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Assessment of Infected *Biomphalaria alexandrina* Snails by Detecting *Schistosoma mansoni* Antigen and Specific Gene.

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Abstract: To control spread of *Schistosoma mansoni* infection, rapid and accurate investigation of infected *Biomphalaria alexandrina* snails that surveyed from any suspected area is required. Routine assays for assessment of infected snails are time consuming and may not be able to detect prepatent schistosomal infections. In the present study two methods were evaluated for assessment of infected snails. The first was detection of *S. mansoni* soluble egg antigens (SEA) in snail hemolymph using two murine monoclonal antibodies (MAbs) in sandwich ELISA assay. The *S. mansoni* antigens measured in the hemolymph of infected snails at intervals 1, 2, 3 weeks post exposure to miracidia, at early shedding snails (4,5) weeks and after the infected snails stopped shedding. Although the positivity, sensitivity and specificity were 100% in the infected control group of snails, the detection of antigen (s) was only possible after the second weak of miracidial infection. In the second method, genomic DNA of infected snails in addition to non infected (as negative control) were subjected to nested polymerase chain reaction PCR using primers specific to *S. mansoni* fructose -1,6- bis phosphate aldolase (SMALDO) gene. PCR was able to detect infection (100% sensitivity) at the 3rd day post infection. In spite of the superiority and the higher specificity of the immunodetection for larg scale detection of prepatency of *B. alexandrina* snails infected with *S mansoni*, the nested PCR assay revealed much higher sensitivity which enables 100% detection of *S. mansoni* infection down to 3 days post infection. So this assay provided higher efficiency for determination of infection prevalence in snails and schistosomiasis transmission.

Key words: *Biomphalaria alexandrina*, hemolymph, sandwich ELISA, monoclonal antibodies, nested PCR, *Schistosoma mansoni.*

INTRODUCTION

The fresh water snail *Biomphalaria* species (*Planorbidae, Basommatophora*) serves as one of the most important intermediate hosts for a widespread pathogen of humans, the digenetic trematode, *Schistosoma mansoni* (Morgan *et al*. 2001). *Biomphalaria alexandrina* have been known to be the major snail vector for *S. mansoni* in Egypt (Yousif *et al.,* 1996).

In schistosomiasis transmission studies and control programs, identification of active transmission sites by community surveys and by studying human behavior was found to be expensive. On the other hand, the detection of infected snails in different sites can be more frequent and allows more rapid control of the intermediate host of schistosomiasis infection (Hamburger *et al*., 1989). This requires collection of snails from many geographic areas and detection of the infection. Snails collected from the field should be transported to the laboratory using proper storage conditions (Malek, 1985).

The detection of *S. mansoni* infected *Biomphalaria* snails is routinely determined by detection of cercarial shedding induced by exposure of snails to artificial light (Webbe,1965), or by detection of sporocysts and developing cercariae through squeezing the snails between two glass slides and examination under microscope (Chu and Dawood, 1970). However, these methods are not able to detect the parasite neither in dead snails nor in the prepatent periods (Caldeira *et al.,* 2004). Therefore, development of specific and accurate methods to identify *Biomphalaria* snails infected with *S. mansoni*, should be useful for epidemiological studies to control the prevalence of the infected intermediate host, *B. alexandrina* (Schmitt *et al*., 2002). This follows the recommendation of the World Health Organization "the research on schistosomiasis should focus on the development and evaluation of new strategies and tools for control of the disease" (WHO 2004).

The employment of suitable monoclonal antibodies (MAbs) has been suggested to be a possible efficient tool for specific detecting and evaluation of the infection in snails (Schmitt *et al*., 2002). Some authors

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demonstrated the presence of common carbohydrate epitopes which might show a cross reaction between *S.mansoni* trematode and its intermediate host (Humberger *et al*., 1989 and Leher *et al*. 2007).

Also, the application of molecular approaches increased the efficiency of parasite detection (Balabat *et al.,* 1996 and Hanelt *et al*.1997). Hamburger *et al*.(1987) detected the prepatent *S. mansoni* infection in snails after one week of infection using dot hybridization. Similarly, detection of prepatent infection in *B. glabrata* snails have been achieved using polymerase chain reaction (PCR) for the mitochondrial DNA minisatellite region of *S. mansoni* (Jannotti-Passos *et al*., 1997) and for repetitive elements in the genome of *S. mansoni* (Hamburger *et al.,* 1998).

