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Full Length Research Paper

Parasitological assessment of potential competitor immunizations in Schistosoma mansoni-contaminated mice

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This study expects to evaluate the prophylaxis of diverse single and joined unrefined antigens utilized as hopeful antibodies as a part of a test model of Schistosoma mansoni contamination. Cercarial antigen planning (CAP), solvent worm antigen readiness (SWAP), dissolvable egg antigen (SEA) and joined antigens (CAP + SWAP + SEA) against schistosomiasis consolidated with either Freund's adjuvant or with Bacillus Calmette-Guérin (BCG) were tried for antibody adequacy in Albino mice by catching their consequences for worm load and tissue egg number (liver and digestive system). The information acquired demonstrated that, the consolidated antigens was the most defensive with huge decrease in the worm trouble (90.28%), and tissue egg load (liver and digestive system: 93.67 and 93.98%), separately. Accordingly, consolidating these diverse antigens (CAP, SWAP and SEA) gives enlargement of the defensive safety contrasted with every segment managed separately. The mix in this manner emerges as a potential competitor worth considering in the advancement of an attainable immunization against schistosomiasis

Key words: *Schistosoma mansoni*, vaccine, egg count, worm burden, contamination, prophylaxis.

INTRODUCTION

Schistosomiasis is the most important of the human helminthiases associated with subtle but persistent morbidities and mortality (Lambertucci et al., 2007). Schistosomiasis infects approximately 700 million people in 74 endemic countries. Those infected are 207 million, with 85% of the cases occurring in Africa (WHO, 2010).

Current schistosomiasis control strategies are mainly based on Praziquantel (PZQ) , but in spite of decades of mass treatment, the number of infected people remains constant (Hotez et al., 2010). Furthermore, efficient drug delivery can require a substantial infrastructure to regularly cover all parts of an area of endemicity, this can make chemotherapy an expensive and often impractical approach, although there is not yet clear-cut evidence for the existence of PZQ resistant schistosome strains, decreased susceptibility to the drug has been observed (Doenhoff and Pica-Mattoccia, 2006; Gryseels et al., 2006; James et al., 2009). The best long-term strategy to control schistosomiasis is through immunization with an anti-schistosomiasis vaccine combined with drug treatment (Martin et al., 2012).

Vaccination can be targeted either towards the prevention of schistosome infection or to the reduction of parasite fecundity. A reduction in worm numbers is the gold standard for antischistosome vaccine development, but because schistosome eggs are responsible for both pathology and transmission, a vaccine targeted at parasite fecundity and egg viability is also relevant (McManus and Loukas, 2008).

The selection of a suitable adjuvant to aid in the stimulation of the appropriate immune response is a critical step in the path to the development and employment of successful antischistosome vaccines, and the approaches are needed to be assessed carefully (Khalifa et al., 2011).

The Bacillus Calmette-Guérin (BCG), a live attenuated

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Mycobacterium bovis strain that has been used for the prevention of human tuberculosis for decades, is considered a promising candidate for the development of live vector systems for the delivery of foreign antigens to the immune system (Ohara and Yamada, 2001). Several advantages are associated with the use of BCG as an antigen-presenting system, including its known adjuvant properties, its ability to elicit humoral or cellular immune responses toward heterologous antigens, its thermo-stability which eliminates the need for a cold chain, and most importantly the possibility of obtaining an efficient immune response by using a single dose (Himmelrich et al., 2000).

Schistosoma mansoni vaccine must be safe, effective, stable, easily administered, and affordable by the target population. The goal in developing the schistosome vaccine under discussion is to produce a vaccine that protects people against infection and the consequent morbidity and mortality associated with *S. mansoni* (Todd and Colley, 2002).

The aim of this study was to design an experimental and a comprehensive model system consisting of individual and combined antigens that can provide prophylaxis to the infection by *S. mansoni*, through parasitological evaluation of these different vaccines by detecting their effects on worm burden and tissue egg deposition (liver and intestine). This system may have the advantage of resisting *S. mansoni* infection.

