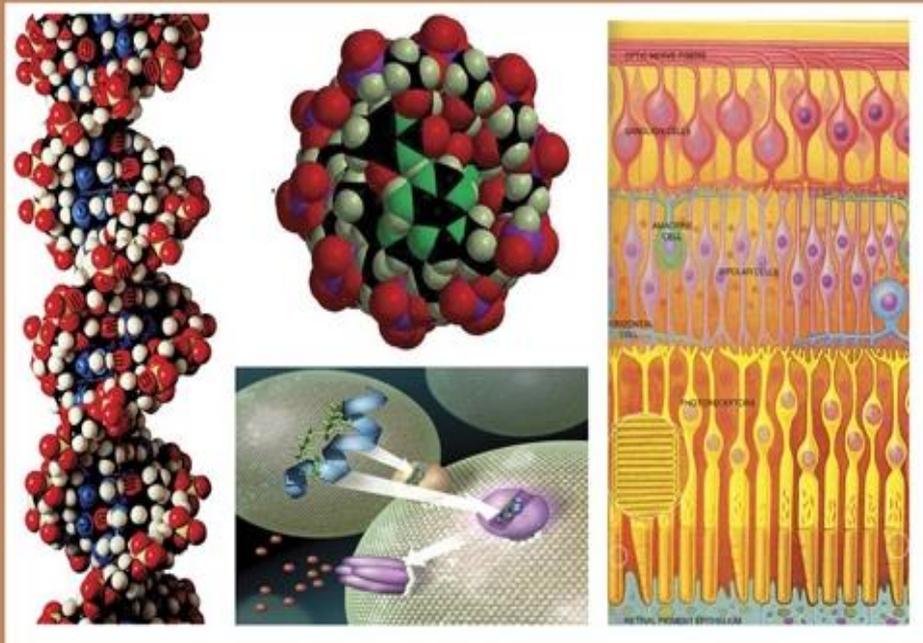




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Associations Between Levels of Liver Enzymes and Circulating Antigens in Individuals Infected with Schistosomiasis *Heamatobium*

Ibrahim A. E. Zahran¹, Samir A. M. Zaahkouk¹, Ibrahim R.B. Aly², Alaa A. M. Samn¹, and Sawsan A.M. EL-Shamy³

1-Zoology Department, Faculty of Science, AL-Azhar University,

2-Department of Parasitology, Theodor Bilharz Research Institute,

3- General Biology, Basic Science Center, Misr University for Science and Technology

E.mail: Szaahkouk@yahoo.com

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ABSTRACT

Current estimates of the prevalence of schistosomiasis depend on the use of well-established, but imperfect, diagnostic tests where specimens are processed by routine parasitological methods and parasitological cure is judged by inspection of a cohort of children before and after Praziquantel (PQZ) treatment. In this study, in order to evaluate the different immunodiagnostic antigen detection assays, the selection of a proper antigen and its purification followed by propagation of its specific antibodies and purification were mandatory. Measurement of Liver function and kidney function were performed and its relation to *S. haematobium* infection in different age and sex individuals were studied. Serum samples from an infected human with *S. haematobium* gave a strong reaction against *S. haematobium* SWAP antigen and no cross-reacting with other parasites. The produced anti-*S. haematobium* SWAP IgG-pAb diluted 1/250 in PBS/T buffer gave strong reactivity to *S. haematobium* SWAP. The highest percentages of *S. haematobium* infected patients were in the age group 21-30 years (25%) and the least percentages of patients were in the age group of 51-55 years (2.9%). Levels of ALT, AST and ALP in the serum of humans among schistosomiasis group recorded a highly significant increase compared to the healthy control group. In conclusion strong association between biochemical parameters and serum circulating antigens among schistosomiasis infection.

INTRODUCTION

Schistosomiasis is one of the most widespread parasitic infections of man and is second to malaria in socioeconomic and public health importance in tropical and subtropical areas. Schistosomiasis affects 230 million of the world's poorest people through 77 countries in tropical Asia, Africa, South America and Caribbean. However those at most risks of infection are in 52 countries (King and Dangerfield-Cha, 2008; WHO, 2018). In Egypt, urinary schistosomiasis is still representing a serious health problem to deal with. Due to control programs over the last decade, a decline in the prevalence of human schistosomiasis in Egypt has been reported, however, the disease is still endemic in many foci (EL-Khoby *et al.*, 2000; Bergquist, 2009; ELshazly *et al.*, 2016). So, early diagnosis is necessary for prompt treatment before non-repairable health damage occurs. The diagnosis of urinary schistosomiasis can serve two purposes; medical treatment of individual patients and screening of groups of people for mass treatment or epidemiological studies.

