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Comparative sensitivities of various tests for diagnosing early *Schistosoma mansoni* infection in mice

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Abstract

Objective: We compared the diagnostic values of cercarial antigen preparation, cercarial secretions, soluble worm antigen preparation and worm vomit prepared from the parasite *Schistosoma mansoni*.

Methods: Enzyme linked immunosorbant assay was used to detect IgG in plasma from *Schistosoma mansoni* infected mice. In parallel, specific primers for the parasite genome was used to detect *S. mansoni* DNA in plasma and urine from infected mice and hemolymph and tissues of infected Biomphalaria alexandrina snails by Polymerase chain reaction.

Results: The results showed that all the above diagnostic approaches enabled infection to be diagnosed as early as three days post mice exposure to parasite cercariae.

Conclusion: Cercarial secretions and worm vomit represent new useful economic crude antigens for preliminary detection of parasite active transmission or response to therapy in an endemic setting. Also, it was found that the detection of *Schistosoma mansoni* DNA in urine from infected mice was the most sensitive and specific (although expensive) method for infection diagnosis than all the others.

Keywords: *Schistosoma mansoni*; Cercarial secretion; Worm vomit; Soluble worm antigen preparation; Polymerase chain

Introduction

Diagnosis of schistosomiasis infection is conventionally carried out by Kato Katz technique [1-4] or serologic methods [5-6]. Using schistosome genome specific primers, allowed detection of the parasite DNA by polymerase chain reaction (PCR) in biological samples from infected subjects with superior specificity and sensitivity than the parasitological or the serological methods [7-14], as demonstrated by the capacity of the PCR to detect parasite DNA as early as 7 days post infection. While, the levels of the anti-worm IgG remained high at 23 weeks post-treatment, the PCR results turned negative at week 10 post-treatment [14].

Herein we compared the diagnostic values of several crude antigens prepared from the cercarial and the adult worm stages of the parasite *S. mansoni* to detect IgG in sera from infected mice with the same parasite kinetically at regular time intervals post infection. Of these antigens cercarial secretaions (CS) and worm vomit (WoV) were previously evaluated for their diagnostic value to detect IgG in sera from infected humans [15]. In parallel, and in a trial to improve the sensitivity and specificity of the diagnosis, a PCR based on specific primers for the parasite genome was evaluated for its capacity to detect infection in plasma and urine from infected mice.

Materials and Methods

Infected mice groups and samples collection

Fifty Swiss albino mice were divided into 10 groups each of 5 and each mouse received 100 cercariae by tail immersion. During the acute phase (the initial 30 days post infection), every 3 days starting from day 3 post infection urine was pooled from each group and individual blood samples were collected on EDTA. Plasma was separated and stored at -20°C till being used. Plasma and urine were collected from 20 uninfected mice to be used as negative controls. Tissues and hemolymph were collected from infected B. alexandirina for detection of *S. mansoni* DNA by PCR. Tissues from uninfected snails were examined as means of negative controls.

Molecular detection

gDNA extraction: DNA was extracted from different stages of *S. mansoni* (worm, cercaria and schistosomula) and tissue from infected *B. alexandrina* snails using lysis buffer containing 2% hexadecyltrimethyl-ammonium bromide (CTAB) with the method reported by Abdel-Hamid et al. [16]. DNA was also extracted from plasma and urine of infected mice using wizard gDNA purification kit (Promega).

Bioinformatics analysis of the used primers: The used forward (ACAGTGCGCGCGTCGTAAGC) and reverse (GAGATCAAGTGTGACAGTTTTGC) primers were previously reported to specifically amplify *S. mansoni* gDNA [10]. The sequences of both primers were compared by multiple alignments against the online available sequences in the GenBank (http://www.ncbi.nlm.nih.gov/) using the basic nucleotide BLAST analysis.

Amplification of gDNA: PCR reactions were carried out in a

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final volume of 50µl. The amplification program included an initial denaturation at 94oC for 5 min followed by 35 cycles; each consisted of denaturation at 94oC for 45 sec, annealing at 58oC for 45 sec and extension at 72oC for 60 sec. The program included a final extension step at 72oC for 10 min. PCR products were resolved by electrophoresis on 1.5% agarose gel containing ethidium bromide [17] parallel to 100 bp DNA ladder (promega, Germany). The amplified *S. mansoni* fragment was visualized using an UV-transilluminator and photographed using a gel documentation system.

