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Evaluation of Gamma Radiation-Attenuated *Schistosoma mansoni* Cercarial Antigens for Immunodiagnosis

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DEVELOPMENT of sensitive and specific diagnostic tools for early diagnosis and treatment has an important necessity. This study focuses on identifying and evaluating immunodiagnostic potential of irradiated *Schistosoma mansoni* cercarial antigens by immunoblot analysis using infected human sera. Forty two serum samples were divided into: 14 sera from *S. mansoni* infected patients (Group I), 18 sera of patients with other parasitic diseases (Group II) and 10 sera from healthy individuals (Group III). Characterization of normal and 0.4Gy gamma radiation-attenuated cercarial antigens (Cer. Ags) was conducted using Gel Electrophoresis (SDS- PAGE). Normal Cer. Ags was resolved into six individual bands of 205, ≈160, 97, 66, 43 and ≈38kDa while irradiated Cer. Ags. showed same bands except 66kDa, in addition to two more bands which were ≈50 and 24kDa. Immunoblot analysis of irradiated cercarial antigens using pooled sera of patients with schistosomiasis *mansoni* identified four bands two like normal one, 97kDa and ≈32kDa, in addition to ≈50kDa and 43kDa bands. Total IgG immunoblotting using irradiated cercarial antigens with individual sera of patients with schistosomiasis and other parasites revealed that higher diagnostic accuracy was achieved by both 97kDa and 24kDa with 78.6%. Both 97 and 43kDa bands gave a higher sensitivity of 64.3% while 24kDa bands achieved the highest specificity of 89.3%. It could be concluded that total immunoblotting using irradiated cercarial antigens at 97kDa and 24kDa proved to be valuable as diagnostic tests for human schistosomiasis.

Keywords: Cercarial antigen, Gamma radiation, Immunodiagnosis, *Schistosoma mansoni*.

Introduction

Schistosomiasis is a common infection that affects over 290 million individuals. Estimates show that at least 220.8 million people required preventive treatment in 2017 according to world Health Organization (WHO, 2019). Control programs may lead to a significant reduction of the prevalence and parasite burden in endemic population. Diagnosis by detection of specific antibodies is likely to be more sensitive than diagnosis by the traditional parasitological techniques which depend on parasite burden as those releasing low numbers of eggs that may not be detected (Lindholz et al., 2018; Oliveira et al., 2018).

However, antibody-based serological methods are generally unable to distinguish between current and past infections (Coelho et al., 2016) as imperfect techniques and cross reactions may result in false-positive results, hence, accurate diagnostic techniques are essential for prevalence determination and identification of positive cases

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(Fonseca et al., 2013; Van Dam et al., 2015).

It is not unusual to find common molecules between species of various parasites such as enzymes, hormones and receptors that have been conserved during evolution and is known as antigenic community. This sharing is able to elicit immune responses amongst different species of various genera and is responsible for antigenic cross-reactivity**.** To overcome this cross-reactivity, an immunoblotting for serodiagnosis which is dependent on using a high quality antigen has been applied (Hussien et al., 2004; El-Ossily et al., 2016).

Antigenic preparations have been used for establishment of reliable processes used for immunodiagosis or immunoprotection through vaccination. When *Schistosoma* larvae exposed to attenuating dose of radiation (gamma or UV) very large number of antigenic load will be delivered. As irradiation affects the peptide compounds of DNA of microorganisms, some surface antigen proteins shows significant differential expression in the parasite (Yang et al., 2009; Moawad et al., 2016).

The present study aims at characterizing and evaluating the antigenic immunodiagnostic potential of normal and gamma radiation-attenuated *S. mansoni* cercariae through using WB techniques.