MATERIALS AND METHODS

The procedures were performed at the laboratories of Parasitology, Faculty of Medicine, Zagazig University and Theodor Bilharz Research Institute, Giza, Egypt. This is a case control experimental study.

Infective cercariae

Laboratory bred *Biomphalaria alexandrina* snails were purchased from the Shistosome Biological Supply Unit, Theodore Bilharz Research Institute (Giza, Egypt). After exposure to light for at least 4 h, *S. mansoni* cercariae shed from the snails were used to infect the experimental mice through the subcutaneous route.

Experimental animals

One hundred and ten laboratory bred Albino male mice (aged 6 to 8 weeks and weighing 18 to 20 g each), were used in this study. Mice were fed on standard diet with free accessibility to water at the Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute, Giza, Egypt.

Ethical aspects

All procedures related to animal experimentation in the present study met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences and approved by ethics committee of the Faculty of Medicine, Zagazig University.

Schistosomal antigens preparation

Cercarial antigen preparation (CAP), soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) were prepared at the SBSP at Theodor Bilharz Research Institute (TBRI). CAP was prepared according to the method of Carter and Collely (1979), SWAP was prepared according to the method of Salih et al. (1978) and SEA was prepared according to Boros and Warren (1970).

Adjuvants preparation

Freund's adjuvant (Adj) {Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA)} was obtained from Sigma Chemical Co., St Louis, Mo, USA and emulsified in phosphate-buffered saline (PBS) at a ratio of 2:1 (v/v).

BCG was obtained from Veterinary Serum and Vaccine Institute, Bacteria Diagnostic Research Department, Abasia, Cairo, and emulsified in PBS at a ratio of 2:1 (v/v).

Vaccination procedure

The total protein content of each antigen preparation was determined by the method of Bradford (1976). The vaccination schedule was performed according to the method of Nabih and Soliman (1986). Each mouse was sensitized with an initial subcutaneous injection of 200 µl of the extracted antigen with total antigen concentration contained 30 µg protein. After two weeks, a second subcutaneous injection of 200 µl of the same antigen was given containing 20 µg proteins; hence, each mouse received a total antigen dose of 50 µg protein.

The antigen was combined with either Freund's adjuvant (complete for the first injection, incomplete for the boost) subcutaneously at a 1:1 ratio (v/v), that is boosted twice at 2-weeks interval (Smithers et al., 1989), or with BCG 100 µl intradermally with the booster dose for each mouse (Boulanger et al., 1999).

Infection procedure

Infection was done by subcutaneous injection into each mouse (vaccinated and unvaccinated) with ±80 *S. mansoni* cercariae suspended in 0.2 ml solution after 3 weeks from the first vaccination (Peters and Warren, 1969).

Experimental design

Mice were divided into eleven groups (10 animals each). Three control groups: (i) infected only, (ii) infected and supported by Freund's adjuvant and (iii) infected and supported by BCG. Eight groups were vaccinated by different antigens: CAP, SWAP, SEA and combined (cocktail) antigens (CAP + SWAP + SEA); these antigens were supported by Freund's adjuvant or with BCG as shown in Table 1.

Assessment of the vaccine preparations effects

Animals were sacrificed by cervical dislocation 10 weeks post infection and parasitological evaluation were done.

Parasitological study

Worm burden recovery: Perfusion of adult worms from the liver and porto-mesenteric system was performed 10 weeks after infection according to Duvall and Dewitt (1967). The degree of protection (% reduction of worm) by the various vaccination preparations was calculated as follows:

$$P\ (\%R) = C - V/C \times 100$$

where P is the percentage protection, C is the mean number of parasites recovered from infected mice and V is the mean of parasite recovered from vaccinated mice (Tendler et al., 1986).