Serological detection

Preparation of antigens: CAP AND SWAP WERE PREPARED ACCORDING TO THE METHOD DESCRIBED BY JASSIM ET AL [18]. CS AND WOV WERE PREPARED ACCORDING TO THE METHOD OF BAHGAT ET AL [15,19].

Detection of IgG levels in plasma from infected mice against CAP, SWAP, CS and WoV [20]: ELISA plates were coated overnight at room temperature with 50 µl/well of CAP (62.5 µg/ml), CS (20 µg/ml), SWAP (250 µg/ml) and WoV (200 µg/lm) in coating buffer. Plates were washed 3 times with PBS-0.05% T20 and blocked against non specific binding with 100µl/well of PBS-0.05% T-5% fetal calf sera (FCS) for 2 h at 37°C. After three washes, individual plasma samples were diluted 1/100 in the blocking buffer and 50 µl of each were applied to individual wells. Plates were incubated at 37°C for 2 h. After washing, diluted peroxidase labeled anti-mouse IgG (1/500) in PBS-0.05% T-5% FCS was then applied 50 µl/well and plates were incubated 2 h at 37°C followed by 3 washes. For visualizing the antigen antibody binding, plates were dried and the peroxidase specific colorimetric substrate O-phenylenediamine dihydrochloride (Sigma, St. Louis, Mo, USA) appropriately diluted in the substrate buffer in presence of H_2O_2 was applied 50µl/well.

Statistical analysis: All obtained data were analyzed using the Student's t-test application of the Graph Pad InStat Soft ware.

Results

Detection of IgG levels by ELISA in plasma from *S. mansoni* infected mice: As presented in Table 1, using CAP coated plates, IgG levels in infected mice plasma (IMP) showed no significant increases at 3, 6 and 15 days post infection, (1.4, 2.2 and 1.9, folds respectively) compared with IgG levels in uninfected (UIMP), while at 9, 12, 18, 21, 24, 27 and 30 days IgG levels in IMS were significantly higher (P< 0.05) than in UIMP. Using CS coated plates, IgG levels in IMP were significantly higher (P< 0.05) than in UIMP in all intervals except at 15 day post infection, IgG levels showed no significant increases (1.96 fold). On the other hand, using SWAP coated plates, at all time intervals IgG levels in IMS were significantly higher (P< 0.05) than in UIMP while, Using WoV coated plates, IgG levels in IMP were significantly higher than in UIMP at all intervals except at 21 day, where, IgG levels in IMP showed no significant increases (1.59 fold).

Mean percentage IgG reactivities in IMP against CAP, CS, SWAP and WoV over 30 days post infection: IgG reactivities in IMP against the used antigens were considered positive if they exceeded the cut off values for each antigen that were calculated as the means of the UIMP IgG reactivities + 2X Standard deviation (S.D.) and were 0.27, 0.32, 0.39 and 0.52 for CS, SWAP, CAP and WoV respectively (Table 1).

Molecular analysis: Using the basic nucleotide blast application, results showed 100% homology to the highlighted termini of the used primers suggesting their exact annealing positions on the parasite template that yields amplification products of molecular size 338bp (Figure 1). Such analysis confirmed the specificity of the used primers

