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Comparative Immunomic Analysis of *Schistosoma japonicum* Soluble Egg Antigens Reacting with Patient Sera Before and after Praziquantel Treatment

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Abstract: Schistosomiasis ranks as the second most serious parasitic disease worldwide after malaria. More than 250 million people are infected with schistosomes in the tropics or subtropics. The treatment and control of schistosomiasis which is a major neglected tropical parasitic disease, depends almost exclusively on chemotherapy with Praziquantel (PZQ). Current serologic diagnostic assays have shown that schistosome specific antibodies in human serum may remain for at least 1 year after cure. Repeated administration of PZQ for a long time might induce drug resistance to the parasite which is a big challenge for strategizing for the prevention and control of schistosomiasis. As schistosome eggs represent the most pathogenic form causing the disease, it is essential to determine if and how the level of antibodies against schistosome Soluble Egg Antigens (SEA) is affected by PZQ treatment. In this study, researchers carried out an immunomic analysis to profile *Schistosoma japonicum* SEA reacting with pooled human serum samples of pre and post treatment with PZQ by two dimensional electrophoresis combined with Western blotting. A total of 67 protein spots that were serologically recognized by serum samples were successfully subjected to mass spectrometric analysis. Of them, 37 different characterized proteins were successfully identified. Furthermore, of 67 protein spots, the reactivity of 49 protein spots to sera was reduced 20 weeks after PZQ treatment whereas only 5 spots showed increases in the intensity of recognition by post treatment sera. The present study suggested that chemotherapy with PZQ mainly affects the intensity of serological recognition of *S. japonicum* SEA. The immunomic proteins that were identified may facilitate a better understanding of the egg induced pathogenesis of schistosomiasis and host-parasite interplay and may provide potential targets for the diagnosis and evaluation of treatment for the disease as well.

Key words: *Schistosoma japonicum*, immunomic analysis, soluble egg antigens, praziquantel, protein, China

INTRODUCTION

Schistosomiasis remains a major neglected tropical parasitic disease affecting >200 million people in 76 countries. About 120 million have symptoms and 20 million suffer severe illness (Chitsulo *et al*., 2000; Hotez *et al*., 2008). Currently due to the absence of vaccines, efficient vector controls and water sanitation, the treatment of schistosomiasis still depends almost exclusively on Praziquantel (PZQ) although several recent drug candidates have exhibited potential anti-schistosomal properties in the laboratory such as oxadiazoles (Sayed *et al*., 2008), cysteine protease inhibitor K11777 (Abdulla *et al*., 2007) and mefloquine (Keiser *et al*., 2009). PZQ was first synthesized in the 1970s (Seubert *et al*., 1977) and subsequently introduced for the treatment of schistosomiasis. Owing to its safety, broad anthelmintic spectrum and reasonable cost, >100 million people have been treated with PZQ in schistosomiasis endemic areas (Cioli, 2000). Because PZQ targets the adult worm but has only minor activity against the young developing stages (i.e., schistosomula)

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(Xiao *et al*., 1985, 1987), retreatment is recommended to kill the parasites (Renganathan and Cioli, 1998). However, its mechanism of action has yet to be fully elucidated (Greenberg, 2005).

Diagnosis as well as chemotherapy is central to the control of schistosomiasis. Although, stool examination is the gold standard for diagnosing or monitoring the response to treatment in schistosomiasis cases, it is labor intensive and time-consuming. In addition, the sensitivity of this method is poor in the endemic areas with low prevalence. Immunodiagnostic techniques are highly sensitive, easy to perform and are useful epidemiological tools for the screening of target populations in endemic areas. However, most immunodiagnostic assays currently applied in China have indicated that the anti-schistosome antibody level has remained high for at least 1 year after cure and in many cases much longer (Zhu, 2005) which leads to the problem that a positive sero-test may be found to be fecal egg negative. Repeated administration of PZQ for a long time might induce drug resistance to the parasite (Doenhoff *et al*., 2002; Doenhoff and Mattoccia, 2006) which is a big challenge for strategizing for the prevention and control of schistosomiasis. Therefore, it is critical to identify the immunogenic antigens that could distinguish the cured cases from those with an ongoing infection.