The choice of one method or another depends on many factors; the objective of the diagnosis, financial constraints, the number of persons to be examined, the prevalence and intensity of the disease, the laboratory equipment available, skill and experience of the technical personnel and the test characteristics (WHO, 2002; Doenhoff *et al.*, 2004; WHO, 2005; Taman and El-Beshbishi, 2019). Immunological diagnosis of schistosomiasis was used most widely to detect the antibodies. However, *Schistosoma* antibody detection often lacks specificity. Soluble worm antigen preparation (SWAP) is considered important diagnostic target antigens, which are highly reactive with sera from schistosomiasis patients and do not cross-react with sera from patients. The magnetic bead immunoassay combines the use of magnetic beads with a high binding capacity as a solid phase and the rapid reaction kinetics of solutions with the simple separation of bound and unbound materials on the solid phase, which provides the chance of enhancing the sensitivity of antigen detection (Gundersen *et al.*, 1992; Ndhlovu *et al.*, 1995). The pathophysiological impact of *Schistosoma mansoni* infection has been mainly attributed to granuloma formation induced by the host cell-mediated immune response to soluble antigens secreted by parasite eggs trapped in the walls of the hepatic and intestinal vessels of infected hosts (Weber *et al.*, 2019). While the degree of *Schistosoma*-associated hepatic dysfunction varies and is dependent on parasite load and host background, liver fibrosis is a common finding in advanced schistosomiasis, mostly at the sites of ongoing granulomatous reactions (Ndhlovu *et al.*, 1995). Nonetheless, a wide range of liver damage has been described in both human and animal infections. These include impaired protein synthesis, reduced lipoprotein concentrations, decreased erythrocyte counts and blood indexes, neutropenia, lymphopenia, and eosinophilia, increased circulating liver enzymes levels, and hemostatic abnormalities (Doenhoff *et al.*,

2004). Despite the variability in clinical signs, it remains unclear whether infected individuals without clinical complaints or symptoms present a detectable degree of liver dysfunction. Therefore, the aim of this work to investigate the associations between *S. haematobium* infection and alterations in levels of circulating liver enzymes in individuals infected with Schistosomiasis haematobium in Egypt.

MATERIALS AND METHODS

Viable worms of *S. haematobium* were bought from Theodor Bilharz Research Institute (TBRI). The worm was washed six times in phosphate-buffered saline (PBS), to eliminate traces of hamsters' tissues & secretions (pH 7.3) and incubated for 16 h at 37°C in Roswell Park Memorial Institute (RPMI) 1640 medium, PH 7.3; containing 2% glucose, in 30 mM HEPES (hydroxyethyl piperazine-N-2-ethanesulfonic acid) and 25 mg of Gentamycin Per ml. Following incubation, the medium was removed and centrifuged at 14900 xg for 30 min, and the supernatant termed Excretory-Secretory (ES) products was then collected into fractions and stored at -20 C° (Weber *et al.*, 2019).

Collection of Samples From Human Subjects:

Group (A) (patients infected with *S. haematobium*) (n= 34): urine and serum samples were collected from patients confirmed parasitologically to have *Schistosomiasis haematobium*.

Group (B) (patients infected with other helminthic parasites) (n= 32): stool, urine and serum samples were collected from patients confirmed parasitologically infected with other helminthes (eg. *H. nana* (8), *Fasciola* (8), *Ascaris* (8), and Hookworms (8)).

Group (C) (control group) (n= 20): urine and serum samples were collected from healthy subjects with no parasitological evidence of having a helminthic parasitic infection. Medical staff at TBRI served as a parasite-free-healthy negative control.

Assessment of Specificity of the Prepared pAb by Indirect-ELISA:

This method was performed, with

some modifications from the original method of **Engvall and Perlmann (1971)** ^[13] (Engvall and Perlman, 1971).