Tissue egg load: One gram of liver and of intestine from each mouse was weighed and put into a test tube containing 2 ml of 5% KOH, then left for 18 h at room temperature for complete digestion without egg destruction. The second day, all test tubes were put in the incubator at 37°C for 6 h. Each tube was shaken well for 5 min, and then 0.1 ml of the digest was examined microscopically for counting *S. mansoni* eggs. The process of counting was repeated five times, to calculate the mean number of ova per 0.1 ml. Mean number of ova per gram of tissue (liver and intestine) = mean number of ova in 0.1 ml × 100 according to the procedure by Cheever (1968).

Oogram pattern: Following perfusion for the recovery of the schistosomes, fragments of the intestine (from the middle loop of intestine till the terminal ileum) from each animal were separated and transferred to the Petri dishes containing saline (0.9%). The intestines were opened lengthwise and the excess mucus was removed. One-centimeter fragments were weighed and placed between a glass slide and a glass cover. The preparation was inverted and pressed on a rubber surface padded with filter paper. The percentages of immature, mature, and dead ova in the small intestines were computed from a total of 100 eggs per intestinal segment and classified according to the categories previously defined by Pellegrino et al. (1962).

Statistical analysis

Data were entered, checked and analyzed using statistical computer program Statistical package for Social Sciences (SPSS version 16 windows). Data are 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.

RESULTS

One hundred and ten laboratory bred albino male mice, each mouse was sensitized with an initial subcutaneous injection of the extracted antigen. After two weeks, a second subcutaneous injection of the same antigen. The antigen was combined with either Freund's adjuvant (complete for the first injection, incomplete for the boost) subcutaneously, that is, boosted twice at 2-weeks interval, or with BCG intradermally with the booster dose for each mouse. Infection was done by subcutaneous injection into each mouse (vaccinated and unvaccinated) with ±80 *S. mansoni* cercariae after 3 weeks from the first vaccination.

The effect of vaccination with different schistosomal antigens depend on the degree of the protection in mice infected with *S. mansoni* cercariae; protection was investigated by comparing the results of the parasitological evaluation (worm burden, tissue egg count, and oogram studies), in the different groups vaccinated with CAP, SWAP, SEA or combined compared to that in the control groups [Mice group (10 mice) of combined + BCG antigens died early before the time of sacrificing (two mice died 2 weeks post infection, 5 mice died 4 weeks post infection and the rest died 5 weeks post infection). The reason was not known, but we suggested that, this may be due to the higher level of activation of immune system, it need more investigations].

Worm burden

The result showed that the most effective antigen with significant reduction in the mean worm burden at $p<0.001$ was combined + Freund's percent reduction of 90.28%, followed by SEA with BCG with percent reduction of 69.91%, SWAP with BCG with percent reduction of 66.67%, then SEA + Freund's with percent reduction of 64.35% and SWAP + Freund's with percent reduction 62.5%. While, the least effective vaccines with significant reduction at $p<0.01$ are noticed in CAP with BCG with percent reduction of 56.02% followed by CAP + Freund's with percent reduction of 51.85%. On the other hand, insignificant reduction at $p>0.05$ detected in C2 and C3 with percent reduction of 13.43 and 5.09%, respectively compared to C1 (the control infected group) (Figure 1) [percent reduction (%R) = % protection = comparing mean worm burden in vaccinated (V) and control (C) where % = (C-V)/C×100.

Tissue egg load

The result detected that the most effective antigen with significant reduction in tissue egg load (the mean egg count/gram tissue – liver and intestine) in combined + Freund's with percent reduction of 93.67 and 93.98%, respectively, followed by SEA + BCG with percent reduction of 72.22 and 73.93%, SWAP + BCG with percent reduction of 69.52 and 69.95%, then SEA + Freund's with percent reduction of 64.78 and 66.78% and SWAP + Freund's with percent reduction of 59.66 and 60.68% (Table 2 and Figure 2).

