity was 2.6–3.1 IU/ml). As the infection progressed, DNA quantity was increased (Log quantity was 6.29 IU/ml). Initial increase of circulating free DNA was observed 10 days post treatment (40 days p.i.) (Log quantity was 7.38 IU/ml). That was followed by a progressive decrease in DNA level by the end of 21st day, post treatment (51 p.i.) (Log quantity 4.35 IU/ml). In conclusion, circulating free *S. mansoni* DNA is a reliable marker in the diagnosis of schistosomiasis and for assessing drug efficacy and monitoring the impact of control interventions.

Keywords *Schistosoma mansoni* · Circulating free DNA · Diagnostic · Prognostic · SYBR green · PCR

Introduction

Schistosomiasis is a parasitic disease caused by trematode flatworms of the genus *Schistosoma*. According to WHO, at least 249 million people received preventive treatment for schistosomiasis in 2012. Preventive treatment reduces morbidity in endemic countries with moderate to high transmission (WHO 2014). Different diagnostic techniques have been employed in the diagnosis of schistosomiasis. However, they are far from ideal. Microscopic detection of *Schistosoma* eggs in stool or urine samples and the detection of circulating antigens lack sensitivity in low-prevalence and posttreatment conditions, while antibody detection lacks specificity (Rabello et al. 2002). The current status of schistosomiasis in highly endemic areas (where ovum detection is frequently used to determine infection) is difficult to be assessed because of the superficially low parasite load after mass drug administration, whereas the parasite transmission rates are still high (Kato-Hayashi et al. 2014).

Polymerase chain reaction (PCR) methods have been tried to improve the direct detection of *Schistosoma*. These tests can utilize different sample types such as urine, stool, or organ biopsy, and involve the extraction of DNA from eggs prior to PCR amplification (Sandoval et al. 2006a; Pontes et al. 2003). The key limitation is the small sample size that can be processed for DNA extraction, and its dependence on chance whether the processed sample contains eggs or not. Real-time PCR is a powerful tool for high-throughput detection of parasite DNA and is

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increasingly replacing conventional-PCR and microscopy for diagnosis of several parasitic infections (Vinkeles Melchers et al. 2014). It can be used to monitor amplicon formation throughout the PCR reaction and to estimate the initial concentration of target DNA in samples (Bell and Ranford-Cartwright 2002). This method has been used for genome numbers estimation and diagnostic detection of parasites, such as *Plasmodium* spp. (Perandin et al. 2004) and *T. gondii* (Buchbinder et al. 2003).

Recently, several biomarkers were tried to improve parasitic infection detection such as MicroRNAs in Host serum (Hoy et al. 2014) as well as cell free circulating DNA (Wagner 2012). The existence of cell free DNA in the human circulatory system has been known since the 1950s, however, intensive research in this area has been conducted for the last years (Wanger 2012). Occasional earlier reports suggested the existence of circulating nucleic acids, but the potential clinical implication was not realized until 1996, when DNA with tumor-specific characteristics was demonstrated in the plasma/serum of cancer patients. This finding opened up possibilities for non-invasive diagnosis (Chan et al. 2003). There has been an upsurge of interest in the analysis of circulating nucleic acids (DNA and/or RNA) in blood plasma or serum as a clinical diagnostic tool. Parasitic cell or its free circulating parasitic DNA can be shed from parasites into blood and excreta which may allow its detection without the whole parasite being present within the portion of clinical sample used for DNA extraction (Wong et al. 2014). With the application of new techniques such as next generation sequencing, digital PCR and mass spectrometry, it is now possible to detect very small amounts of specific DNA in the presence of excess of other nonspecific nucleic acids (Wanger 2012). As an extension of this rationale, we reasoned that it might also be possible to confirm both diagnosis of different stages as well as the elimination of *Schistosoma* free circulating DNA after successful treatment. To prove this hypothesis, *Schistosoma*-specific real-time PCR was established and optimized for detection of parasite DNA. A mouse model of schistosomiasis was used to study the levels of *Schistosoma* free circulating DNA in mice serum during infection, as well as after therapy.