Wells of polystyrene microtitre plates (96-flat bottomed wells) were coated with 100 µl/well of the purified anti-*S. haematobium* SWAP IgG-pAb at a concentration of (2.5, 5, 10, 20 and 30) µg/ml 0.60 M carbonate buffer (capture antibody), pH 9.6 and incubated overnight at room temperature. The plates were washed 3 times with washing buffer, (0.1 M PBS, pH 7.4), then blocked with 200 µl /well by 0.1% of Bovine serum albumin (BSA) (Sigma) in 0.1 M PBS, pH 7.4 for 1 hr at 37°C. Finally, the plates were washed with washing buffer 5 times. 100 µl of infected human sera with *S. haematobium*, *Fasciola*, *H. nana*, *Ascaris* and Hookworms diluted 1/250 in washing buffer were added to each well and incubated for 1 hr at 37°C. The plates were then washed 3 times with washing buffer. 100 µl/well of the purified anti-*S. haematobium* SWAP IgG-pAb peroxidase conjugate diluted in washing buffer (1/250) (detecting antibody) was dispensed and the plates were incubated for 1 hr at 37°C. The plates were washed 3 times with washing buffer. 100 µl of substrate solution was added to each well and the plates were incubated in

the dark at room temperature for 30 min. 50 µl/well of stopping solution was added to stop the enzyme-substrate solution. The absorbance was measured at 492 nm using ELISA reader (Bio-Rad microplate reader, Richmond, Co.).

Assessment of Biochemical Parameters:

The Biodiagnostic kits (Dokki, Giza, Egypt) were used for the determination of serum aminotransferase enzymes (AST & ALT) activities (Reitman and Frankel, 1957), alkaline phosphatase (ALP) activity according to the method of (Belfield and Goldberg, 1971), estimation of urea level was determined according to the method of Azra,(2014) and the level of creatinine according to Krishnegowda *et al.*, (2017).

RESULTS

Preparation of *S. haematobium* SWAP Antigen:

One hundred viable worms of *S. haematobium* were incubated for 16 h at 37°C in Roswell Park Memorial Institute (RPMI) 1640 medium, 50 ml of supernatant were collected containing crude extract of SWAP. The protein content of the crude extract of SWAP was 2.6 mg/ml. The total protein content was 2.6x50 equal 13.0 mg as measured by Bio-Rad protein assay (Fig. 1).

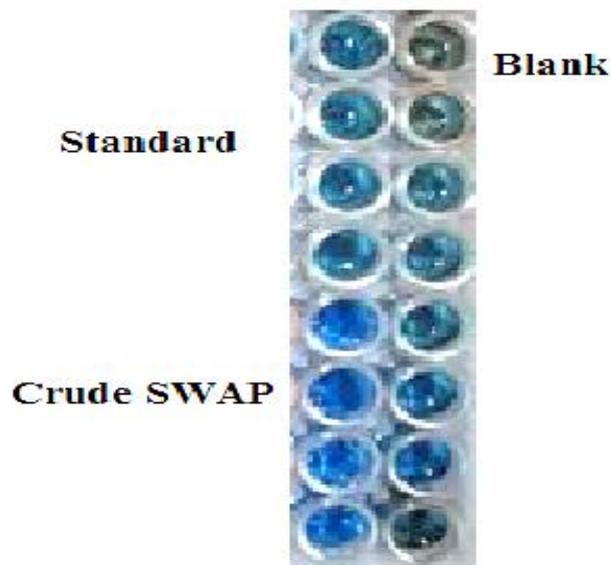


Fig. 1: Serial dilutions from the standard and crude SWAP antigen.

Protein Content Determination of The Purified *S. haematobium* SWAP Antigen:

The protein content of purified SWAP antigen of *S. haematobium* was measured again after gel filtration chromatography. The total protein was 5.3 mg/ml of total protein as measured by Bio-Rad protein assay (Bradford, 1976)..

Assessment of Reactivity And Specificity Of The Prepared SWAP *S. haematobium* Antigen by Indirect-ELISA:

The antigenicity of the SWAP antigen was tested by indirect ELISA technique. Serum samples from an infected human with *S. haematobium* gave a strong reaction against *S. haematobium* SWAP antigen with mean OD reading equal to 1.69 and no cross-reactions were recorded with sera of patients infected with other parasites e.g., *H.nana* (8), *Fasciola* (8), *Ascaris* (8), and Hookworms (8). (Table 1).