In the present study, two methods were evaluated for detection of *S. mansoni* infected *B. alexandrina* snails. In the first one, two monoclonal antibodies (MAbs) were chosen from a panel of different MAbs produced against soluble egg antigens (SEA) and were employed in sandwich ELISA to determine specific *S .mansoni* antigens in the hemolymph of infected snails (of the genus *Biomphalari)* during different time intervals after infection. In the second method, nested PCR using *S. mansoni* specific primers was employed for detection of *S. mansoni* infected snails.

MATERIALS AND METHODS

Snail Infection:

Biomphalaria alexandrina snails were exposed individually for 3 hours to 8- 10 freshly hatched miracidia in 1ml dechlorinated water. Exposed snails were maintained under standard laboratory conditioned and then examined for infection starting from the 1st day post exposure to six week (Prah and James. 1977). *Schistosoma mansoni* miracidia (Egyptian strain), were supplied by the Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute, Cairo, Egypt. Groups of snails were kept uninfected as negative control.

Collection of Hemolymph:

Clean and infected snails at different intervals of infection (1,2,3,4 weeks, shedding , after shedding and after the snails stopped shedding) were separately punctured near the innermost coil of the shell and hemolymph was drawn out by a mouth operated suction tube with attached needle, each snail with a separate needle (Figuerido *et al*.,1973). The hemolymph aliquots were stored at -70ºC.

Monoclonal Antibody Production (Mansour et al., 1998):

Spleen cells from Balb/c mice immunized with *S. mansoni* SEA were fused with non-secreting murine myeloma cells (P3X63Ag.8) in the presence of 43% polyethylene glycol (PEG) (Sigma). Hybridomas were screened for anti-*Schistosoma* antibodies by indirect ELISA, and high reactive hybrids were cloned by limiting dilution method, using a splenocytes feeder layer. Specificity determination was done by indirect ELISA using *Fasciola hepatica* and *Echinococcus granulosus* antigens. Only MAbs showing no cross reactivity with other parasites were chosen. Hybridoma cells were intraperitoneal injected into Balb/c mice for ascites production. Two anti-SEA IgG1 MAbs showing a high reactivity towards a 88 kDa glycoprotein molecule were chosen for purification and employment in sandwich ELISA (El-Bassiouny *et al*., 2005). Produced MAbs were purified by ammonium sulfate treatment, then by ion exchange chromatography according to Goding (1986).

Immunoassay Detection of Infected Snails:

One of the chosen MAbs was labeled with horse-radish peroxidase according to Tijssen and Kurstak (1984). The other MAb was used as antigen capture MAb. The lower detection limit of the used MAbs was evaluated according to El-Bassiouny *et al*. (2005). Sandwich ELISA was standardized according to Demerdash *et al.(*1995) with slight modification. Briefly, wells of microtiter plates (Falcon, Becton Dikenson Labware CA) were coated with 100ul (0.1ug/well) Ag-capture MAb diluted in carbonate buffer pH 9.6 and left overnight at room temperature. The plates were washed with PBS pH 7.2 and blocked with 5% bovine serum albumine (BSA) for 2hrs, then washed with PBS, then 100 µl/well of hemolymph (diluted 1:10 in PBS) was applied and left overnight at 4ºC. After washing with PBS, the peroxidase labelled MAb was applied in concentration of 1/200. After 1hr. incubation at 37ºC the reaction was stopped by adding 8M H_2SO_4. Optical density (OD) readings were taken at 492 nm. Cut-off value was measured as mean of negative controls + 2SD. OD values above cut-off value were considered positive and OD values below cut-off value were considered negative. The sensitivity and specificity of immunodetection test were determined by testing snails shedding cercariae at different week's intervals, together with normal control snails.