While, the least effective antigens are CAP + BCG with percent reduction of 56 and 55.79% followed by CAP + Freund's with percent reduction of 46.37 and 48.4%. On the other hand, insignificant reduction detected in C2 and C3 with percent reduction of 1.56 and 9.02 and 0.515 and

Table 1. Animal groups.

Group	Explanation
Control group	C1 (control infected group): Mice were unvaccinated and infected by ±80 *S. mansoni* cercariae. C2 (control infected + BCG): Mice were injected by BCG and infected by ±80 *S. mansoni* cercariae. C3 (control infected + Freund's): Mice were injected by Freund's adjuvant and infected by ±80 *S. mansoni* cercariae acting as.
Vaccinated group	CAP+BCG: Mice were vaccinated by CAP+BCG and infected by ±80 *S. mansoni* cercariae. CAP+Freund's: Mice were vaccinated by CAP+Freund's and infected by ±80 *S. mansoni* cercariae. SWAP+BCG: Mice were vaccinated by SWAP+BCG and infected by ±80 *S. mansoni* cercariae. SWAP+Freund's: Mice were vaccinated by SWAP+Freund's and infected by ±80 *S. mansoni* cercariae. SEA+BCG: Mice were vaccinated by SEA+BCG and infected by ±80 *S. mansoni* cercariae. SEA+Freund's: Mice were vaccinated by SEA+Freund's and infected by ±80 *S. mansoni* cercariae. Combined+BCG: Mice were vaccinated by cocktail antigen preparation (CAP+SWAP+SEA)+BCG and infected by ±80 *S. mansoni* cercariae. Combined+Freund's: Mice were vaccinated by cocktail antigen preparation (CAP+SWAP+SEA) + Freund's and infected by ±80 *S. mansoni* cercariae.

Table 2. The effect of vaccination with different antigens on the tissue egg load (mean egg count/gram tissue – liver and intestine) and oogram of the studied mice groups.

Group	Tissue egg load (Mean egg count/gram tissue)						Oogram		
	Liver eggs			Intestinal eggs			Immature (%)	Mature (%)	Dead (%)
	Mean±SD	R (%)	t test (p)	Mean±SD	R (%)	t test (p)	Mean±SD	Mean±SD	Mean±SD
C1	1029.8±74.28	-	-	1173.90±115.53	-	-	27.3±2.4	67.5±3.2	5.2±0.9
C2	1024.5±20.88	0.515	3.14	1106.0±225.26	5.78	0.729	27.2±2.1	67.1±2.9	5.9±1.2
C3	1013.7±24.35	1.56	0.688	1068.0±317.51	9.02	0.879	26.9±2.2	66.4±2.8	6.7±1.4
CAP+BCG	453.1±121.39***	56	10.29	518.9±24.00***	55.79	15.59	18.1±1.8*	45.2±2.4*	36.7±2.1**
CAP+Freund's	552.3±46.37***	46.37	14.53	605.7±34.05***	48.4	13.87	22.4±1.9	47.7±2.6*	29.9±2.3*
SWAP+BCG	313.9±43.97***	69.52	65.31	352.8±24.45***	69.95	21.852	13.4±1.7**	40.8±2.2	45.8±2.5**
SWAP+Freund's	415.4±27.25***	59.66	22.93	461.6±33.59***	60.68	17.33	17.3±1.8**	42.9±2.6*	39.8±2.3**
SEA+BCG	286.6±42.22***	72.22	34.19	306.00±130.06***	73.93	18.621	12.7±1.6**	39.7±2.1*	47.6±2.7**
SEA+Freund's	362.7±25.84***	64.78	24.34	390.00±140.24***	66.78	16.132	16.2±1.5**	41.2±2.1*	42.6±2.4**
Combined+Freund's	65.2±21.22***	93.67	44.81	70.70±15.97***	93.98	29.28	5.6±1.2***	2.4.2±1.9***	70.2±2.3***

*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.

5.78%, respectively compared to C1 (control infected group).