IgG reactivity against CAP at different time intervals of infection											
	C	3D	6D	9D	12D	15D	18D	21D	24D	27D	30D
Mean	0.22	0.31	0.49	0.53	0.47	0.42	0.64	0.622	0.602	0.538	0.75
SD	0.08	0.02	0.26	0.09	0.01	0.21	0.22	0.12	0.21	0.07	0.11
P- value		0.08 NS	0.09 NS	0.001*	0.006*	0.12 NS	0.01*	0.001*	0.013*	0.0005*	0.0001*
Cut off	0.39	0%	60%	100%	80%	20%	80%	100%	80%	100%	100%
IgG reactivity against CS at different time intervals of infection											
	C	3D	6D	9D	12D	15D	18D	21D	24D	27D	30D
Mean	0.25	0.37	0.6	0.46	0.36	0.49	0.61	0.65	0.679	0.638	0.79
SD	0.01	0.1	0.02	0.16	0.07	0.28	0.25	0.21	0.14	0.1	0.27
P- value		0.03*	0.003*	0.01*	0.007*	0.07 NS	0.007*	0.001*	0.001*	0.001*	0.001*
Cut off	0.27	80%	100%	100%	80%	100%	100%	100%	100%	100%	100%
IgG reactivity against SWAP at different time intervals of infection											
	C	3D	6D	9D	12D	15D	18D	21D	24D	27D	30D
Mean	0.24	0.68	0.69	0.43	0.47	0.6	0.51	0.537	0.747	0.57	0.55
SD	0.06	0.17	0.22	0.05	0.1	0.19	0.08	0.15	0.09	0.13	0.11
P- value		0.0007*	0.002*	0.001*	0.003*	0.0005*	0.0006*	0.005*	0.0001*	0.001*	0.0007*
Cut off	0.32	100%	100%	100%	100%	80%	100%	100%	100%	100%	100%
IgG reactivity against WoV at different time intervals of infection											
	C	3D	6D	9D	12D	15D	18D	21D	24D	27D	30D
Mean	0.32	0.53	0.6	0.64	0.49	0.57	0.54	0.51	0.83	0.81`	0.87
SD	0.1	0.12	0.12	0.1	0.07	0.14	0.12	0.2	0.09	0.13	0.14
P- value		0.01*	0.006*	0.002*	0.01*	0.01*	0.01*	0.1 CNS	0.0001*	0.0002*	0.0001*
Cut off	0.52	40%	80%	80%	20%	80%	60%	60%	100%	100%	100%t

*Significant value P< 0.05
Cut off: 2mean ± SD for control
N.S.: non significant
(P< 0.05)

Table 1: Detection of IgG reactivity at different time intervals post S.mansoni infection against different antigens.

to *S. mansoni* genome as they did not show any extent of homology to other existing human parasites in Egypt (Figure 1).

PCR amplification of the target sequence on the gDNA from different *S. mansoni* stages using the species specific primers: Specific amplification products from gDNA of adult *S. mansoni* worms, schistosomula, cercariae (lanes 4, 5 & 6), infected snail's tissues (lanes 7, 8) as well as hemolymph (lanes 9, 10) were visualized at 338 bp (Figure 2) that were not detected in the negative control lanes (2, 3).

PCR amplification of the target *S. mansoni* sequence from gDNA extracted from IMP and mice urine samples: DNA extracted from IMP then subjected to PCR amplification demonstrated 338 bp amplification products (Figure 3) as early as 3 days post infection that were persistently detectable over 30 days post infection. On the other hand, all gDNA preparations from collected mice urine samples at the 3rd, 9th, 12th, 15th, 18th, 21st and 30th days post infection were consistently PCR positive for the 338 bp fragment. At the 6th, 24th and 27th days post infection PCR was not carried out due to the insufficient collected urine volume (less than 100 µl) for gDNA extraction (Figure 4).

Discussion

A diagnostic tool capable of detecting *Schistosoma* infections in its acute phase would be of great value permitting early treatment that could prevent the pathology associated with chronic infections.

In the present work, the recorded high IgG levels against CAP at different time intervals post infection agrees with the previously reported high IgG levels in sera of *S. mansoni* infected patients predominantly against the abundant carbohydrate epitopes in the cercarial antigens [21], an increase in IgG at all intervals post infection against SWAP agrees with the previously reported high sensitivity of such antigenic preparation to detect IgG in sera from patients infected with *S. mansoni* [22].

The antigenicity of the SWAP might be attributed to the carbohydrate epitopes on glycoconjugates either presented or excreted by adult worms and elicit strong humoral immune response or the O-glycans of the major gut-associated CAA [23].

GA GAT CAAG TGTGACAG TTTTGCTCTGAGCT ACCCTGGA TCGGGTTG T

TTGTGAA TGCAGCCCAAAGTGGGTGG TAAACTCCA TCA GGC TAAATACTT

ACACGAGTCCG ATAGCAAACAAGT ACCGGGAAAGTTGAAAGT ACTTTG

AAGAGAGAGT AAACAGTGCGTGAACCGCT AAAGGT AAACGGGTGGAG T

TGAACTGCAAGCTCTGGGAA TTCAGCTGA TGAGTGTGA TTTGT ACTTGGG

CATACTGGCCGCCTTCAGTGTCTGTTT AACCGCAGGTGCCTTCCTTTTTGG

TGGG TATGTGTGAA TCGTTTGCTT ACGACGCGCGCACTGT

Figure 1: Deduce target *S. mansoni* sequence for the PCR amplification using the previously published primers (Sandoval, *et al.*, 2006 a & b). Sequences of both the forward and reverse primers were compared with the published schistosome sequences on the GenBank (http://www.ncbi.nlm.nih.gov) using the basic nucleotide blast application for and results showed 100% homology to the highlighted termini of the below mentioned previously *S. mansoni* sequence suggesting their exact annealing positions on the parasite template.