PCR Detection of Infected Snails:

Genomic deoxyribonucleic acid (DNA) was extracted from *B. alexandrina*- infected snails (one day, three days, one week and shedding snails in addition to uninfected snails as negative control) using Puregene genomic DNA purification kit (Gentra, Germany). DNA integrity and concentrations were estimated by comparison with molecular weight standard on 0.7% agarose gel electrophoresis. Genomic DNA of different groups were subjected to nested polymerase chain reaction (PCR) using primers (SMA F1 and SMA B1 in the 1st PCR and SMAF2 and SMA B1 in the second PCR) specific for the *Schistosoma manoni* fructose -1,6-bisphosphate aldolase gene (SMALDO) (El-Dabaa *et al*., 1998). A total PCR reaction volume of 100 µl containing: 500 ng genomic DNA (or 5 ul of 1st reaction in 2nd PCR), 200 µM dNTPs, 1 µM of both 5` and 3` primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (w/v) and 2.5 units *Taq* DNA polymerase was prepared. PCR was done for 50 µl of the reaction mix in PTC100™ system using the following cycling conditions (95˚C 5 minutes (94˚C 1 minute, annealing 1 minute (different temperatures were tested), 72˚C 1 minute, 35 cycles), 72 ˚C 10 minutes) for both 1st and second PCR reactions (Innis *et al*., 1990). As internal control for amplification, primers (AITS1F and AITS1R) designed to specifically amplify 310 bp of *B. alexandrina* nuclear rDNA internal transcribed spacer1 (ITS1) were added to the other 50 µl of the previously prepared amplification reaction for SMALDO and the PCR control reactions were done using the following cycling conditions (95˚C for 1 min, 62˚C for 2 min, and 72˚C for 1 min followed by 30 cycles of 95˚C for 30 sec, 62˚C for 30 sec 72˚C for 1min and final extension step 72∘C for 7 mins).(Lotfy *et* al., 2005). PCR reactions were analyzed through electrophoresis using 1.5% agarose gel electrophoresis.

Primers

SMA F1: 5' CAGATCGGCGTTGAAAATAACG 3'
SMA F2: 5' GCCGCTTCGCTAAGTGGC3'
SMA B1: 5'GCAACGAACAATGATTTGTCTCC 3'
AITS1F1; 5'TTGCTATCGACGATAACAGCAC3'
AITS1R1; 5'AGGGGCATAGGTACCCTGGAAC3'

RESULTS AND DISCUSSION

Immunodetection of Infected Snails:

A total of 348 *Biomphalaria alexandrina* snails, including 88 normal snails, 85 mature S*chistosoma mansoni* infected snails and 175 snails at different intervals after infection have been tested for infection. *Schistosoma* infection was detected in the hemolymph of snails using the described sandwich ELISA technique. After two weeks of infection, the normal control snails showed 0% positively, while the infected control group showed 100% positively, sensitivity and specificity. After l week post exposure to the miracidial penetration the positivity was 0%, while after 2, 3 weeks, snails maintained positivity of 35.3%, 82.6% respectively. At early shedding (4 weeks) positivity was 87.5% and at maximal shedding (5, 6 weeks) the positivity was 96.5% and 89.5% respectively. Late patency showed only 15% positivity, while snails when stopped shedding, the positivity was 3.1% (Table 1).

Molecular Detection of Infected Snails:

Several optimization trials have been done to got sensitive and specific (one specific band, approximately, 0.65 kb) PCR reactions in both 1st and 2nd PCR using shedding snails and non infected ones as control. Reaction components mentioned above and 55˚C were found to give the optimum results. In 62˚C (annealing temperature for snail specific primers), no SMALDO band obtained. PCR for the specific SMALDO gene in snails infected for different intervals following the optimum conditions was able to detect SMALDO gene and consequently infection in 100% of shedding snails, one weak and 3 days post infection (100% sensitivity) but not uninfected snails (Fig.1a and table, 2). The assay was able to detect infection in one day infected snails with only 30% sensitivity). On the other hand amplification control reactions using primers specific for *B. alexandrina* AITS1, were all positive (Fig 1b).

Discussion:

Conventional methods used for the detection of Schistosome infected snails have limitations in situations such as, low parasite burden, prepatent infections, aborted development of sporocysts, and death of the molluscs after collection, resulting in underestimation of the true prevalence of infection. In addition, discrimination of *S. mansoni* cercariae from other trematodes can be problematic (Barbosa 1992 and Favre *et al*. 1995).