Patients and Methods

Patients of the present study were selected from the outpatient clinics of Menoufiya University Hospitals and Mansoura University Hospitals, Egypt over the period 2013-2014. A total of forty two individuals were classified into three groups. Group I: included 14 patients who were positive for schistosomiasis as examined by three different stool samples on three consecutive days by Direct wet mount method (WHO, 1991), Formol ether sedimentation (Garcia, 2001) and Kato- Katz technique (Katz et al., 1972) and negative for other parasitic infections. Group II: included 18 patients negative for schistosomiasis (three separate samples collected in three different times) and positive for other parasitic infections; 3 fascioliasis, 5 amoebiasis, 7 toxoplasmosis and 3 hydatidosis. Group III: included 10 healthy individuals (parasitologically and serologically diagnosed).

Preparation of the serum

After obtaining oral consent, 5ml blood was ethically taken from each individual enrolled in this work, centrifuged at 1000xg for 5min then sera were withdrawn, aliquted in 0.5ml eppendorf tubes and stored at -80ºC until being used.

Preparation of the cercarial antigens

Cercariae of *S. mansoni* were purchased from Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI). From the cercarial suspension, 0.1ml was pipetted and placed drop by drop in small petri dish. A drop of 1% iodine was added to kill and stain the cercariae. Using a dissecting microscope, the average number of cercariae in the 0.1ml of suspension was determined by doing 3 counts and taking their mean (Moore et al., 1977). 6 x 10^3 (1000 cercariae per ml) of cercariae were used. Half of them were irradiated by exposure to 0.4gray gamma irradiation at a dose rate 2.5kGy/ hour at intensity of 400µw/cm² for one minute at the National Center for Radiation, Research and Technology (NCRRT), Atomic Energy Authority (Shi et al., 1990).

Extraction of the selected normal or irradiated cercarial antigens of *S. mansoni* was done through homogenization in extracting buffer (0.15ml PBS, pH 7.5, 0.02 % w/v NaN3 and 2ml phenyl-methyl sulphonyl fluoride, PMSF) and then were subjected, on ice, to three 20 (60) seconds bursts of sonication (Sanyo MSE, London, U.K., Soniprep 150). The destroyed cercariae were centrifuged at 18,000rpm for 1h at 4ºC (Abdel-Rahman et al., 2014). The supernatant was transferred into fresh tubes and were filtered using 0.45µm filter to get rid of the cell debris. The supernatants was assayed for its protein content, aliquoted and stored at -20°C until use.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE)

The components of resolving gel (4.9ml H_2O, 6ml 30% Acrylamide/bis-Acrylamide, 3.8ml 1.5M Tris (pH 8.8), 0.15ml 10% SDS, 0.15ml 10% ammomiium persulphate and 0.006ml TEMED) was mixed, poured and left for 45-60min at room temperature until being completely polymerized. Then stacking gel Tris-glycin SDS-PAG (5.5ml d H_2O, 1.0ml 30% Acrylamide/bis-Acrylamide, 0.75ml M Tris (pH 8.8), 0.06ml 10% SDS, 0.06ml 10% ammonium persulphate and 0.006ml TEMED) was prepared and loaded on the surface

of the resolving gel until reaching the upper border of the short plate. The clean comb was then placed to construct the wells. The gel was left for about 30-45min to be polymerized, then comb was removed. The gel cassettes were placed in the electrode assembly and put inside the tank filled with SDS-PAGE running buffer (3.03g Tris base, 14.4g Glycine and 1g SDS Complete to 1L with distilled H_2O). From each protein sample, 15µl was mixed with 5µl 5% mercaptoethanol loading buffer and boiled at 95°C for 5min to insure protein denaturation. Unstained protein molecular weight marker was applied to the first well and used as standard for SDS-PAGE; wide range (Jena-Bioscience). The power was applied to the cell and the electrophoresis was run using 120V constant until the front dye reaches the lower end. After the end of the gel electrophoresis, gels were immersed immediately in the Coomassie Blue solution (600mg Coomassie Blue R250 (Sigma), 260ml water, 240ml methanol and 100ml acetic acid) in a plastic box with a lid and left for 1h on a rocking platform at room temperature. The excess stain was removed and a de-staining solution (30% methanol, 10% acetic acid) was poured over the gel. The de-staining solution was changed every ten minutes for three times. Then the gel was kept in 50 % de-staining solution overnight on the rock platform in a tightly sealed box to prevent the gel from drying. The gel was examined for seeing the band of increasing intensity and then scanned (Laemmli, 1970).