The most effective antigen with remarkable changes inthe oogram with significant reduction in immature ova and mature ova and significant increase in dead ova in combined + Freund's, followed by SEA + BCG, SWAP + BCG, then SEA + Freund's and SWAP + Freund's, respectively. The least effective antigens are CAP + BCG with

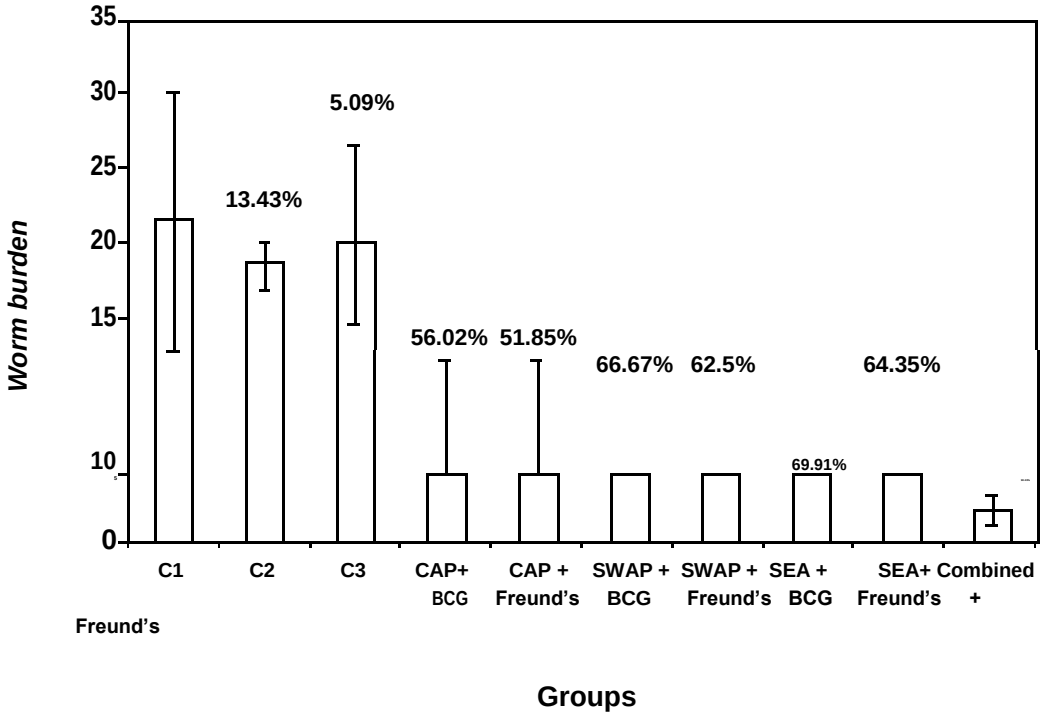

Figure 1. The effect of vaccination with different antigens on the mean worm burden of the studied mice groups [% = %R (% protection)]**.**