Schistosome eggs are the primary cause of pathogenesis in schistosomiasis and egg antigens play a critical role in regulating host immune responses to the helminth (Pearce and MacDonald, 2002). In addition, there is evidence that treatment with PZQ could lead to changes in the Soluble Egg Antigen (SEA)-induced immune responses (Martins-Leite *et al*., 2008) and variations in specific anti-egg antibody levels which could reflect the current antigen levels (Mutapi *et al*., 1998). Also, the average number of eggs produced by *Schistosoma japonicum* per day is much more than other schistosome species (Moore and Sandground, 1956). Hence, knowledge of the effects of PZQ on the level of antibodies against *S. japonicum* SEAs is essential for evaluating chemotherapy efficacy which may also be a useful complementary way to carry out surveillance of schistosomiasis in epidemic areas (Wang *et al*., 2008). Furthermore, such knowledge is able to facilitate an understanding of egg induced pathology during schistosomiasis.

Crude antigens are valuable for detecting general patterns of immune responses in infected populations. However, single antigens permit a better definition of humoral and cellular responses under investigation (Mutapi, 2001). The advent of the omics era has yielded an avalanche of helminth omics data that provide valuable resources for defining those immunogenic proteins and investigating the intriguing biology of helminth parasites (Ju *et al*., 2010). A traditional expression library screening method has its limitations in the detection of those proteins with post translation modifications but a combination of 2-Dimensional Electrophoresis (2DE) and Western Blotting (2D-WB) can circumvent this problem and cover a wider range of antigenic targets (Wilson *et al*., 2004). A number of *S. haematobium* worm antigenic proteins recognized by patient serum samples before and after curative PZQ treatment were identified by 2DE, Western blotting and Mass Spectrometry (MS) (Mutapi *et al*., 2005). The combination of proteomic and serological techniques was subsequently used to analyze the components of immunogenic proteins from the tegument and excretory-secretory products of adult *S. bovis* (Perez-Sanchez *et al*., 2006). More recently, several proteomic investigations have addressed schistosomal egg-associated proteins including SEA (Curwen *et al*., 2004), Egg Secreted Proteins (ESP) (Cass *et al*., 2007) and the soluble constituents in developed and undeveloped eggs (Mathieson and Wilson, 2010). In addition, the identification of schistosome specific proteins will also profit from comprehensive genome analysis of the blood flukes *S. japonicum* (Liu *et al*., 2009b) and *S. mansoni* (Berriman *et al*., 2009) such as an investigation of vaccine candidate immunomics screening with *S. mansoni* protein microarrays (Driguez *et al*., 2010). To date, there have been no reports determining the effect of treatment with PZQ on *S. japonicum* egg immnunogenic antigen profiles, however such investigations would be valuable for a better understanding of the egg induced pathogenesis of schistosomiasis and host parasite interplays, provide potential targets for diagnosis and evaluation of treatment for the disease as well.

To identify and characterize the immunogenic proteins of *S. japonicum*, researchers combined 2DE and Western blot analysis to determine the proteins of SEA that were recognized by pooled serum samples of patients with *S. japonicum* before and after PZQ treatment and researchers compared the difference in serological recognition between them.

MATERIALS AND METHODS

Ethics statement: Ethical clearance was granted by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai, P.R. China (Ref No.: 20100525-1). The objectives, procedures and potential risks were orally explained to all participants. A signed written consent

form was also obtained from each participant or their proxy. All experimental uses of animals for the studies described in this study were also approved by the Institutional Animal Care and Use Committee.