Western blotting (WB)

A piece of nitro-cellulose paper (Bio-rad, 162-0097) was cut slightly larger than the gel and marked by pen on the surface that will face the gel. In a large plate contained transfer buffer the Gel Holder Cassette was opened and on its black cathode site, the sandwich was formed in the following order: one Fiber pad, filter paper, the SDS-PAGE gel, nitrocellulose membrane, the other filter paper and finally the other Fiber pad. Then the cassette was closed and transferred into the tank full of the transfer blotting buffer (2.9g Glycine, 5.8g Tris, 0.37g SDS, 200ml Methanol and Completed with d H_2O to one litter). A frozen Bio-Ice cooling unit was put alongside the gel holder to cool the system while running. The current was adjusted to 300 milliampire for 45-60min. After that, the membrane was removed from the cassette and transferred into the blocking buffer (5% BSA in PBS-T) for 30min at room temperature on a shaking platform. Then the membrane was washed three times with washing buffer (PBS-T), cut into strips of 4mm wide and placed in multichannel tray. Each strip was incubated with a human serum diluted at 1:200 in washing buffer for 2h at room temperature. Then the strips were washed three times with the washing buffer, and anti-human IgG horse–radish peroxidase conjugate (1:1000 dilution in washing buffer) (Sigma) was added and left for 1h at room temperature, then washed three times. AEC substrate reagent was prepared in test tube (by mixing 4 ml deionized water, 2 drops Acetate Buffer, 1 drop AEC Chromogen and 1 drop 3% hydrogen peroxide immediately before use) and incubated for 5-10min until clear red insoluble signal developed. Finally, the strips were washed in several changes of deionized water. In dark room, the membrane with its previously marked protein side was incubated with X-ray film (Kodak, Z35, 847-9) in the film cassette for 1-5min. The film was put in the developer solution (AGFA, G153A) and shaken until the signal appeared, then it was transferred to the fixer solution (AGFA, G153B). The film was put again on the membrane in the light to mark the bands of the pre-stained standard protein marker on the film. The film was washed with running tap water and left in the room temperature until getting dried (Towbin et al., 1979).

Statistical analysis

Statistical study was performed with Student's t-test using computer software package Graph Pad Prism 5.02 version to compare groups and using one way ANOVA using statically package for social science version 15 (SPSS) computer programs (2002). The diagnostic parameters were calculated as follows:

Sensitivity= (a÷(a+c))* 100, Specificity= (d÷(b+d))* 100, Positive predictive value (PPV)= (a÷(a+d))* 100, Negative predictive value (NPV)= (d÷(c+d))* 100, Diagnostic accuracy= (a+d) ÷ (a+b+c+d)* 100

where (a) is true positive, (b) is false positive, (c) is false negative, (d) is true negative (Gonzalez-sapienza et al., 2000).

Ethical approval

All procedures were performed according to the standards guidelines for researches and after taking an approved experimental protocol regarding human objects-patients by Research

Ethics Committee of the National Center for Radiation Research and Technology (REC-NCRRT) (Authorization number 4H/18).

Results

SDS-PAGE profile

Protein analysis of normal and irradiated Cer. Ags is demonstrated in Fig. 1. Both antigens were resolved under reducing conditions and the gel was stained with comassie stain. It was found that the normal Cer. Ags revealed the presence of six polypeptides ranging from 205 to 38kDa. The major individual bands were 205, ≈160, 97, 66, 43 and ≈ 38kDa (Lane A). All these proteins were shared with irradiated Cer. Ags except 66 in addition to two more bands (≈ 50 and 24kDa Lane B).