Table 1: Reactivity of purified SWAP antigen by indirect ELISA

Serum Samples	OD readings at 492 nm (m ± SD)
<i>S. haematobium</i>	1.69 ± 0.129
<i>H.nana</i>	0.09 ± 0.01
<i>Fasciola</i>	0.201 ± 0.06
<i>Ascaris</i>	0.05 ± 0.01
Hookworms	0.19 ± 0.04

OD= optical density, M= mean, SD= standard deviation.

Assessment of Specificity Of The Purified pAb by Indirect ELISA:

Specificity of anti-*S. haematobium* SWAP IgG-pAb against *S. haematobium* SWAP antigen and other parasite antigens (*H.nana* (8), *Fasciola* (8), *Ascaris* (8), and Hookworms) were assessed by indirect ELISA. The produced anti-*S. haematobium*

SWAP IgG-pAb diluted 1/250 in PBS/T buffer gave strong reactivity to *S. haematobium* SWAP. The OD means reading at 492 nm for *S. haematobium* SWAP was 0.98 compared to 0.13, 0.22, 0.16 and 0.05 for *H.nana* (8), *Fasciola* (8), *Ascaris* (8), and Hookworms infected sera, respectively (Table 2).

Table 2: Specificity of rabbit anti-*S. haematobium* SWAP IgG-pAb against different parasitic antigens by indirect ELISA

Parasitic antigen	OD readings at 492 nm (m ± SD)
<i>S. haematobium</i>	0.98 ± 0.03
<i>H.nana</i>	0.13 ± 0.01
<i>Fasciola</i>	0.22 ± 0.01
<i>Ascaris</i>	0.16 ± 0.02
Hookworms	0.05 ± 0.01

OD= optical density, M= mean, SD= standard deviation.

Samples Collection And Processing:

Study Population:

In the present study, a number of 86 individuals were enrolled. They were classified into three groups;

Group A: *Schistosoma haematobium* infected group (n=34).

Group B: Other helminthic parasites infected groups (n=32). It included 8 patients infected with *H. nana*, 8 with *Fasciola*, 8 with *Ascaris* and 8 with *Hook worms*.

Group C: Healthy control group (n=20).

Group A consisted of 21 males (61.7%) and 13 females (38.3%). They ranged from 8-55

years old. The frequency distribution of the patients in the different age groups is demonstrated in Table (3). It shows that the highest percentages of *S. haematobium* infected patients were in the age group 21-30 years (25%) and the least percentages of patients were in the age group of 51-55 years (2.9%).

Table (3): Frequency/percentage distribution of age and sex within group A.

Age group (Years)	Males	Females	Total	Frequency (%)
8-10	4	1	5	14.7%
11-20	8	3	11	32.4%
21-30	9	3	12	35.3%
31-40	2	1	3	8.8%
41-50	1	1	2	5.9%
51-55	1	0	1	2.9%
Total	25	9	34	100%

OD= optical density, M= mean, SD= standard deviation

Ethical Considerations:

The study was conducted according to ethical standards for human experimentation. The ethics committee for Theodor Bilharz Research Institute (TBRI) approved the protocol and written consents were filled.

Urine Examination:

According to the parasitological

examination and the number of *schistosoma* egg count in urine samples of the 34 infected patients using Nuclepore Membrane Filtration Technique is shown in table (4). The table shows that 29 patients (67.4 %) had a mild infection, while 8 patients (18.6%) had a moderate infection and only 6 of the patients (14%) had a heavy infection.

Table (4) : Intensity of *Schistosoma* infection in relation to egg count in urine samples

Egg count (no. of eggs/10ml)	Males	females	Total	Frequency (%)
Light (1-10 eggs)	29	5	24	67.4%
Moderate (11-20 eggs)	8	1	7	18.6%
Heavy (>20 eggs)	6	0.0	6	14.0%
Total	43	6	37	100%

Stool Examination:

The result of Merthiolate-Iodine-Formaldehyde concentration technique (MIFc) method (Blagg *et al.*, 1955) in stool samples

of the 32 infected cases of group B is shown in Table (5). The table shows that 8 patients were infected with *H. nana*, 8 with *Fasciola*, 8 with *Ascaris* and 8 with *Hook worms*.