Serum samples: Human serum samples were obtained from the residents living in Miaoling village in Anhui province of China where *S. japonicum* is endemic. Diagnoses were confirmed by the improved Kato-Katz stool examination (Katz *et al*., 1972) in which two fresh stool samples collected from each subject on two consecutive days were used to prepare six slides, coupled with a hatching test (Kassim and Gibertson, 1976). Following the initial examinations, all infected residents who had never been treated for any helminth infection agreed to receive chemotherapy with PZQ at the standard dose of 40 mg kg^{-1} body weight. Serological samples were collected from the residents that were confirmed as egg negative 20 weeks after treatment. Of these, 10 residents that donated both pre and post treatment sera were selected for the study. The sex ratio (males vs. females) of these compliant participants (19-54 years old) was approximately 1.5. Sera from persons free of *S. japonicum* from non endemic areas were included as normal controls. Almost no cross reaction with *Clonorchis sinensis* and *Fasciola buski* were found in all the studied serum samples. The treatment was performed in the schistosomiasis non-transmission season to avoid re-infection (Table 1).

Preparation of soluble egg antigens: The Anhui strain of *S. japonicum*, maintained in *Oncomelania hupensis* snails at the National Institute of Parasitic Diseases (Shanghai, China) was used for the experiments. Schistosome eggs were collected from the livers of New Zealand white rabbits that had each been percutaneously exposed to 800~1000 *S. japonicum* cercariae 42~45 days earlier using a method to isolate schistosome eggs free of host cells and debris (Dalton *et al*., 1997). Eggs were ground in a mortar and then sonicated (Boros and Warren, 1970) 8 times for 5 sec in lysis buffer (9.5 M urea, 65 mM DTT, 4% CHAPS and 0.2% IPG buffer) containing protease inhibitors, followed by centrifugation for 45 min at 12,000 g and 4°C. The SEA were produced from the supernatant and protein concentrations were determined using the Bradford assay. The samples were then stored at -80°C until needed.

Table 1: The information of sera samples in Miaoling village

ID	Sex	Ages	EPG (pre-treatment)	EPG (post-treatment)
400801	Male	19	4	0
806402	Female	35	16	0
702401	Male	28	96	0
703501	Male	47	8	0
700401	Male	54	12	0
702502	Female	33	28	0
702501	Female	21	80	0
703405	Male	36	8	0
703401	Male	44	16	0
702701	Female	29	4	0

2-Dimensional Electrophoresis (2-DE): 2-dimensional separation gels with SEA were performed in parallel; 100 and 800 μg SEA were loaded onto analytical and preparative gels, respectively. Isoelectric Focusing (IEF) was performed in 13 cm IPG strips (Amersham) with a range of pH 3-pH 10. The IEF was accomplished using the Ettan IPGphor Isoelectric Focusing System (Amersham). The soluble antigens were mixed with 250 μL rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer and 18 mM DTT. After passive rehydration for 12 h, IEF was performed at a step voltage of 500 V for 1 h, 1000 V for 1 h and 8000 V for 5 h. Following IEF separation, the gel strips were equilibrated for 2×15 min in equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS and 30% glycerol. About 1% DTT was added to the first equilibration buffer and in the second equilibration buffer, DTT was replaced with 4% iodoacetamide. The 2nd-dimensional electrophoresis was performed on 12.5% SDS-polyacrylamide gels with the SE 600 System (Amersham) at 30 mA per gel for 40 min and then at 60 mA until the bromophenol blue reached the end of the gel. Each sample was repeated three times. The analytical gels were stained with silver as previously described (Hochstrasser *et al*., 1988a, b) and the preparative gels were stained with coomassie blue.