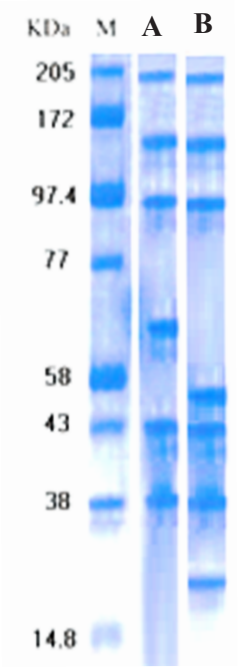

Fig. 1. The electrophoretic profile of the irradiated Cer.Ag and normal Cer.Ag, Irradiated Cer. Ag [M: Marker; Lane A: Normal cercarial antigens and Lane B: Irradiated cercarial antigens].

Immunoblotting (Western blot)

S. mansoni-infected patient sera were tested using the fractionated normal and irradiated Cer. Ags. Two antigenic bands were identified by the serum from the normal antigen; 97kDa and ≈ 32kDa. While four bands (two of them like normal one; in addition to ≈ 50 and 43kDa), were identified from irradiated antigens (Fig. 2).

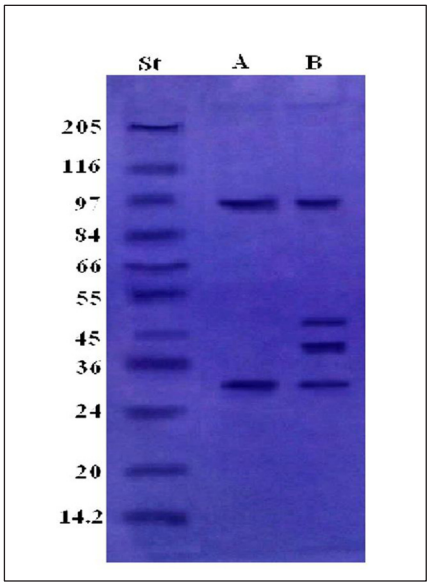

Fig. 2. Immunoblot analysis of normal and irradiated cercarial antigen using pooled sera of patients with *schistosomiasis mansoni* [St: Molecular weight standards, Lane A: Normal Cerc. Ag and Lane B: Irradiated Cer. Ag].

Polypeptides separated from irradiated Cer. Ags. were electrotransferred and immunoblotted with *S. mansoni* positive sera. It was found that 97k and 43kDa reacted with nine *S. mansoni* infected sera giving sensitivity of 64.3% while 66kDa reacted with only four sera giving lowest sensitivity of 28.6 %. There was a band which was not recognized by the pooled serum, a 24kDa, reacted with eight *S. mansoni* infected sera with sensitivity of 57.2% (Fig. 3 and Table 1).

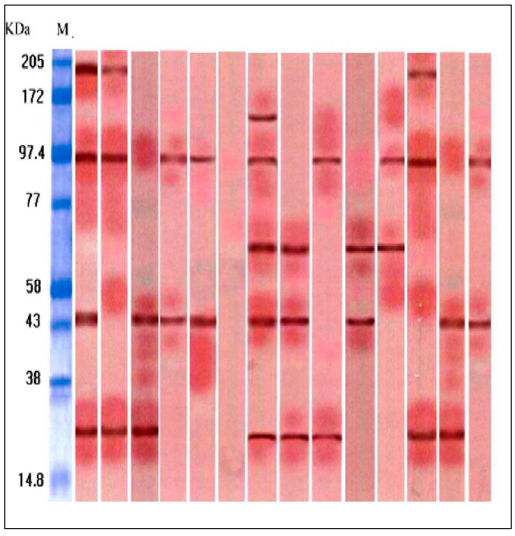

Fig. 3. Immunoblot analysis of irradiated cercarial antigen using total IgG with individual sera of patients with schistosomiasis *mansoni*

TABLE 1. Positive different molecular weight (MW) bands recognized by immunoblot analysis of irradiated cercariae against different sera.

