A molecular Approach for Evaluation of Experimental Trials of Anti Schistosomal Vaccination in Murine Models

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Background and study aim: Current schistosomiasis control strategies are mainly based on chemotherapy, but many researchers believed that the best long term strategy to control schistosomiasis is through immunization with anti-schistosomiasis vaccines. This study aims at assessment of the efficacy of different potential anti-schistosomal vaccines (as crude soluble egg antigens (SEA), soluble worm antigen preparation (SWAP) and combined SEA & SWAP) by parasitological and molecular studies in experimental murine models.

Materials and Methods: Sixty male laboratory bred Swiss Albino mice were used and divided into six groups; control normal (G1), control infected by ± 80 cercariae by S.C. route (G2), Freund's adjuvant (adj.) received then infected (G3), SEA+adj. received then infected (G4), SWAP+ adj. received then infected (G5) and combined (SEA+SWAP) + adj. received then infected (G6). A schedule of sensitization, immunization and schistosomiasis challenge were followed and performed on different mice groups. Mice were euthanized 10 weeks post-infection. Potential vaccine efficacy was investigated by parasitological and molecular studies including egg count/gram stool using modified Kato thick smear**,** liver egg load, oogram pattern in the liver and stool PCR to detect *S. mansoni* egg DNA in stools of studied mice.

Results: The combined (SEA+SWAP) vaccine caused the highest significant reduction in the fecal egg count followed by SWAP then SEA antigens. On the other hand, the highest percentage reduction in eggs/gram liver tissue was attributed to the combined (SEA+SWAP) followed by SEA then SWAP antigens. Regarding oogram results, the combined (SEA+ SWAP) antigens were more efficient in increasing the number of dead ova with highly significant reduction in the number of mature & immature ova, followed by SEA then SWAP antigens. The lowest percentage of *S. mansoni* egg DNA detected by PCR in stool samples was encountered with the combined (SEA+ SWAP), followed by SEA then SWAP antigens.

Conclusion: The parasitological and PCR-based assessment studies denoted that the combined (SEA+SWAP) vaccine candidate was the most effective in protection against schistosomiasis challenge. The results of parasitological and molecular studies were nearly similar but the molecular study was more sensitive, definite and accurate.

INTRODUCTION

Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma.* After malaria and intestinal helminthiasis, schistosomiasis is the third most destructive tropical disease in the world, being a major source of morbidity and mortality for developing countries in Africa, South America, the Caribbean, the Middle East, and Asia **[1]**. Mature patent schistosome infections are associated with chronic local inflammatory response to schistosome eggs trapped in host tissues **[2].** In the context of schistosomiasis, an effective anti-schistosome vaccine would contribute greatly to a decrease in morbidity

associated with schistosomiasis via protective immune responses leading to reduced worm burdens and decreased egg production [3]. *Schistosoma* parasites secrete & excrete a number of different antigens into circulation of the host; these antigens are classified according to the stage of development of the parasite into cercarial; adult worm and egg antigens [4]. In order to develop an accurate immunization procedure, many different antigens have been prepared and tested as adult worm and egg antigens [5] and irradiated cercarial antigen [6]. The combination of two vaccines provides augmentation of the protective immunity and reduction of hepatic immunopathology [7].

Microscopic examination of stools remains the gold standard test for diagnosis of *schistosoma mansoni* infection, either by using a direct fecal smear or after the application of a concentration technique. *Schistosoma mansoni* eggs are easy to be detected and identified on microscopy owing to their characteristic size and shape with a lateral spine [8].

Kato technique is widely used in field studies and recommended by WHO for diagnosis of intestinal schistosomiasis when the intensity of infection is high, as it could detect the egg load as low as 40 eggs per gram stool [9]. The Kato-Katz technique was found to be more sensitive than the formalin ether concentration technique and the wet smear preparation [10].