Immunoblotting: Proteins from the same two gels were electrotransferred synchronously to two hybond-C nitrocellulose membranes (Amersham Biosciences) using the Trans Blot cell (Bio-Rad) for 90 min at 0.22 A. Following the transfer, the membranes were stained with ponceau S solution (Ameresco) to check the transfer efficiency and then were incubated in blocking solution (1% BSA in 1 × PBS) overnight at room temperature. The two membranes were subjected to 3 separate 15 min washes with 0.05%-PBST after blocking. Then, one membrane was incubated at room temperature for 2 h in a pool of pre-treatment serum samples (diluted 1:100 in PBST) from ten randomly selected individuals as mentioned before. The other membrane was simultaneously treated in the same way with a pool of post-treatment serum samples (confirmed to be negative 20 weeks after PZQ treatment) from the same ten individuals as for pre-treatment (related demographics of these serum samples are in supplementary 4). After washing in PBST (3 × 15 min), HRP conjugated goat anti-human IgG (1: 2000 in 0.05% PBST, Sigma, USA) was

added. The membrane was incubated at room temperature for 1 h and then washed 3 times in 0.05% PBST. The membrane was developed using H_2O_2 and 3,3'-Diaminobenzidine (DAB) in PBS. A pool of ten samples of normal human sera served as a control and was treated in the same way. The gel electrophoresis and Western blotting were repeated twice for all samples for confirmation.

Image analysis and mass spectrometry: Images from the immunoblot patterns were obtained by scanning with a Bio-Rad GS710 scanner and image analysis was accomplished using Image Master Software (Amersham) to match the reacted spots. Predicted matches were also visually verified. Protein spots on the coomassie blue stained gel that matched those on the Western blots were excised from the preparative gels and then destained with 100 mM NH_4HCO_3 in 30% ACN. After removing the destaining buffer, the gel pieces were lyophilized and rehydrated in 30 μL of 50 mM NH_4HCO_3 containing 50 ng trypsin (sequencing grade; Promega, Madison, WI, USA). After overnight digestion at 37°C, the peptides were extracted three times with 0.1% TFA in 60% ACN. Extracts were pooled together and lyophilized. The resulting lyophilized tryptic peptides were kept at -80°C until MS analysis. A protein-free gel piece was treated as above and used as a control to identify autoproteolysis products derived from trypsin.

MS and MS/MS spectra were obtained using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster city) operating in a result dependent acquisition mode. Peptide mass maps were acquired in a positive ion reflector mode (20 kV accelerating voltage) with 1000 laser shots per spectrum. Monoisotopic peak masses were automatically determined within the mass range 800-4000 Da with a signal to noise ratio minimum set to 10 and a local noise window width of 250 m/z. Up to five of the most intense ions with a minimum signal to noise ratio of 50 were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. In the MS/MS positive ion mode, spectra were averaged, collision energy was 2 kV and the default calibration was set. Monoisotopic peak masses were automatically determined with a signal to noise ratio minimum set to 5 and a local noise window width of 250 m/z. The MS together with MS/MS spectra were searched against Schistosoma NCBI 2010-3-12 using the Software GPS Explorer 3.6 (Applied Biosystems) and MASCOT 2.1 (Matrix Science) with the following parameter settings: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine allowed as variable modification, peptide mass tolerance set to 100 ppm, fragment tolerance set to ±0.3 Da and the minimum ion score confidence interval for MS/MS data set to 95%.

RESULTS AND DISCUSSION

2-DE: In this study, researchers carried out 2-DE analysis of the proteins of SEAs as described before. The total proteins from SEA were separated on 2-DE gels with a pH gradient from pH 3-10 (Fig. 1). The 2-DE gels were stained with coomassie blue and >300 discrete spots were displayed. Most of them were located between pH 4 and 8. Each sample was subjected to triplicate runs.