Table (5): Distribution of different infections of patients in group B (other parasites infected group) in stool samples

Other parasitic infections	Males	Females	Total
<i>H.nana</i>	5	3	8
<i>Fasciola</i>	6	2	8
<i>Ascaris</i>	7	1	8
Hookworms	5	3	8
Total	23	9	32

Biochemical Study:

Figures (2,3 and 4) demonstrated that the effect of *Schistosoma* infection on liver enzymes. Levels of ALT , AST and ALP in the serum of humans among schistosomiasis

group recorded a highly significant increase when compared to the Healthy control group. While the levels of ALT, AST and ALP in groups infected with other parasites recorded a non-significant increase.

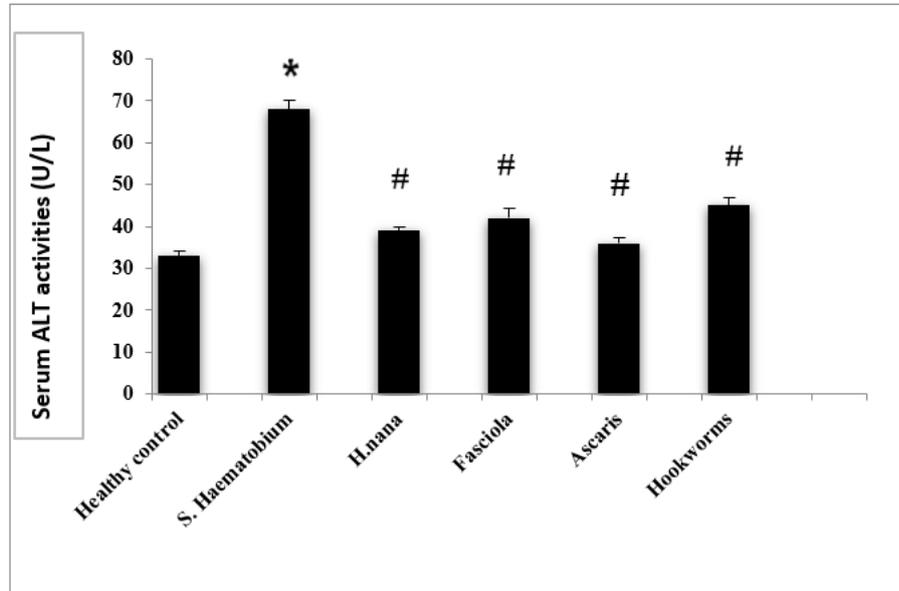


Fig. 2: Mean serum alanine amino transferase (U/L) activities in the control and various studied groups. Each bar with vertical line represents the mean \pm S.D. * Highly statistically significant ($P < 0.01$) # statistically insignificant ($P > 0.05$) when compared to the healthy control group.

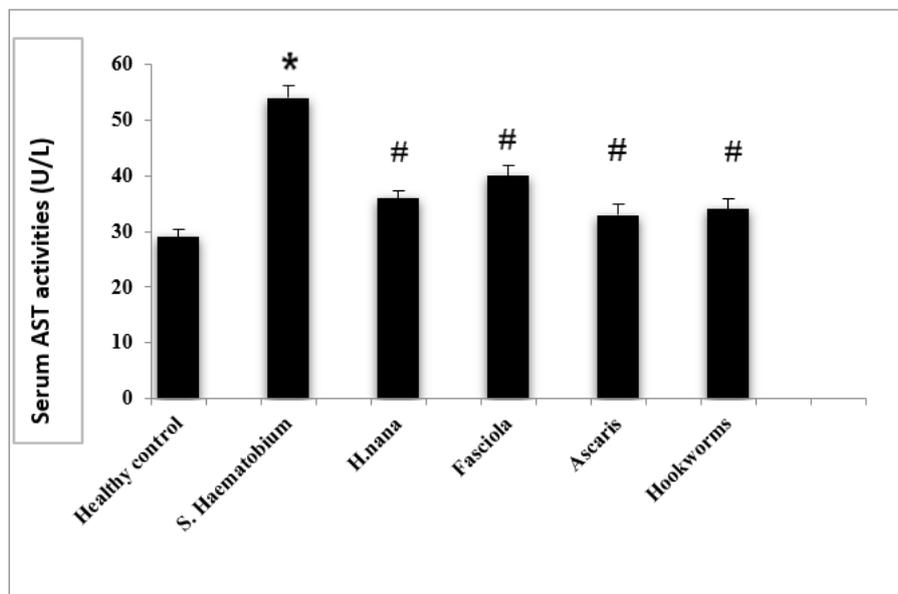


Fig. 3: Mean serum aspartate amino transferase (U/L) activities in the control and various studied groups. Each bar with vertical line represents the mean \pm S.D. * Highly statistically significant ($P < 0.01$) # statistically insignificant ($P > 0.05$) in comparison with the healthy control group.