Polymerase chain reaction (PCR) is a relatively simple technique that can detect a nucleic acid fragment and amplify its sequence, with high sensitivity and specificity [11]. Pontes et al. reported the first use of PCR for the diagnosis of *S. mansoni* DNA in fecal samples [12].

The current study aims at assessment of the efficacy of different potential antischistosomal vaccines as crude SEA, SWAP and combined SEA & SWAP by parasitological and molecular (PCR) studies experimentally, in murine models.

MATERIALS AND METHODS

This experimental study was conducted during the period between February 2013 and May 2014 at the Departments of Medical Parasitology and Biochemistry, Faculty of Medicine, Zagazig University, and Theodor Bilharz Research Institute, Imbaba, Giza, Egypt.

Infective cercariae: According to Liang et al. [13], *S. mansoni* cercariae (Egyptian strain) were obtained from infected laboratory bred *B. alexandrina* snails which were purchased from Schistosome Biological Supply Center at Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. After exposure to light for at least 4 hours, *S. mansoni* cercariae shed from the snails were used to infect the studied experimental animals (± 80 cercariae/mouse) by subcutaneous injection.

Animals: The current work was carried out on male laboratory-bred, parasite-free Swiss albino mice, about eight weeks's old age and 18-20 grams in weight for each mouse at the beginning of the experiment. Mice were obtained from Schistosome Biological Supply Center at Theodor Bilharz Research Institute (TBRI). The mice were maintained on a standard commercial pelleted diet with free accessible water and in an air-conditioned animal house at 20-22°C all over the time of study.

Materials:
- Schistosomal antigen preparations: Schistosomal crude antigens (SWAP and SEA) were obtained from Schistosome Biological Supply Center at Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt.
- Adjuvants: Freund's complete adjuvant (FCA) was obtained from Sigma Chemical Co., St Louis, Mo, USA and emulsified in phosphate-buffered saline (PBS) at a ratio of 2:1.

Experimental design:
Sample size:
Sixty albino mice. Mice were divided into six groups (of ten mice each).

Preparation of Antigens:
SWAP was prepared according to the method of Salih et al. [14], and SEA was prepared according to the method of Boros and Warren [15], the protein content was estimated using Bio-Rad kit (Bio-Rad Laboratories, Hercules, California, USA) [16] and the final concentration was adjusted with PBS to a concentration 50 μg / ml and stored at -70°C until use.

Antigen administration regimens:
The immunization schedule was performed according to Nabih and Soliman [17]. Each mouse was sensitized with an initial S.C. injection of 200 μl of the extracted antigen with a total antigen concentration of 30 μg protein. After two weeks, a second S.C. injection of 200

μl of the same antigen was taken and diluted to contain 20 μg protein. The antigen was combined with complete Freund's adjuvant at a 1:1 ratio and injected S.C. **[18].**

Infection of mice:

Infection of mice was done by subcutaneous injection with about ±80 *S. mansoni* cercariae/ mouse, suspended in 0.2 ml solution (cercariae in distelled water) 3 weeks after the initial S.C. antigen injection. The suspension was injected into the loose skin of the back of the mouse using an insulin syringe (1 cm length) **[19].**

Animal groups:

Group 1: control non infected group.

Group 2: control infected group (infected by ± 80 *S. mansoni* cercariae/ mouse by subcutaneous injection).

Group 3: mice were subcutaneously injected with complete Freund's adjuvant (CFA) and then infected by ±80 *S. mansoni* cercariae (adj. + infected group).

Group 4: mice were subcutaneously injected with soluble egg antigen (SEA) + complete Freund's adjuvant, and then infected (SEA+ adj. + infected group).

Group 5: mice were subcutaneously injected with soluble worm antigen (SWAP) + complete Freund's adjuvant, and then infected (SWAP+ adj. + infected group).

Group 6: mice were subcutaneously injected with SWAP and SEA +complete Freund's adjuvant, and then infected (SEA+ SWAP + adj. + infected group).