Antigenic bands	Schistosomiasis (n= 14)		Fascioliasis (n= 3)		Toxoplasmosis (n= 7)		Hydatidosis (n= 3)		Amoebiasis (n= 5)		Controls (n= 10)	
	+Ve	%	+Ve	%	+Ve	%	+Ve	%	+Ve	%	+Ve	%
97kDa	9	64.3	1	33.33	1	14.3	1	33.33	1	20	0	0
66kDa	4	28.6	1	33.33	2	28.57	1	33.33	0	0	0	0
43kDa	9	64.3	1	33.33	2	28.37	2	66.66	1	20	0	0
24kDa	8	57.2	1	33.33	0	0	1	33.33	1	20	0	0

Immunoblotting of irradiated Cer. Ags. with sera infected with parasites other than schistosoma is shown in Fig. 4, Table 1. The 24kDa band reacted only with 3 sera fascioliasis, amoebiasis and hydatidosis. The 43kDa band was recognized by the highest number of parasitic sera (6 sera) that were one fascioliasis, two toxoplasmosis, two hydatidosis and one amoebiasis sera. The 66kDa reacted with one fascioliasis, two toxoplasmosis and one hydatidosis sera while 97kDa was identified by only one fascioliasis, one toxoplasmosis, one hydatidosis and one amoebiasis sera. None of these bands reacted with any of the negative control sera (Fig. 5).

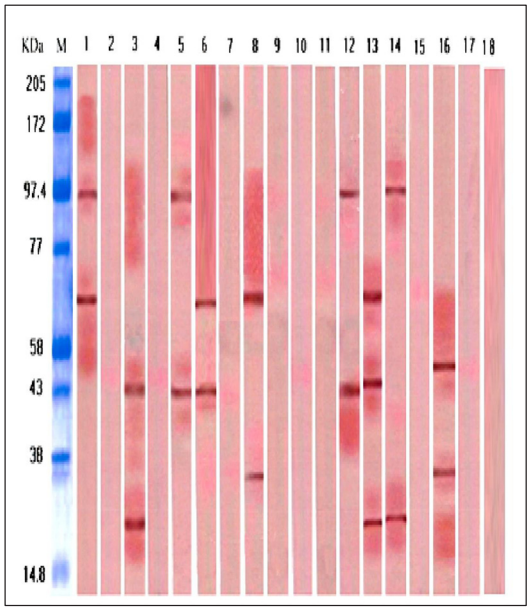

Fig. 4. Immunoblot analysis of irradiated cercarial antigens using total IgG with sera patients with other parasites than schistosomiasis (1-3= facioliasis, 4-10= toxoplasmosis, 11-13= hydatidosis, 14-18= amoebiasis.

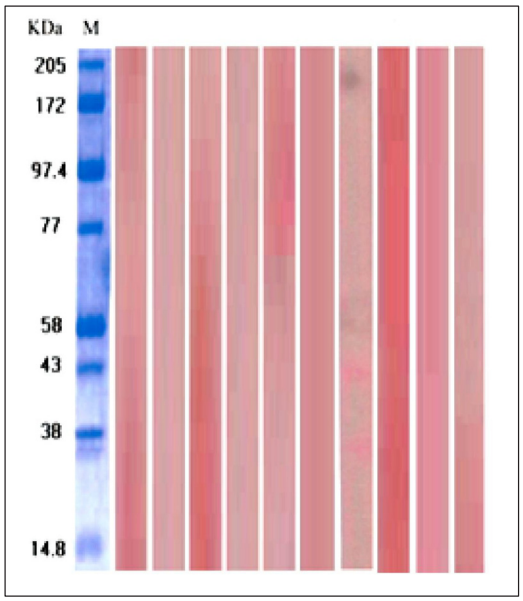

Fig. 5. Immunoblot analysis of irradiated cercarial antigens using total IgG with sera of negative control individual.