Western blot analysis: To investigate whether the components of *S. japonicum* SEA could be recognized by the pooled human serum samples collected before and after PZQ treatment, Western blot assays were performed on the basis of the 2D gels in the range pH 3-10. Compared with the hybridized membrane of normal human sera (Fig. 2), researchers observed that >80 strong

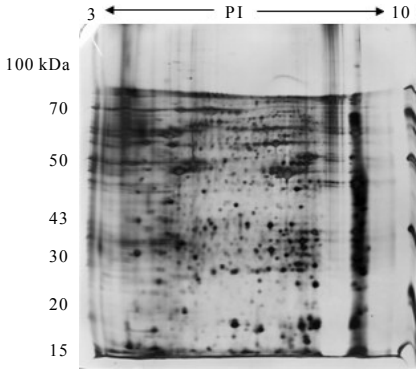

Fig. 1: 2-dimensional image of SEAs from *S. japonicum*. Coomassie blue stained gels with a range of pH 3-10. Molecular mass markers (kDa) are given on the left

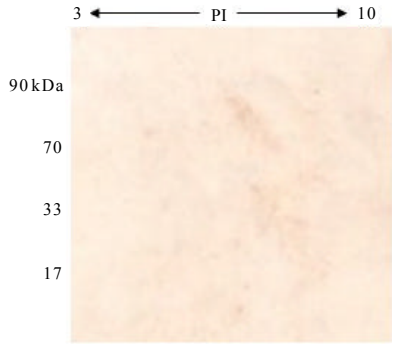

Fig. 2: Normal control for *S. japonicum* SEA. Molecular mass markers (in kDa) are given on the left

reactive spots displayed on the membranes hybridized with the pre treatment and post treatment human sera. There were differences between these two immunoblot patterns (Fig. 3A and B) while no significant immunoreactive spots were recognized by the normal human sera. Image analysis of 2D-WB showed that a total of 84 protein spots were recognized in the pre and post treatment sera.

MS/MS analysis: After matching the immunoblots with their homologous coomassie blue stained gels of SEA with Image Mater 2D gel software (Amersham), researchers localized the spots that corresponded to the antigenic proteins in the 2D gels. In total, 84 matched spots were excised from the coomassie blue stained gels, digested by typsin and were further analyzed by LC/MS-MS (Fig. 4). The MS/MS raw data were used to search *S. japonicum* and *S. mansoni* protein public databases with the SEQUEST search program. Finally, 67 spots were successfully identified. The identified spots with their summary protein scores, isoelectric points (pI) and molecular mass (kDa) are shown in Table 2. MS/MS analysis suggested that some different spots were derived from the same proteins. For example, spots 48, 49, 50, 51, 56 and 57 all referred to Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) as these spots corresponded to the same proteins but with different NCBI accession numbers.

For the reason above, the 67 identified protein spots were grouped into 37 different proteins by molecular functions which included chaperone proteins, motor proteins, catabolic activity, binding proteins and others (Table 3). Furthermore, 1 unknown protein and 3 hypothetical proteins without functional annotation are also shown in Table 3. The raw data of peptide mass flngerprints and secondary MS are shown in supplementary 1 and 2. The peptide data (sequence, ion score and C. I. %) are listed in the supplementary 3.

The effect of PZQ treatment on serological recognition: Comparative immunomic analysis of *S. japonicum* SEA recognized by the patient sera of pre and post treatment with PZQ was performed to investigate how chemotherapy altered responses to the antigens. The results suggested that treatment with PZQ mainly affected the intensity of serological recognition of *S. japonicum* SEA. The reactivity of most protein spots to sera was reduced by chemotherapy with PZQ after 20 weeks (Table 2). A total of 49 spots showed reduced reactivity. The most apparent spots included spots 25-27 (ATP synthase alpha subunit mitochondrial and 2 hypothetical proteins), 34-37 (branched chain amino acid aminotransferase, lactate dehydrogenase A and heterogeneous nuclear ribonucleoprotein A2 homolog 1) and 44-58 (actin, glyceraldehyde-3-phosphate dehydrogenase, troponin T, major egg antigen P40, phosphatase 2A inhibitor I2PP2A, tubulin beta 2, ester hydrolase C11orf 54 homolog and an unknown protein). However, only five spots (1, 3, 17, 18 and 19) showed increases in the intensity of recognition after treatment. They were the dentin sialophosphoprotein precursor, KH type splicing regulatory protein and dihydropyrimidinase like 2.