Table 1: Detection of *Schistosomamansoni* infection in *Biomphalaria alexandrina* snails using sandwich ELISA

Snail group	No. of snails	Weeks post exposure	No. of positive snails	% positivity in sandwich ELISA
Normal	88	--	0	0%
Infected control	85	>4	85	100%
Post exposure	14	1	0	0%
Post exposure	17	2	6	35.3%
Post exposure	23	3	19	82.6%
Early shedding	24	4	21	87.5%
Shedding	26	5	25	96.5%
Maximum shedding	19	6	17	89.7%
Late shedding	20	8	3	15%
Stop shedding	32	<10	1	3.1%

Table 2: Sensitivity of detection of infected snails by SMALDO PCR in one day, 3 days and one weak post infection (P.I.) with miracidia of *Schistosoma mansoni.*

Group	No. tested	No. positive	% positive
One day P. I.	10	3	30%
Three days P. I.	10	10	100%
One week P. I.	10	10	100%
Sheding snails	10	10	100%

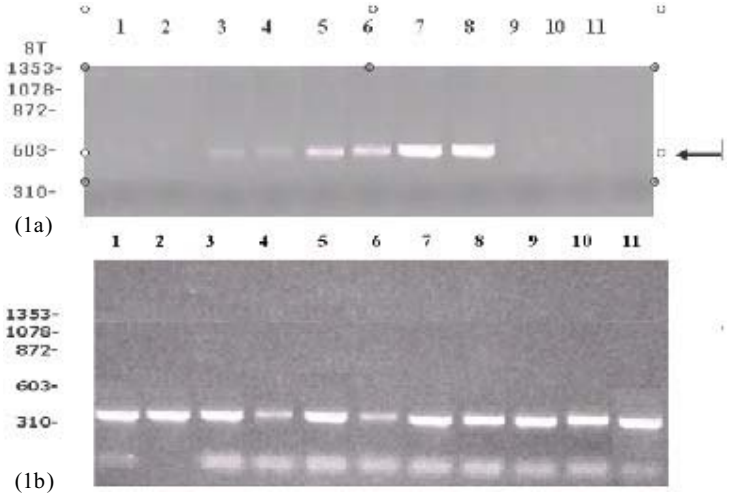

Fig. 1: 1a: Agarose gel electrophoresis for nested PCR of SMALDO gene in one day (1and 2), 3 days (3 and 4), one week (5 and 6) post infection, shedding snails (7 and 8), non infected snails (9 and 10) and negative reaction control (11). St: Molecular weight standard. Arrow indicates the amplified band. 1b: Agarose gel electrophoresis for PCR of the same samples using *Biomphalaria alexandina* ITS1 primers as amplification control.

Two specific monoclonal antibodies which recognized a 88 kDa *S. mansoni* SEA glycoprotein, were employed in the immunodetection of *S. mansoni* infection in the hemolymph of *B alexandrina* snails. These MABs have no cross reactivity with snails' antigens. Leher *et al.* (2007), found a common carbohydrate epitope between *S. mansoni* parasite and the hemolymph of its intermediate host *B. glabrata* and was characterized to be of N-glycans. It was extracted from fresh snail to detect *S. mansoni* glycoconjugates.

Two MAbs (10F, 5E) raised against *S. mansoni* antigen were employed by Bayne *et al.* (1987) . Both MAbs recognized the same epitopes on the surface of *S. mansoni* miracidia. The 10F and 5F MAbs are species- specific, which recognized only *S. mansoni* and showed no cross- reactivity with other *Schistosoma* species or any parasite.

In the present study, infected laboratory snails were classified according to the stages of shedding period with regard to the weeks post exposure to the infection. The presence of schistosomal antigens in snail's hemolymph collected at 1-6 weeks after exposure to miracidia was detected using sandwich ELISA. Schistosomal antigens were first detectable in the snails' hemolymph two weeks post infection. This time coincides with the release and formation of daughter sporocysts from the infected snails. Results (Table,1)

indicated that the quantity of the detected antigens increases as infection progresses from prepatency to patency and reached the highest values at 5 and 6 weeks post infection. These results were also explained by Humberger *et al*. (1989) who used a MAb for detecting schiosomal antigens in the hemolymph of infected *B. glabrata*. The rate of infection determined by conventional shedding test was 9.8%, while in combined patent and prepatent, the infection rate was 22.9%. This difference was attributed to the resistance of the snail, resulting in destruction of the parasite at very early prepatency (Meuleman *et al*., 1987). By using monoclonal antibodies in the study of Humberger *et al*. (1989), the rate of detection reached 87.5% at 4 weeks.