Vaccination efficacy assessment:

Animals were sacrificed by cervical dislocation 10 weeks post infection. Efficacy of the vaccination was assessed by parasitological and molecular studies.

1- Parasitological studies:

a) Egg count/gram stools using the modified Kato thick smear **[20]** daily starting from the 7th week post-infection, the average egg count for each mouse and group was calculated and tabulated.

b) Tissue (liver) egg load **[21]**:

c) Oogram pattern in the liver **[22]:** for each liver specimen, 100 eggs were microscopically counted & classified into viable (either immature or mature) and dead.

2- Molecular studies:

Detection of *S. mansoni* egg DNA in stool samples of studied mice by Polymerase chain reaction "PCR" **[12]**:

(A) DNA extraction :

In this study, Extraction of DNA from stool samples was done using **QIAamp® DNA stool mini Kit .Cat. No. 51504 (QIAGEN, GmbH, Hilden, Germany)**.

(B) DNA amplification and PCR reaction:

The PCR was done using a forward primer (5′-GAT CTG AAT CCG ACC AAC CG-3′) and reverse primer (5′-ATA TTA ACG CCC ACG CTC TC-3′) that were designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni*. Briefly, for a 25 μL final volume of PCR mixture, 5 μL of DNA extract was used as template, 12.5 μL 2X QiagenTag PCR Master Mix (0.05 u/μL Taq DNA Polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTPs), 1.5 μL of each primer and finally 4.5 μL of molecular biology grade water. The amplification reaction was carried out for 35 cycles, with each cycle consisting of a denaturation step at 95 °C for 40 s, an annealing step at 60 °C for 30 s and an extension step at 72 °C for 1 min. The first cycle had an extended denaturation step for 5 min and the reaction was ended with an extension step at 72 °C for 5 min. Amplified PCR products were then analyzed by electrophoresis in 2.5% agarose gels and detected by UV trans-illumination after staining with ethidium bromide. Stool samples positive for *S. mansoni egg* DNA showed bands at the level of 121bp in the DNA ladder, while Stool samples negative for *S. mansoni egg* DNA showed <u>no</u> bands at the level of 121bp in the DNA ladder **[12].**

Statistical analysis:

Data were entered, checked and analyzed using statistical computer program Statistical package for Social Sciences (SPSS version 16 windows). Data were expressed as the mean ± standard deviation (SD). Comparison between the mean values of different parameters in the studied groups was performed using one way analysis of variance (ANOVA) test, with paired (t) test for comparison between means of two groups. Chi square test was used for comparing between the qualitative data.

RESULTS

I- Results of the parasitological studies:

Table (1): The results of Kato technique, Liver egg load and Liver oogram in the different groups

Group	No. of eggs/g stool Mean ± SD	Liver egg load Mean ± SD	Liver oogram		
			Immature Mean ± SD	Mature Mean ± SD	Dead Mean ± SD
G2	361.83±37.66	1036.17 ± 72.43	25.3 ± 3.2	70.5 ± 1.6	4.2 ± 0.7
G3	234.83±27.25	1010.83 ± 21.25	24.6 ± 5.6	69.1 ± 2.9	6.3 ± 1.6
G4	64±7.4***	356.83 ±36.18***	18.1±2.1*	35.5± 1.8**	46.4 ± 2.3***
G5	56.33± 8.62***	462.83 ±41.22***	19.6±1.3*	44.1 ±2.4**	36.3 ±1.8**
G6	35.17 ± 8.2***	108.8 ± 34.13***	6.3±1.7***	21.3± 2.8***	72.4± 3.1***

*Significant difference from infected control at p<0.05.
**High significant difference from infected control at p<0.01.
***Very high significant difference from infected control at p<0.001.