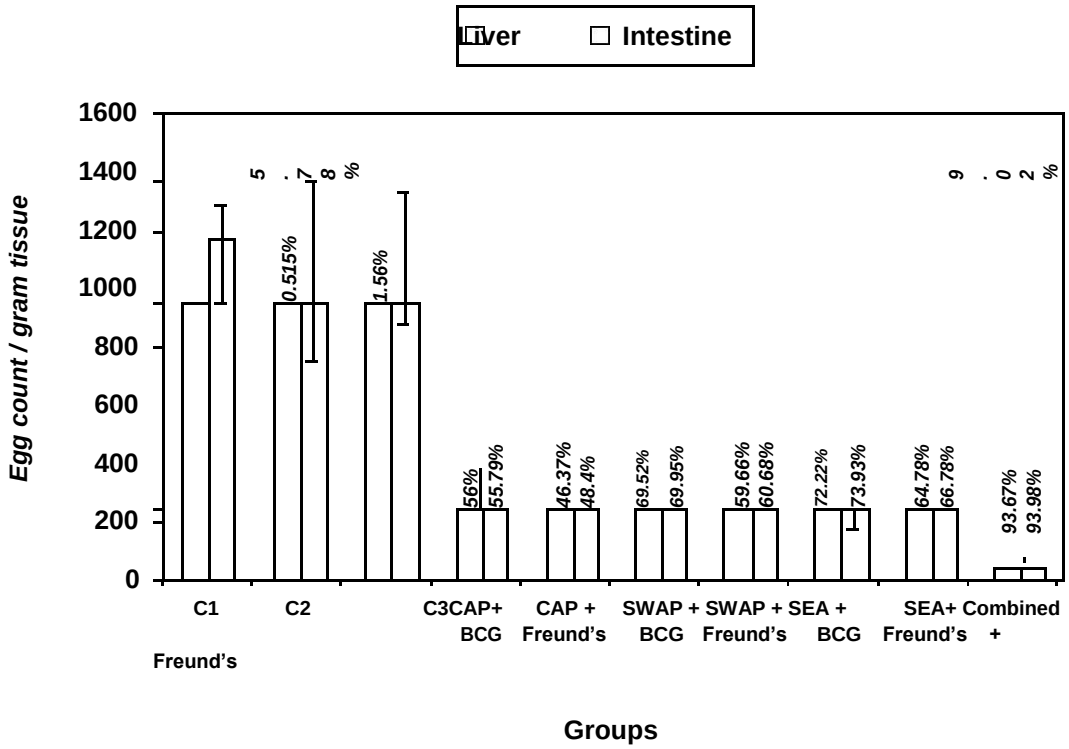

Figure 2. The effect of vaccination with different antigens on the tissue egg load (liver& intestine) of the studied mice groups. %= %R (% protection).

significant reduction in immature ova and significant reduction in mature ova and high significant increase in dead ova and CAP + Freund's with significant reduction in mature ova and significant increase in dead ova. On the other hand, insignificant oogram changes detected in C2 and C3 compared to C1 (Table 2 and Figure 2).

DISCUSSION

Development of a vaccine for schistosomiasis is far fetched; it has been targeted as a priority by researchers and the World Health Organization (WHO, 2000).

Waine and McManus (1999) decided that the attenuated vaccines for schistosomiasis are considered neither safe nor practicable for human use, and therefore, other approaches must be considered. In the current experimental study, the crude antigens were used in mice, because it was suggested that these antigens were good representative to all *Schistosoma* stages present inside the human body. In addition, Ashour et al. (2004) reported that schistosomes are multicellular parasites with differentiated tissues, and therefore with antigen complex.

A vaccine that induces even a partial reduction in worm burdens could considerably reduce pathology and limit parasite transmission (Chitsulo et al., 2004). Since the levels of protection elicited by a single antigen were low, development of novel cocktail vaccine formulations is necessary to enhance protection levels to at least induce protection ranges from 70 to 80% (Khalifa et al., 2011).

In this context, the results of the current study showed that combined antigens, displayed the best protection with the highest level of decrease in the mean worm burden compared to unvaccinated infected control group (C1). The result was closely related to Ismail (2005) who reported that the reduction in worm burden was 88% in mice which were vaccinated with combined antigens (Adj + CAP + SWAP + SEA) compared to unvaccinated infected control group. In addition, Ashour et al. (2004) detected that worm burden protection percentage was 60.91, 78.16 and 64.77% for (SEA + CAP), (SEA + SWAP) and (CAP + SWAP), respectively.

Nascimento et al. (2002) decided that as an antigen, candidate for vaccine development against schistosomiasis should be given consistently at least 40% of protection. The results support those of Nascimento et al. (2002) in which there were other vaccinated groups with significant decrease in the mean worm burden, but lesser than combined group as SEA + BCG, SWAP + BCG, SEA + Freund's, SWAP + Freund's, CAP + BCG, CAP + Freund's, respectively, compared to C1 (infected control group).