Materials and methods

Experimental *Schistosoma mansoni* infection

Animals and infection

A batch of Swiss albino mice CD-1, weighing 18–22 g each, were provided by the Schistosome Biology Supply Center (SBSC), Theodor Bilharz Research Institute

(TBRI). The animals were maintained under environmentally-controlled conditions (temperature, 25 °C; humidity, 70 %; 12-hour light and 12-hour dark cycle). Each mouse was infected with 90 ± 10 *S. mansoni* cercariae using the body immersion technique (Liang et al. 1987). Starting from the second day (p.i.), and every 3 days meantime 30 days post infection (p.i.), two or three mice were randomly chosen from the infected animals, sacrificed, and blood was collected by cardiac puncture.

Thirty days p.i., all the remaining animals were treated with a single oral dose of mefloquine (each mice received 0.2 ml equivalent to the dose of 400 mg/kg) (Eraky et al. 2010; Fakahany et al. 2014). Two or three mice were sacrificed 5, 10 and 21 days post treatment (35, 40 and 51 days p.i.).

Serum of each mouse was separated by centrifugation ($1,500 \times g$ for 15 min). Collected sera from each two/mice, in the same time period were pooled and used for PCR. All serum samples were kept frozen at -80 °C until use.

Parasite DNA extraction

DNA from serum and from a saline solution containing *S. mansoni* eggs were extracted using the QIAamp DNA Blood and Body Fluid Mini Kit (Qiagen, Germany), according to the manufacturer's instructions, 200 μ l of the DNA were eluted. Following extraction, the concentration and purity of the eluted DNA were determined by absorbance measurements at A260 and A280 using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) to determine the suitability of samples for PCR, and to exclude any possible contamination with protein or RNA.

Amplification and detection

For the quantitative detection of circulating parasite DNA using SYBR green real-time PCR, DNA samples were amplified using the forward (5'-GATCTGAATCCGACCAACCG-3') and reverse (5'-ATATTAACGCCACGCTCTC-3') primers designed by Pontes et al. (2002), which generate a 110-base pair product following the amplification procedure. These primers were designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni* described by Hamburger et al. (1991). The repetitive fragment constitutes at least 12 % (600,000 copies/cell) of the *S. mansoni* genome. All DNA samples had a concentration of 1–3 ng/ μ L. For amplification, 2 μ L of DNA (served as PCR template) were added to 20 μ L of reaction mixture containing, 0.6 μ L of each primer, 10 μ L Super-Real PreMix Plus Ready Mix (SYBR Green) and 2 μ L Rox dye. The mixture was incubated under the following conditions: 95 °C for 15 min, followed by 40 cycles of 30 s of

denaturation at 95 °C and 1 min of annealing at 55 °C and extension at 72 °C for 30 s. All real time PCR reactions were conducted on the Applied Biosystems® 7900HT Fast Real-Time PCR System (Molecular Biology unit, Medical Biochemistry Department, Benha University). The qPCR amplicons were subjected to gel electrophoresis analysis to confirm their specificity. Ten µl of each amplified DNA and DNA ladder (Gene Ruler™ 100 bp plus DNA Ladder, 100–3000 bp supplied by Fermentas-Germany) were separated on 2 % agarose gel containing 0.3 µg/ml of ethidium bromide. The bands were visualized using UV transilluminator (254 nm), photographed and analyzed to estimate band sizes.

Interpretation of results was carried out through the amplification plot curve. Through this curve, the cycle threshold value (C_t) was detected. The C_t (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). C_t levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the C_t level the greater the amount of target nucleic acid in the sample).

Ethical consideration

Animal experimental studies and sacrifice procedures were complied with ethical guidelines approved by the Ethical Committee of the Federal Legislation, the National Institutes of Health Guidelines in the USA, and with the current laws and regulations of the Egyptian Ministry of Higher Education and Scientific Research.

Results and discussion

Results are shown in Table 1, Figs. 1, 2 and 3.