II- Results of the molecular (PCR) study:
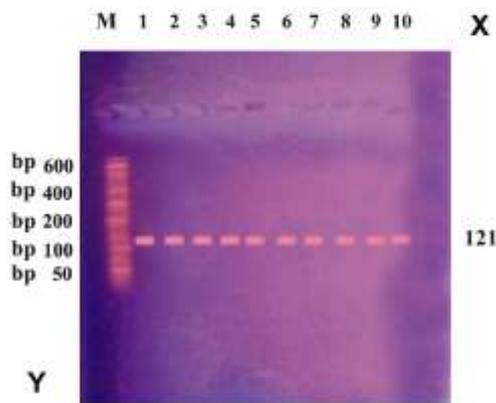

Figure (1): PCR results of (G2) showing that all fecal samples were positive for *S. mansoni* egg DNA (10 bands at 121 bp).
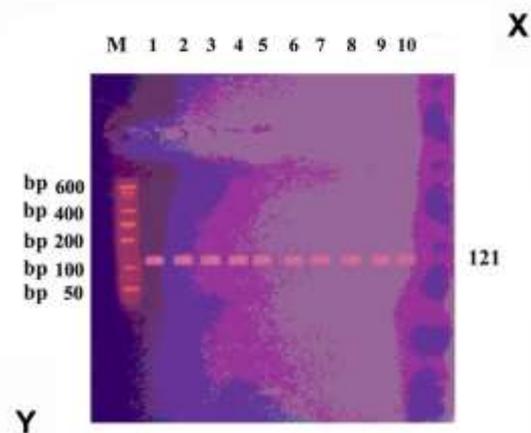

Figure (2): PCR results of (G3) showing that all fecal samples were positive for *S. mansoni* egg DNA (10 bands at 121 bp).
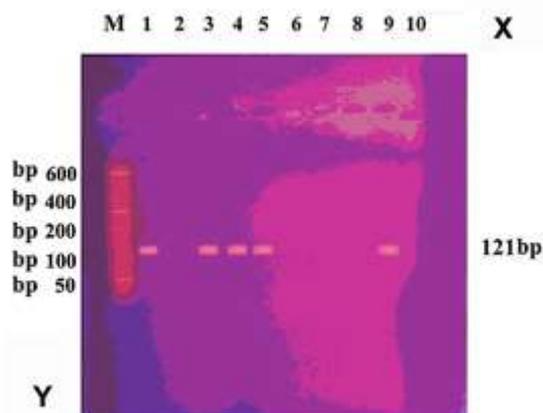

Figure (3): PCR results (G4) showing that **five** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).
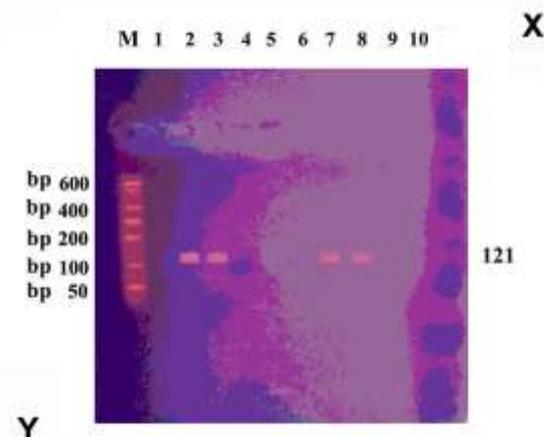

Figure (4): PCR results of (G5) showing that **four** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).
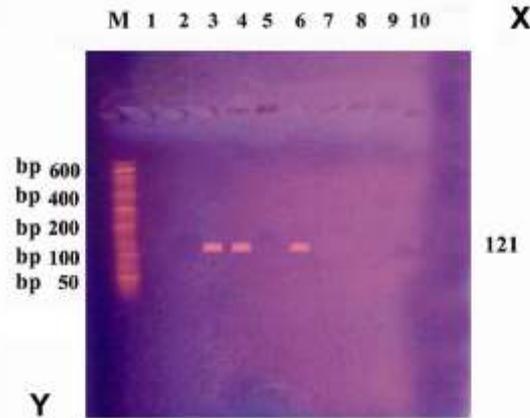
Figure (5): PCR results of (G6) showing that **three** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).