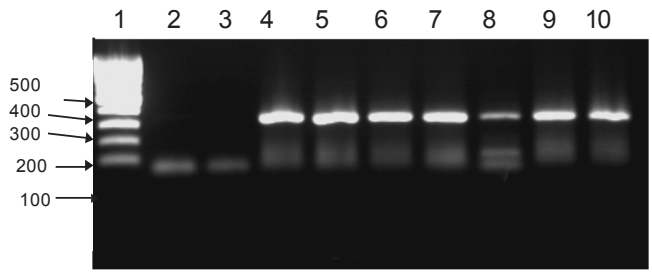

Figure 2: Electrophoresis of amplification products from genomic DNA of various parasite developmental stages. PCR products of adult *S. mansoni* worms, schistosomula, cercariae (lanes 4, 5 & 6), infected *B. alexandrina* snail tissues (lanes 7 & 8) as well as hemolymph (lanes 9 & 10) were subjected for electrophoresis on 1.5% agarose gel containing ethidium bromide parallel to 100 bp DNA ladder (lane 1) and products from a negative control of PCR reaction (lanes 2 & 3) where no DNA was added in the master mix were included on the same gel. Specific amplification products at 338 bp were visualized in all developmental stages that were not evidenced in the negative control lanes.

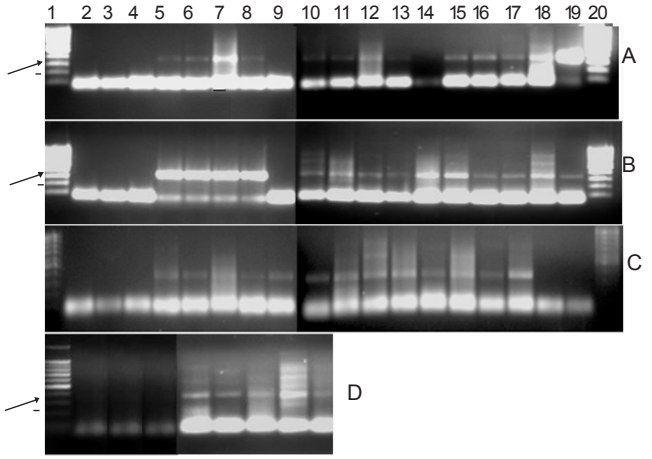

Figure 3: Electrophoresis of amplification products from genomic DNA of infected mice plasma (IMP) at different time points post infection. At 3, 6 and 12 days post infection 4 out of the tested 5 IMP samples at each of the 3 time points were PCR positive (A; lanes 5-8 & 10-13, B; 6-9) while, 1 sample at each of the 3 time points was PCR negative (A; lanes 9&14, B lane 5). At 9, 15, 18, 21, 24 and 30 days post infection all the 5 tested IMP samples at each of such time points were uniformly PCR positive (A; lanes 15-19, B; 10-14&15-19, C; 5-9&10-14, D; 5¬-9). At 27 days post infection, 3 samples were PCR positive (C; lanes 15-17), while, two were negative (C; lanes 17&18). That were subjected for electrophoresis on 1.5% agarose gel containing ethidium bromide parallel to 100 bp DNA ladder (lane 1& 20), products from a negative control of PCR reaction (lane 2), and products from control of UIMP (3 &4), where 338 bp specific amplification products were visualized in all positive samples that were not evidenced in the negative control lane.