Diagnosis of schistosomiasis is difficult as it involves a wide range of symptoms and stages. Sensitive and specific diagnostic methods of schistosomiasis at an early stage of infection are important to avoid egg-induced irreversible pathological reactions. Chemotherapy has been used on a large scale in countries where *Schistosoma* is endemic. This has led to a lower intensity of infections and consequently lower diagnostic values of commonly used diagnostic tests like serology and Kato-Katz stool smear (Lier et al. 2006). The cell-free circulating schistosome DNA consists of the fragments of parasite-derived DNA that exist in the host's bodily fluids (Wichman et al. 2009, 2013). In this study we have explored the utility of detecting cell-free *S. mansoni* DNA in serum of experimentally infected mice as an alternative diagnostic and prognostic technique to egg detection in stool, urine, biopsies currently applied immunological tests. Several

repetitive parallel samplings are necessary to increase the clinical sensitivity of laboratory diagnostics (Garcia and Palmer 1999; Pontes et al. 2003). This problem applies not only to microscopy, but also to conventional PCR on stool or urine samples (Sandoval et al. 2006a, b; Pontes et al. 2002). In conventional PCR on stool or urine samples, there are additional issues such as PCR inhibition in stool samples.

Our rationale was that schistosomiasis infection implicates incessant parasite turnover, liberating DNA from decaying parasites that would reach the blood. Unlike eggs in stool or urine, cell-free *S. mansoni* DNA in blood would not undergo random sampling effects that complicate diagnostics (Wichman et al. 2009). Although this period in mouse challenge, is not exactly equivalent to that of human infections in clinical course, it could be considered as an early acute infection in patients as regards parasite migration and development.

The first confirmed finding and application of our study is the very early detection of DNA in serum of mice as early as the 2nd day post infection associated with persistent continuous increase in the DNA level throughout the progression of infection (until the beginning of treatment 30 days p.i.) (Fig. 1). The initial detection cycle threshold (C_t) of DNA in first four samples was 39.8–38.24 (Log quantity was 2.6–3.1 IU/ml). This result agrees more or less with Kato-Hayashi et al. (2010) who found that schistosome DNA (*S. mansoni*, *S. haematobium*, *S. japonicum*, and *S. mekongi*) could be detected from day 1 p.i. in experimentally infected animals. Hussein et al. (2012) could also detect free *S. mansoni* DNA in sera of experimentally infected mice at the 3rd day p.i. using conventional PCR. Furthermore, a PCR assay for the detection of *S. japonicum* DNA detected *S. japonicum* DNA in the sera of experimentally infected rabbit at the first week p.i. (Xia et al. 2009). This relatively delayed detection in the last study compared to our work may be attributed to the different detection method used in both studies, as they detected DNA using conventional PCR technique while our study detected free DNA using SYBR green Real time PCR which is more sensitive than conventional methods. Another explanation is the different *Schistosoma* strains. *Schistosoma mansoni*-specific DNA has been detected in urine 7 days p.i. in an experimental model (Sandoval et al. 2006b), suggesting the possibility of excretion of free circulating pathogen DNA in urine. Similarly, other parasites, free circulating DNA was detected by PCR during early infection periods in the experimental models for *Trypanosoma cruzi* (Kirchhoff et al. 1996), filariasis (Albers et al. 2014) and *Toxoplasma gondii* (Weiss et al. 1991; Wang et al. 2012), thus allowing the diagnosis of infection earlier than when using conventional diagnostic techniques as microscopy or serology.

Fig. 1 Amplification plot of the amplified targeted gene 121-bp tandem repeat DNA sequence of *S. mansoni* from ABI 7900 fast real time machine

Fig. 2 Changes in the cycle threshold value (C_t) with the progression of infection (before and after treatment): Free circulating *S. mansoni* DNA was detected from the 2nd day p.i. (first sample) (C_t was 38.48) then gradually increased (C_t was 27.73) at the 30 days p.i. (10th sample). Ten days post-treatment, marked increase in free DNA (C_t was 24.09) then remarkably decreased 21 days post-treatment (51 days p.i.) (C_t was 34.2)

The dynamic of free DNA level during the next samples showed continuous increase in the DNA level throughout the infection progression (until the beginning of treatment 30 days p.i.) As the infection progressed, DNA quantity increased so as the C_t decreased to 27.73 by the end of the 30 day p.i. (10th sample) (Log quantity 6.29 IU/ml) (Table 1, Figs. 1, 2, 3).