Analyzing most significant molecular weight bands of irradiated Cer. Ags. were detected as shown in Table 2. The 24kDa band achieved the highest specificity with 89.3%, while 43kDa revealed the lowest specificity (78.7%). The other two bands showed the same specificity (85.7 %). Also, the 24kDa revealed the highest PPV (72.7%) followed with 97kDa (69.2%), then 43kDa which achieved 60%, while 66kDa recorded the least PPV (50 %). Highest NPV was achieved by 97kDa (82.7%) followed by 43kDa (81.5%) then 24kDa achieved 80.6%, while 66kDa recorded the least NPV (70.6 %). Regarding the highest diagnostic accuracy, it was achieved by both 97kDa and 24kDa (78.6%), where 43kDa revealed diagnostic accuracy of 73.8% and 66kDa showed the lowest accuracy with 66.6%.

TABLE 2. Analysis of the most significant molecular weight bands in western blot of irradiated Cer. Ags.

Antigenic bands	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
97kDa	64.3	85.7	69.2	82.7	78.6
66kDa	28.6	85.7	50	70.6	66.7
43kDa	64.3	78.7	60	81.5	73.8
24kDa	57.2	89.3	72.7	80.6	78.6

Sensitivity= (a÷(a+c))* 100, Specificity= (d÷(b+d)) * 100, Positive predictive value (PPV)= (a÷(a+d)) * 100, Negative predictive value (NPV)= (d÷(c+d)) * 100, Diagnostic accuracy= (a+d) ÷ (a+b+c+d) * 100, where (a) is true positive, (b) is false positive, (c) is false negative, (d) is true negative.

Discussion

Accurate diagnostic tools for schistosomiasis are crucial for epidemiological surveys, improved patient management, and evaluation of community-based intervention studies. In areas of rebounding infections or after effective control measures the intensity of infection will be low and therefore likely to be missed by traditional microscopic techniques, so highly sensitive and specific diagnostic tools are required for effectively assessing the impact of control (Coulibaly et al., 2016; Utzinger et al., 2015).

As for the aim of the study to characterize the antigenic epitopes of both normal and irradiated cercarial antigens, SDS-PAGE revealed that the normal Cer. Ags resolved into six individual bands ranged from 205 and ≈ 38kDa while irradiated Cer. Ags showed the same bands are similar to the normal one except band 66, in addition to two more bands which were ≈ 50 and 24kDa. This coincides with the study of Sulahian et al. (2005) that revealed that WB profiles allowed the identification of six well-defined bands their masses ranged from 65 to 120kDa. Analysis *S. mansoni* Adult Worm Antigen (AWA) revealed the presence of nine polypeptides their MW ranged from 206 to 15kDa. Soluble egg antigens (SEA) revealed the presence of seven visible protein bands their MW ranged from 74 to 15kDa (EL-Ossilyet al., 2016).

To test the diagnostic potentiality, pooling of each antigen with *Schistoma mansoni* serum used in Western blot (WB) identified sharing of two antigenic bands in addition to other two bands in irradiated Cer. Ags. This was in accordance with the study of Yang et al. (2009) who reported expression of different proteins from ultraviolet-attenuated cercariae of *Schistosoma japonicum* than the normal one. This can be explained that attenuation of parasite by radiation leads to expression of different surface protein antigen which could not be detected in the normal un-irradiated.

In the current study, irradiated Cer. Ags. revealed high sensitivity (64.3 %) at 97kDa and 43kDa. Previous studies found that sensitivity of WB analysis on using commercially available antigen strips was 89.5% while it was reported to be recommended bands ranged from 30kDa to 32kDa highly specific for serologic diagnosis of infection using adult worm antigen (Cesari et al., 2005; Pardo et al., 2004).

A 24kDa (not recognized by SDS–PAGE) showed a high specificity (89.3%) and PPV (72.7%) with less cross reaction with other parasitic infected sera. This can be explained in the light that following the exposure to irradiation either gamma or ultra-violet, antigens expression is modified quantitatively and qualitatively (Constant et al., 1990**).**

Conclusion

In conclusion, irradiated Cer. Ag. achieved a high diagnostic value and specificity due to low cross-reactivity with other parasites so it is an effective tool to make a correct diagnosis. Also, immunoblot assay using 24kDa or 97kDa are of great value as a diagnostic test for serodiagnosis of human schistosomiasis.

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Conflict of interest: The authors report no conflicts of interests.

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