In addition, Ismail (2005) concluded that percent reduction in worm burden was 87.3, 71.5 and 76.4% for CAP, SEA, and SWAP, respectively. Although Soliman et al. (2008) used different animal model, our results were closely related to them, they tested *Schistosoma mansoni* SWAP and cercarial antigen (CAP) alone to detect their efficacy against the infection in hamsters, results showed that the worm reduction percentages were 53.8 and 56.4% for CAP and SWAP, respectively.

Our results are higher than those of Ashour et al. (2004) who found that worm burden protection percentage was 42.5, 58.33 and 53.33% for CAP, SEA, and SWAP, respectively; this may be attributed to using of Freund's adjuvant and BCG in our study. Also, the protective level obtained by SEA in our result was higher than that of El-Ahwany et al. (2012) who recorded 49% decrease in worm load on administration of SEA prior to infection.

Human studies of vaccine development are instructive for not only identifying the few antigens directly and exclusively associated with resistance, but also for indicating which of these components can be formulated with adjuvants to generate protective responses in animal models (Bergquist et al., 2008). In this context, we used two different adjuvants BCG and Freund's.

Traditional approaches had declared that Freund's adjuvant is the best to be used when antigens are first being assessed as potential candidate vaccines in the mouse model (McManus and Loukas, 2008). Our results showed that although C2 induced higher levels of protection than C3, but both were statistically insignificant compared to C1 (control infected group), confirming that these substances alone have no effect against *S. mansoni* when used alone, become more effective when used with different antigens. This finding agreed with El-Marhoumy et al. (2009) who reported that BCG acting as a potent adjuvant in association with larval antigens, but was not protective when administrated alone.

In this work, combined (cocktail) antigens, showed significant decrease in the mean egg count/gram tissue (liver and intestine) compared to C1 (control infected) with marked changes in the oogram including significant reduction in immature ova and mature ova and very high significant increase in dead ova. In addition, there were other vaccinated groups with significant decrease in the mean egg count/gram tissue and with oogram changes, but lesser than combined group as SEA + BCG, SWAP + BCG, SEA + Freund's, SWAP + Freund's, CAP + BCG, CAP + Freund's, respectively, compared to C1 (infected control group).

Our results are in confidence with the results of Fallon and Dunne (1999) who reported that reduction in worm burden should result in a corresponding decrease in the number of deposited eggs. Our results also, agreed with Ismail (2005) who reported marked oogram changes with reduction in total ova count in (liver and intestine), 98.02, 96.31, 90.27, 79.11 and 78.93% in mice vaccinated with cocktail antigens, CAP, SWAP, SEA and Freund's adjuvant, respectively. Besides, Teixeira de Melo et al. (2010)

emphasized 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%.

All mice of combined+BCG group died before scarifying, the reason was not known, further investigations will be needed, and this may be attributed to the higher level of activation of immune system as a direct consequence of vaccination.

In this study, administration of single or combined antigens prior to infection resulted in decreased worm load, hepatic and intestinal ova together with change in oogram pattern. This could be due to enhancement of immune response or would be acting as assort of primary infection that somewhat hinders the challenge one.

The dream of a vaccine against schistosomiasis remains a powerful driving force in research (El-Marhoumy et al., 2009). Obviously, further research is required on the development of novel adjuvant vehicles as well as cocktail vaccine formulations to enhance protection levels with the eventual aim of 100% worm reduction (Siddiqui et al., 2011).

Conclusively, the present study has shown that combining different antigens (cocktail antigens) {CAP, SWAP and SEA} provide augmentation of the protective immunity when compared with each component administered individually, and considered a convenient mean to overcome many of the problems associated with the successful implementation of vaccine schedule of schistosomiasis. Adding adjuvants like BCG and Freund's provide more power to the candidate vaccine. This further suggest that immune interference resulting from immunizations with antigenically distinct vaccine targets that may be an important consideration in the development of multicomponent vaccine preparations, as the need for *Schistosoma* vaccines is now more pressing than ever.

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