- **Axis (Y)** represented the number of DNA base pairs in the used DNA marker (ladder).
- **Axis (X)** represented the stool samples of each group.
- **M:** molecular weight marker.
- **Lanes 1 to 10:** represented the PCR results of fecal samples of each group.

Table (2): PCR results of studied control and vaccinated groups

Group	Number of stools samples	+ve stools samples by PCR	-ve stools samples by PCR	% of +ve samples	Chi sequare χ^2	P-value
G1	10	0	10	0 %	G2VsG4=6.7	0.01**
G2	10	10	0	100 %	G2VsG5=8.5	0.003**
G3	10	10	0	100%	G2VsG6=10.8	0.001**
G4	10	5	5	50%	G3VsG4=6.7	0.01**
G5	10	4	6	40%	G3VsG5=8.5	0.003**
G6	10	3	7	30%	G3VsG6=10.8	0.001**
					G4VsG5=0.2	0.6
					G4VsG6=0.8	0.4
					G5VsG6=0.2	0.6

**High significant difference between the groups at P-value <0.01

G2VsG4   **G2VsG5**   **G2VsG6**
G3VsG6   **G3VsG4**   **G3VsG5**
 Insignificant difference between the groups at P-value > 0.05
G4VsG5   **G4VsG6**   **G5VsG6**

Table (3): Comparison between PCR and Kato technique results for evaluation of the efficacy of the different potential antischistosomal vaccines regarding the percentage of positive samples

	No. of samples	No. of +ve samples	No. of –ve samples	% of +ve samples
Kato technique	60	26	34	43 %
PCR	60	32	28	53 %

Table (4): The diagnostic value of PCR technique using (Sensitivity %, Specificity %, PPV %, NPV % and Accuracy %) as compared to Kato technique

PCR				
Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
100 %	82 %	81.25%	100%	90%

Table (5): Parasitological and molecular results in studied groups

Group＼Item	G2	G3	G4	G5	G6
No. of eggs/g stool Mean±SD	361.83±37.66	234.84±27.25	64±7.4***	56.33±8.62 ***	35.17±8.2***
Liver egg load Mean±SD	1036.17±72.43	1010.83±21.25	356.83±36.18***	462.83±41.22***	108.8±34.13 ***
Immature Mean±SD	25.3±3.2	24.6±5.6	18.1±2.1*	19.6±1.3*	6.3±1.7***
Mature Mean±SD	70.5±1.6	69.1±2.9	35.5±1.8**	44.1±2.4**	21.3±2.8***
Dead Mean±SD	4.2±0.7	6.3±1.6	46.4±2.3***	36.3±1.8**	72.4±3.1***
% of +ve stool samples by PCR	100 %	100 %	50 %	40 %	30 %

*Significant difference from infected control at p<0.05.

**High significant difference from infected control at p<0.01.

***Very high significant difference from infected control at p<0.001.

DISCUSSION

Schistosomiasis is a parasitic disease caused by the digenetic trematodes of the genus *Schistosoma* members which are commonly known as blood flukes [23]. It is one of the most prevalent parasitic diseases in the world, second to malaria, it is estimated that schistosomiasis causes about 200,000 deaths per year [24].

In this work, vaccination of mice with different schistosomal antigens (SEA, SWAP and combined SEA & SWAP) showed very high significant results which were supported by previous studies that demonstrated the ability of antigens obtained from different life stages of the parasite to induce protective immune response in mice [25]. On using the crude vaccines, we are putting all the parasite stage components in one preparation because that parasite stage is parasitizing man as one unit by its whole components. So, the current study aimed to evaluate the efficacy of the potential antischistosomal vaccines using crude preparation of (SEA, SWAP and combined SEA+SWAP) by parasitological and molecular methods.