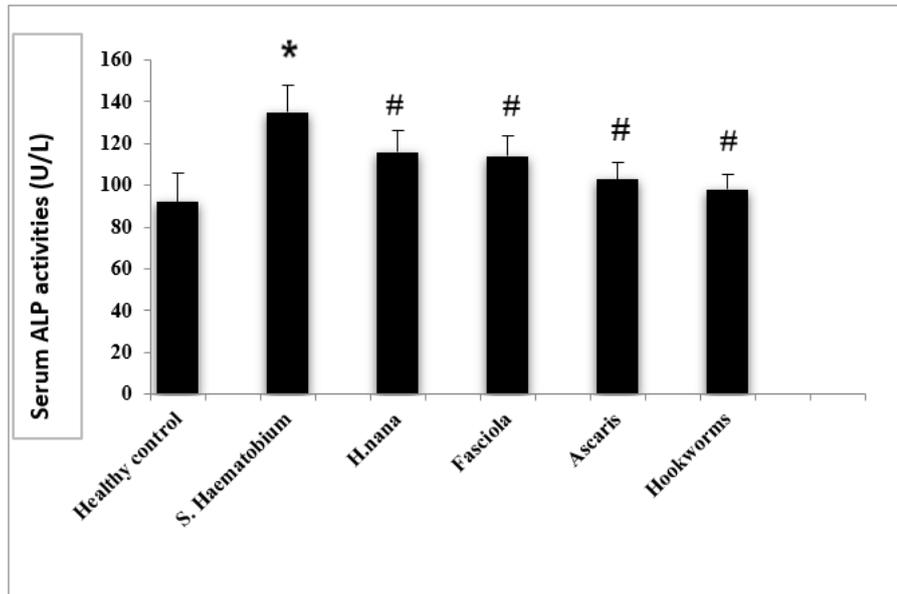


Fig. 4: Mean serum alkaline phosphatase (U/L) activities in the control and various studied groups. Each bar with vertical line represents the mean \pm S.D. * Highly statistically significant ($P < 0.01$) # statistically insignificant ($P > 0.05$) the healthy control group.

Urea levels in serum of schistosoma infected *Schistosoma* infection on creatinine level. human recorded a non-significant increase. Levels of creatinine in the serum of humans compared to the normal control group among schistosomiasis as well as other parasites recorded a non-infected groups recorded a non-significant significant increase compared to the normal increase in the level of creatinine in control group (Fig. 5). comparison with the healthy control group.