Because of the ease of taking blood samples, and in view of the risk contributed by undiagnosed schistosomiasis, it could become a realistic option to integrate

Schistosoma circulating free DNA PCR in routine diagnostic regimens for the clarification of gastrointestinal conditions.

A second field of application is the monitoring of therapy. In order to prevent relapse and long term sequelae from insufficient treatment, it is important to achieve a laboratory confirmation of treatment success (Rabello et al. 1997; Alonso et al. 2006; Khurana et al. 2005; Lambertucci et al. 2000). Patients after therapy as well as patients after a long course of disease with spontaneous healing (“burnt

Fig. 3 Changes in the Log quantity with the progression of infection (before and after treatment): Free circulating *S. mansoni* DNA was detected from the 2nd day p.i. (first sample) (Log quantity 3.07 IU/ml) then gradually increased (Log quantity was 6.29 IU/ml) at the 30 days p.i. (10th sample). Ten days post-treatment, marked increase in free DNA (Log quantity 7.38 IU/ml) then remarkably decreased 21 days post-treatment (51 days p.i.) (Log quantity 4.35 IU/ml)

Table 1 Changes in the cycle threshold (C_t) and Log quantity (IU/ml) before and after treatment

Days p.i.	Before treatment										After treatment		
	2	6	9	12	15	18	21	24	27	30	35	40	51
C_t (cycle threshold)	38.48	39.3	38.24	39.8	36.86	35.8	32.7	35.96	30.23	27.73	31.54	24.09	34.2
Log 10 fold quantity	3.07	2.82	3.14	2.67	3.56	3.89	4.83	3.84	5.54	6.29	5.15	7.38	4.35

out bilharzia”) are difficult to judge based on clinical or laboratory findings (Hamilton et al. 1998; Rabello et al. 1997). We have demonstrated here that, changes of *Schistosoma* circulating free DNA level can be detected very early after treatment. A marked increase of cell free *Schistosoma* DNA concentration was observed shortly after initiation of therapy (10 days after treatment C_t was 24.09 and Log quantity was 7.38 IU/ml). This was followed by decrease in DNA level in the subsequent sample (21 days after treatment C_t was 34.2 and Log quantity was 4.35 IU/ml) (Figs. 1, 2, 3). The average *Schistosoma* circulating free DNA concentration in those mice that still had detectable DNA after treatment was lower than those with pretreatment infection (Table 1).

This is consistent with the hypothesis that circulating *Schistosoma* DNA stemmed from decaying parasites. The circulating DNA in blood is protein-bound (nucleosomal) DNA and circulating DNA has a short half-life (10–15 min) which is removed mainly by the liver. Accumulation of DNA in the circulation can result from an excessive release of DNA caused by massive cell death, inefficient removal of the dead cells, or a combination of both (Pontes et al. 2003).

The decline of *Schistosoma* circulating free DNA concentration before and after treatment may thus become an effective parameter for monitoring patients under therapy (Kato-Hayashi et al. 2013). Similar results were reported by Wichmann et al. (2009) who evaluated the *Schistosoma* circulating free DNA concentration in mice experimentally infected with *S. mansoni* and treated by praziquantel 42 days post infection, they found initial increase followed by progressive decrease till complete clearance in *Schistosoma* circulating free DNA level 138 days post treatment. In human, Wichmann et al. (2009; 2013) purposed that, it may take more than one year until *Schistosoma* circulating free DNA becomes entirely undetectable as inactive eggs may release DNA with very slow kinetics. The greater number of eggs in humans with chronic disease may be responsible for a considerably longer duration until *Schistosoma* circulating free DNA is totally eliminated (Kato-Hayashi et al. 2013).

Based on this study results, it is concluded that, detection of free circulating DNA of *S. mansoni* carries the potential of becoming a novel diagnostic marker for detection of any stage of infection as well as assessment of drug effectiveness. The utility of paired *Schistosoma*

circulating free DNA determinations in individual patients before and after treatment can be applied for confirmation of treatment success. Future studies are recommended to determine the applicability of this marker for diagnosis and treatment follow up in human cases as well as differentiation of mixed infection and other parasitic infections.

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