A significant reduction in the number of *S. mansoni eggs* in stool samples was detected 10 weeks post infection in comparison to the control infected group. The highest percentage reduction "90.3 %" was encountered with the combined SEA & SWAP-vaccinated group. On the other hand, there was insignificant reduction in ova count in (Freund's adjuvant + infected) group when compared to the control infected group.

These results were in agreement with those reported by Gundersen et al. [26] who found a positive correlation between the number of eggs per gram stools (using Kato technique) and the schistosomal antigen level.

The present results were in agreement with those of Ismail [27] who used also crude antigens and reported very high significant reduction in fecal egg count in mice vaccinated by adjuvant and combined schistosomal antigens (CAP + SWAP +SEA). The current results were in accordance with Etewa et al. [28], who found that a significant reduction in the mean egg counts/g stools was recorded by Kato technique in combined (CAP+SEA+SWAP) - vaccinated group, as compared to control infected group. On the other hand, a statistically insignificant reduction in the mean egg counts/g stools was recorded in the adjuvant- received group as compared to control infected group (P>0.05).

The present work cleared that the most effective antigen with very high significant reduction in liver egg load (the mean egg count/gram liver) was the combined vaccine + FCA, followed by SEA + FCA then SWAP + FCA. On the other hand, insignificant reduction was detected in the FCA- received group as compared to control infected group. These results nearly coincided with those of Etewa et al. [29] who detected that

the most effective antigen with significant reduction in tissue (liver) egg load was the combined (CAP, SWAP & SEA) + Freund's adjuvant with 93.67% percentage reduction as compared to control infected group in spite of using an extra antigen (CAP). On the other hand, the current results were not near to those of Romeih et al. [30] who noted that there was a significant reduction (41.53%) in the mean number of eggs/g liver of the multivalent DNA-vaccinated mice group, as compared to the control group. This may be attributed to the difference in antigen preparation. They also added that a significant reduction (29.90%) in the fecundity of female worms was observed after vaccination, denoting that the multivalent DNA vaccine permitted a better growth of mice and reduced the worm burden, egg number and worm fecundity.

In agreement, Fallon and Dunne [31] & Ismail [27] reported that administration of individual or combined schistosomal antigens led to significant reduction in worm burden with subsequent decrease in the number of deposited eggs. Besides, Teixeira de Melo et al. [25] explained that immunization of mice with adult worm tegument (Smteg) together with Freund's adjuvant induced a Th1 type of immune response associated with a significant reduction in eggs trapped in the liver by 65%.

On the other hand, results of the present study were higher than that of El-Ahwany et al. [32] who used SEA without Freund's adj. and Rezende et al. [33] in SWAP vaccinated group; they reported a percentage reduction in the liver tissue egg load of 42.8% and 8.4%, respectively.

This contrary may be explained by the higher protective effect of the combined (cocktail) antigens together with the augmenting role of Freund's adjuvant in nonspecific induction of the immune system.

In this work, the most effective antigen resulting in remarkable oogram changes with highly significant reduction in immature & mature ova and highly significant increase in dead ova was the combined vaccine + Freund's, followed by SEA + Freund's then SWAP + Freund's. On the other hand, insignificant oogram changes were detected in the adjuvant + infected group, as compared to control infected group. The present findings were partially agreed with those of Ismail [27] and Etewa et al. [29] who reported marked oogram changes in the vaccinated groups with highly significant reduction in immature and mature ova together with a highly significant increase in dead ova in combined vaccine (CAP + SEA + SWAP) + Freund's, followed by SEA + Freund's, then SWAP + Freund's.

In the present study, it was noticed that the decrease in *Schistosoma mansoni* egg count in stools of vaccinated groups (G4, G5 and G6) was directly proportional to the decrease in liver egg load, mature and immature egg numbers, while it was inversely proportional to the number of dead eggs in the oogram pattern.