Figure 6. demonstrated the effect of

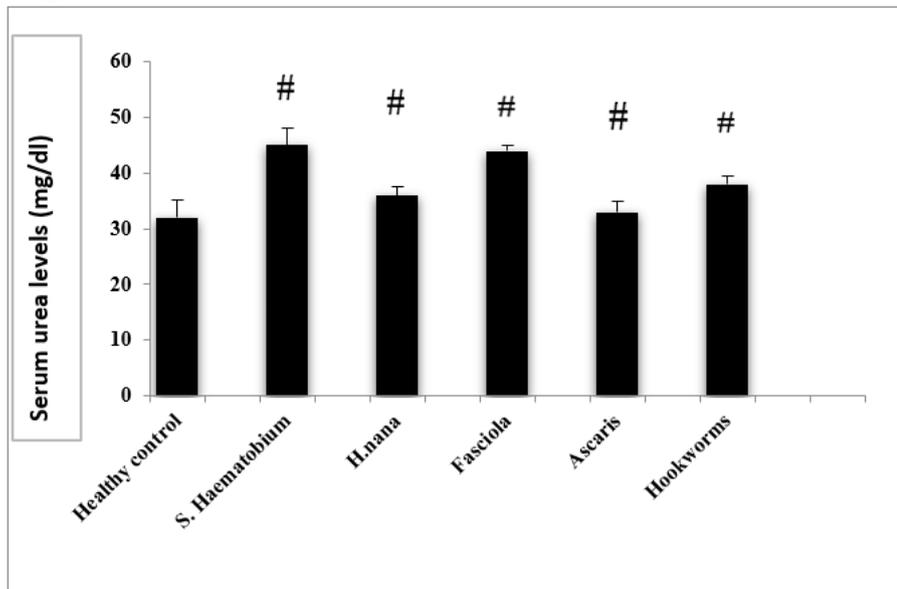


Fig. 5: Mean serum urea levels in the control and various studied groups. # statistically insignificant ($P > 0.05$) when compared to the healthy control group.

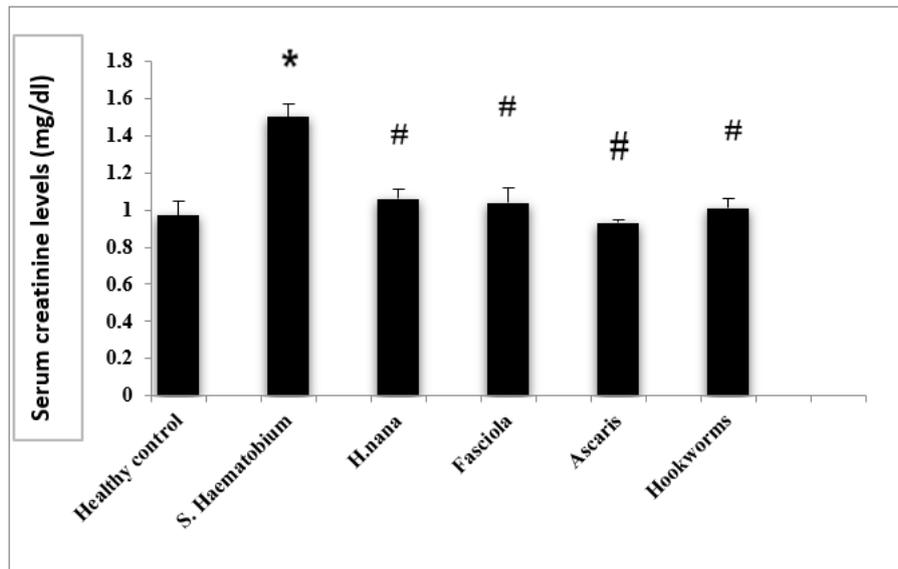


Fig. 6: Mean serum urea levels in the control and various studied groups. # statistically insignificant ($P > 0.05$) in comparison with the healthy control group

DISCUSSION

The gold standard procedure in the diagnosis of active schistosomiasis is the microscopic demonstration of eggs in urine and stool samples of patients. Detection of *S. haematobium* ova in the urine of infected individuals remains the leading routine method for direct diagnosis of the disease. However, a homogeneous distribution of *S. haematobium* ova in urine is difficult to achieve and due to many obstacles, it is not of valuable sensitivity (Corachan, 2002; Zinsou 2020).

To overcome these problems, several immunological tests have been developed for the diagnosis of schistosomiasis. Immunological tests using crude or purified egg and adult worm antigens have been developed in the last decades to detect anti-*S. haematobium* antibodies (Utzinger *et al.*, 2001; Rabello *et al.*, 2002). However, *Schistosoma* antibody detection often lacks specificity due to the persistence of the antibody-response after effective removal of the worms using chemotherapy. So, antibody detection cannot differentiate between active and past infection. Moreover, there is no correlation between antibody level and worm burden or morbidity (Bottieau *et al.*, 2006; Utzinger *et al.*, 2015). In addition they can

also cross-react with other helminthes giving false-positive results (Gryseels *et al.*, 2006; Utzinger *et al.*, 2015). By culturing *S. haematobium* viable ova in vitro, ES products were collected, purified and analyzed for their functional capabilities. *S. haematobium* viable ova were cultured in RPMI 1640 medium. The present study described the immuno-affinity purification of SWAP from ES products of *S. haematobium* viable ova by gel filtration ion chromatography on sephadex A-50 (DEAE column chromatography method) followed by gel filtration chromatography on sephacryl-S-200 HR column. The eluted proteins, from the gel filtration column chromatography analyzed by 12.5% SDS PAGE under reducing conditions. It showed only one band at 27.5 kDa which was SWAP. This study showed a high level of infection in 2 age groups; 11-20 and 21-30 years old with the highest percentage in the age group 11-20 years old (30%). This finding is in accordance with (Mansour and Voge, 1981; Abdel-Wahab *et al.*, 2000; Hajissa *et al.*, 2018) who stated that the peak prevalence of *S. haematobium* infections in Egypt occurs between the ages of 9-15 years old. A positive correlation was found between the number of *S. haematobium* eggs excreted in 10 ml urine