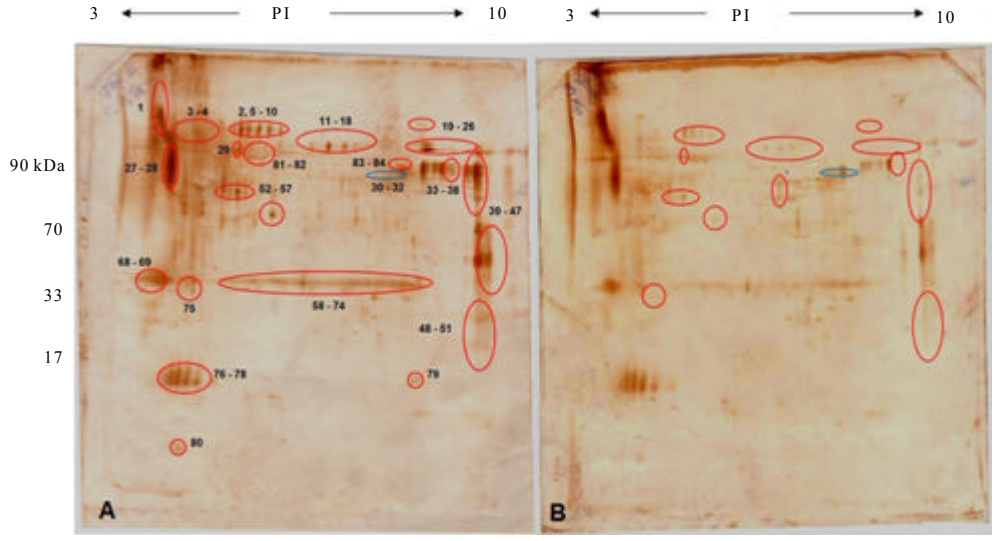

Fig. 3: Immunoblot patterns of *S. japonicum* SEAs recognized by serum samples. A: Spots reacting with pre treatment sera; B: Spots reacting with post treatment sera. Red circles represent the different areas between pre treatment and post treatment. Molecular mass markers (kDa) are given on the left

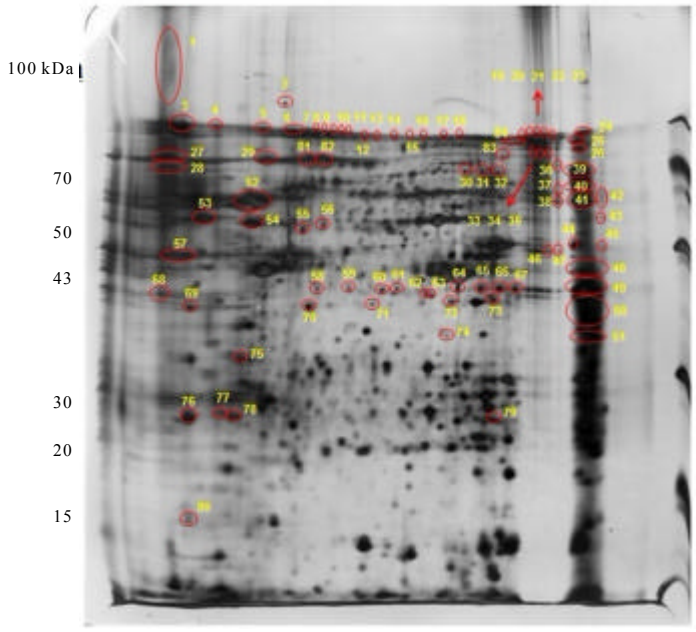

Fig. 4: Coomassie blue stained 2-D gel of *S. japonicum* SEAs showing spots matched to immunoblots. Molecular mass markers (kDa) are given on the left