These results were similar to that of Ismail [27] and Etewa et al. [29] who found marked reduction in fecal egg count, liver egg load, immature and mature eggs number in oogram and marked increase in dead ova in the vaccinated groups which demonstrated positive efficacy of the tested antigens.

Techniques for identifying circulating S. mansoni DNA have been widely used and such methods have demonstrated that it is possible to detect infections as early as one day after parasite exposure [34].

PCR-based diagnosis has been shown to be highly sensitive and specific and should therefore be considered as alternative methods for the diagnosis of *S. mansoni* infections [35].

The present study revealed that the combined (SWAP + SEA) potential vaccine was the most protective regarding detection of *S. mansoni* egg DNA in stools of *S. mansoni* - challenged mice with 70% success, followed by SWAP then SEA antigens with 50% and 40% success, respectively, as compared to control infected group. On the other hand, the adjuvant alone (G3) exhibited no protection and all fecal samples were positive for egg DNA by PCR.

Considering that the Kato technique is the gold standard test, we found that the PCR sensitivity was 100 %, specificity was 82 %, PPV was 81.25% and NPV was 100%. So, PCR was more accurate in evaluating the tested antigens as potential vaccines.

The obtained results are nearly in agreement with Pontes et al. [36] who compared between Kato technique and PCR for diagnosis of *S. mansoni* infection and evaluation of the efficacy of Praziquantel in treatment of infection. They found that the PCR technique showed a sensitivity of 96.7%, a specificity of 88%, PPV

was 78.4% and NPV was 98.3%, using the parasitological examination as the reference test.

Moreover, our results were near to those of Gomes et al., who compared between the Kato technique and PCR for diagnosis of *S. mansoni* in stool samples. They studied 67 cases and found that Kato technique detected infection in 42% of samples while, PCR detected infection in 61% of samples. However, they reported that PCR sensitivity was 92.9% and PCR specificity was 61.5% **[37]**.

The current results were also in agreement with those of Oliveira et al., who studied fecal samples using 2 PCR assays utilizing distinct primer pairs. One of the primer pairs was targeted to a highly repeated 121-base pair sequence of *S. mansoni*, and the other was targeted to *Schistosoma* 28S rDNA. The results obtained with stool samples from individuals with schistosomiasis showed a high sensitivity for PCR as *S. mansoni* DNA was detected in 91% of the samples analyzed **[38]**.

The obtained results are also supported by de Carvalho et al., who reported that the assessment of a single faecal sample by PCR detected more cases of infection than the analysis of one sample with two slides using the Kato-Katz technique, suggesting that PCR can be a useful diagnostic tool, particularly in areas with low endemicity. They found that the PCR sensitivity was 93.8%, specificity was 70%, PPV was 100% and NPV was 99.3 % **[39]**.

The present findings partially differ from those of Carneiro et al., who analysed and compared the results from a conventional PCR-based method with results from serological (ELISA) and parasitological tests. *S. mansoni* was detected by the KK technique in 33.9% of cases. In contrast, the PCR-based method was able to detect *S. mansoni* in 68.4% of cases i.e. more than two-fold increase in sensitivity. However, PCR failed to detect DNA in six samples that were confirmed to be positive by parasitological examination and ELISA **[35]**. Pontes et al. **[36]** reported that missed cases after PCR were certainly misdiagnosed by the DNA amplification assay due to many factors such as: inhibition of the amplification reaction by fecal compounds and/or DNA degradation during transportation from the field, variation in egg output and uneven distribution in feces.

CONCLUSION

On using combined antigens as a potential vaccine, more stimulation of the immune system against different antigenic components will occur, thus increasing the vaccine efficacy. This suggests that development of a multivalent (cocktail of antigens) vaccine may be the way forward, while the molecular method (stool PCR) for vaccine evaluation was more sensitive, accurate and definite.

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