and the SWAP level detected in sera and urine of *S. haematobium* infected patients by sandwich ELISA. The percentage of positivity reached 100% in both serum and urine of the high infection subgroup. In the moderate infection subgroup, the incidence of positivity for SWAP antigen in both serum and urine was 85.7%. Whereas in the light infection subgroup; the incidence of positivity for SWAP antigen in serum was 80% and in urine was 76%.

In our study, schistosomal infection induced increased circulating levels of ALT, AST, and ALP. This comes in agreement with the result of (Aly and Mantawy, 2013; Al-Sayed *et al.*, 2014) who observed high serum enzyme levels of AST and ALT which is largely used during the assessment of liver damage by schistosomal infection (Aly and Mantawy, 2013; Al-Sayed *et al.*, 2014).

Necrosis or membrane damage releases the enzymes into circulation; therefore, they can be measured in the serum. In agreement with the reports of Kadry *et al.* (2013) and Mahmoud and Elbessoumy (2013), the increment of such enzymes in serum may be due to the destruction of hepatocytes by the action of toxins of the parasite eggs leading to their release into the circulation. In addition, Naik *et al.* (2011) reported that hepatocyte membrane damage seems to be the prime culprit for the marked increase in the serum marker enzymes, AST, ALT, and ALP following schistosoma.

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ARABIC SUMMARY

العلاقة بين مستويات أنزيمات الكبد والأنتيجينات السابحة في الأفراد المصابين بداء البلهارسيا البولية.

إبراهيم عبدالحميد السيد زهران¹ - سمير عطيه محمد زعقوق¹ - إبراهيم ربيع بيومي² - علاء عبدالعزيز سمن¹ - - سوسن عبدالمقصود الشامسي³

- 1- قسم علم الحيوان - كلية العلوم - جامعة الأزهر
- 2- . - قسم الطفيليات - معهد تيودور بلهارس للأبحاث
- 3- . - مركز العلوم الأساسية - جامعة مصر للعلوم والتكنولوجيا

تعتمد التقديرات الحالية لانتشار داء البلهارسيا على استخدام اختبارات تشخيصية راسخة ولكنها غير كاملة حيث تتم معالجة العينات بالطرق الطفيلية الروتينية ويتم الحكم على العلاج الطفيلي من خلال فحص مجموعة من الأطفال قبل وبعد علاج (PQZ). البرازقانتل في هذه الدراسة، من أجل تقييم المقاييس المختلفة لتشخيص المضادات المناعية واختيار المستضد السليم وتنقيته تليها نشر الأجسام المضادة المحددة وتنقيته كانت إلزامية. تم إجراء قياس وظائف الكبد ووظائف الكلى وتمت دراسة علاقتها بعدوى بلهاسيا المجاري البولية للأفراد في مختلف الأعمار والأجناس. وكان محتوى البروتين من المستخرج الخام للديدان الذائبة يساوي 2. SWAP ملغ / مل. وكان إجمالي محتوى البروتين 2.6x50 يساوي 13.0 ملغ كما تقاس بـ Bio-Rad البروتين المقاييس. أعطت عينات المصل من الإنسان المصاب ببلهارسيا المجاري البولية رد فعل قوي ضد أنتيجين الديدان الذائبة المخفف بفوسفات المتعادل 1/250 وكانت أعلى النسب المئوية للمرضى المصابين ببلهارسيا المجاري البولية في الفئة العمرية 21-30 سنة (25%) وكانت أقل النسب المئوية للمرضى في الفئة العمرية 51-55 سنة (2.9%). سجلت مستويات انزيمات الكبد للمرضى المصابين بمرض البلهارسيا زيادة كبيرة جدا مقارنة مع مجموعة التحكم الصحية.