Schmitt *et al*. (2002) used two MAbs to identify schistosomal glycoconjugate antigens of *S. mansoni* and *S. haematobium* in the hemolymph of infected snails. Results revealed higher positivity rates in *S. mansoni* infected snails, than in snails infected with *S. haematobium*.

Considering the molecular methods for sensitive and early detection of infected snails especially in prepatency, several methods were developed depending on detection of specific sequences in genomic DNA (Hamburger *et al*., 1991). Some of such methods like dot hybridization using labeled probes (Hamburger *et al*., 1998) was impractical and did not detect early infection. The use of PCR amplification of the minisatellite repeat from *S. mansoni* mitochondrial DNA was proposed to identify infected *B. glabrata* snails from one week after exposure to miracidia, and distinguished *S. mansoni* among other trematode (Jannotti-Passos *et al*. 1997). Calderia, *et al.,* (2004) used the same technique in order to detect *S. mansoni* by obtaining traces of organic material from inside *B. glabrata* shells, artificially dried up to eight weeks.

In this work nested PCR method for detection of infected snails using *S. mansoni* gene specific primers was evaluated for sensitivity. Analysis of the developed SMALDO primer sequences against non-redundent data bases using the Basic Local Alignment Search Tool (BLAST) programs (Altschul *et.al*., 1990) at the National Center of Biotchnology revealed that, these primers are specific only for *S. mansoni* and not cross reactive with other schistosomes or molluscan aldolases. Nested PCR for such gene is sensitive enough to detect one cercariae and did not amplify *S. haematobium* gene (author unpublished data). Although the detection of the transcripts of SMALDO could be more sensitive in detection of prepatent infected snails due to RNA redundancy of such gene (Franco *et al*., 1997), amplification from genomic DNA was adopted. PCR from genomic DNA is more convenient for the conditions available for field surveys and snail collection and storage as DNA is more stable and requires no special storage conditions like RNA.

PCR results (Fig 1 &table 2) revealed high specificity represented in detection of one major band of *S. mansoni* SMLALDO in infected snails only and the complete negativity of non infected snails. The detection of schistosome species is superior to shedding and microscopic examination methods which cannot discriminate the species of infecting schistosomes (Caldeira *et al*., 2004).

Concerning sensitivity, the developed SMALDO nested PCR protocol permits detection of infected snails up to 3 days after miracidial infection with 100 % actual sensitivity in *B. alexandrina* infected with 8-10 miracidia per snail. Positivity of the moluscan specific control PCR in the one day infected snails exclude the false negativity in SMALDO amplification due to PCR inhibition.

Some PCR methods which depend on detection of repetitive sequences using PCR were sensitive enough to detect early infection in *Biomphalaria glabrata*. Hamburger *et al* (1998) amplified a 121 bp repeatitive region from *S. mansoni* and detected its presence in infected *B. glabrata* snails up to one day after infection with actual sensitivity 100%. The produced ladder pattern required using of strict stringent condition and the number of ladder bands varies with change in genomic DNA concentration (Hamburger *et al.,* 1998). One species specific positive band versus negative differentiation between infected and uninfected snails using SMALDO nested PCR should be more practical to attain and interpret than variable ladder pattern which did not tested on *B. alexandrina*.

Development of specific, sensitive and simple methods for detection of infected snails was considered potentially useful for providing extended information on Schistosoma snail epidemiology that may facilitate rapid evaluation of the danger of post control re-infection, and help to make decisions on the time and place of supplementary control measures. In this context, the potential usefulness of such methods is facilitating further catalytic evaluation model representations of Schistosome-snail epidemiology warrants.

In Conclusion:

comparative evaluations of the diagnostic qualities and technical aspects and coasts of these tests, point to the superiority and higher specificity of the immunodetection for larg scale detection of prepatency of snails infected with *S mansoni* compared to conventional methods used for early detection of infection in *B. alexandrina* snails. The adopted specific nested PCR assay revealed much higher sensitivity which enables the detection of *S. mansoni* infected snails down to 3 days post infection. So this assay provided higher efficiency for large-scale determination of prepatent infection prevalence in snails, and the evaluation of schistosomiasis transmission for schistosomiasis control.

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