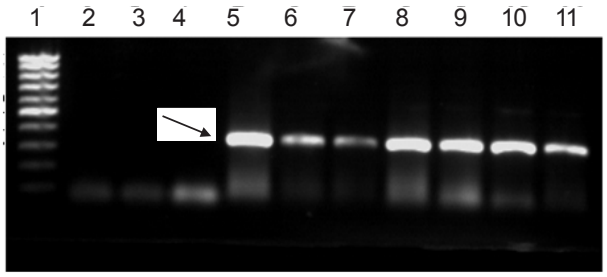

Figure 4: Electrophoresis of amplification products from genomic DNA of infected mice urine at different time points post infection. At 3rd, 9th, 12th, 15th, 18th, 21st and 30th (5, 6, 7, 8, 9, 10 & 11espectively) were subjected for electrophoresis on 1.5% agarose gel containing ethidium bromide parallel to 100 bp DNA ladder (lane 1), products from a negative control of PCR reaction (lane 2) and control of non infected urine (3 & 4) PCR reaction where 338 bp specific amplification products were visualized at all time points that were not evidenced in the both control and negative control lanes.

The present results clearly demonstrated that CS sensitivity to detect IgG in plasma of *S. mansoni* infected mice was generally higher than that of WoV which contrasted the previous study that compared the sensitivity of the two antigens to detect IgG in sera of infected humans residing in an endemic area in Burkina Faso [15]. Furthermore, antibodies against certain antigens present in the worm intestine, may take longer time to disappear [24].

The superior sensitivity of the CS to detect IgG agrees with the previous report [25] that described cercarial elastase (CE) purified from *S. mansoni* larval secretions as marker for human exposure to *S. mansoni* cercariae and not necessarily infection that was later contradicted and clearly demonstrated that natural infection induces antibodies to cercarial antigens present in the CS but not to the CE [26].

Using previously reported primers [10] in PCR, allowed amplification of specific fragments with the expected molecular weight (338 bp) from gDNA extracted from growing sporocysts within infected *B. alexandrina* snails, cercaria, schistosomula and adult *S. mansoni* worms that confirmed the sensitivity of the used primers. Such results match with those reported by [10] to establish PCR allowing specific amplification of a genus-specific product from the 28S ribosomal DNA subunit of *Schistosoma* spp.

The most impressive finding in the present work was that parasite DNA started to be detectable in IMP as early as 3 days post infection using PCR and continued to be detectable for 30 days, reflecting the superior sensitivity of such diagnostic tool for detecting infection at a very early stage.

Six out of ten time points reached 100% sensitivity, three time points declared 80% and only one time point showed 60% sensitivity. These results match with those from previous study [8] in which amplification was achieved with minimum amounts of *S. mansoni* egg template DNA (1fg) in human serum and feces. Also, 97% of PCR sensitivity to detect infection, demonstrating that single PCR survey detected more cases of *S. mansoni* infections than three Kato-Katz stool examinations [9].

The sensitivity of ELISA against the four used antigens reached 100% compared to 60% PCR sensitivity at day 27. However, this indicates that false-positive results might have been observed due to cross-reactivity with other helminth infections but this can be ruled, since the DNA from these worms is not amplified by the *S. mansoni*-specific primers [10,12].

Due to the facts that the collection of blood is invasive and collection of stool may be difficult in some populations, thus hampering diagnostic procedures, therefore that PCR was applied on gDNA extracted from pooled mice urine at different time intervals post infection.

In the present work, parasitic DNA was also detectable by PCR in extracted gDNA from pooled mice urine as early as three days post infection that remained detectable over 30 days reflecting the extremely high sensitivity of such detection. This agrees with a previous study to detect *S. mansoni* infection in mice urine by PCR after 7 days post infection [11]. The obtained 100% sensitivity of detecting *S. mansoni* DNA by PCR in urine in the current study was consistent with a previous study [10].

In the present study, 80µl of plasma and 400µl of urine were sufficient to extract gDNA for detecting *S. mansoni* infection by PCR. However, three ml of human urine sample for parasite detection by PCR [10]. Furthermore, *S. mansoni* infection could be detected from only 2µl of infected mice sera by direct PCR without DNA purification [10].

The general superiority of CS and WoV as new antigenic preparations to detect infection in IMP at different time points post infection clearly demonstrates a more economic and easy to prepare antigen that can be used to detect infection in poor endemic settings, yet, the specificity of detection remains as an opened question. However, PCR seems to be the best in terms of both sensitivity and specificity, yet, the decision on using it will always depend on the cost.

It could be concluded that in the acute infection phase, PCR detection of *S. mansoni* infection in urine samples is non invasive, very sensitive and specific diagnostic method than immunodiagnosis or even PCR detection of infection in sera.

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