Table 2: Identities of *S. japonicum* SEA antigenic proteins recognized by serum samples

Spot No.	Protein name	Species	NCBI Accession No.	Protein score	PI	Daltons	Trend
1	Dentin sialophosphoprotein precursor	*S. japonicum*	gi|226485495	116	4.13	51762.9	↑
2	Actin	*S. mansoni*	gi|256079407	95	5.30	41704.8	↓
3	Dentin sialophosphoprotein precursor	*S. japonicum*	gi|226480886	218	4.28	49969.7	↑
4	Endoplasmin	*S. mansoni*	gi|7673568	86	5.14	90445.1	↓
5	Myosin heavy chain	*S. mansoni*	gi|238665121	83	5.70	222168.2	↓
6	Rootletin (Ciliary rootlet coiled coil protein)	*S. mansoni*	gi|238661382	71	5.92	233729.8	↓
7	Heat shock protein 70 (HSP70)-4, putative	*S. mansoni*	gi|238663484	60	5.43	94404.3	↓
8	SJCHGC04997 protein	*S. japonicum*	gi|76155264	91	5.57	26838.0	↓
9	Tubulin tyrosine ligase related	*S. mansoni*	gi|238667196	62	9.33	156758.0	↓
10	Uracil DNA glycosylase, putative	*S. mansoni*	gi|238663347	58	6.84	34927.8	↓
11	Hypothetical protein	*S. japonicum*	gi|226468334	92	5.92	86311.6	↓
12	Aconitate hydratase	*S. mansoni*	gi|238662901	129	8.51	85529.7	↓
13	Arginine kinase	*S. japonicum*	gi|226481457	171	8.46	80133.0	↓
14	Heat shock protein 70 (HSP70), putative	*S. mansoni*	gi|238667420	431	5.36	68960.1	-
15	Phosphoenolpyruvate carboxykinase, putative	*S. mansoni*	gi|238657216	148	6.43	70345.2	-
16	HSP70	*S. japonicum*	gi|2829289	713	5.12	71442.2	↓
17	Putative KH-type splicing regulatory protein	*S. japonicum*	gi|257205944	247	6.73	54188.8	↑
18	KH-type splicing regulatory protein (FUSE binding protein 2)	*S. japonicum*	gi|226477992	108	7.08	56457.9	↑
19	Dihydropyrimidinase-like 2	*S. japonicum*	gi|226468250	128	7.54	63647.0	↑
20	Heat shock protein 70, putative	*S. mansoni*	gi|238667420	62	5.36	68960.1	-
21	Heat shock protein 70, putative	*S. mansoni*	gi|238667421	125	5.42	69785.6	-
22	Heat shock protein 70, putative	*S. japonicum*	gi|238667420	55	5.36	68960.1	-
23	Alpha tubulin, putative	*S. mansoni*	gi|238665538	98	4.92	48380.7	↓
24	ATP synthase alpha subunit mitochondrial, putative	*S. mansoni*	gi|238656902	104	9.16	59606.7	↓
25	Hypothetical protein	*S. japonicum*	gi|226471882	108	8.67	34871.8	↓
26	Hypothetical protein	*S. japonicum*	gi|257216372	180	8.67	55850.4	↓
27	ATP synthase alpha subunit mitochondrial, putative	*S. mansoni*	gi|238656902	207	9.16	59606.7	↓
28	Putative tubulin, beta, 2	*S. japonicum*	gi|226467269	145	4.79	49802.9	↓
29	Enolase	*S. japonicum*	gi|257123775	61	6.18	47221.0	↓
30	Phosphoglycerate kinase 1	*S. japonicum*	gi|226475588	272	6.76	44252.8	↓
31	Actin, putative	*S. mansoni*	gi|238661234	233	5.30	41706.8	↓
32	Actin, putative	*S. mansoni*	gi|238663895	159	5.30	35366.6	↓

Table 2: Continue

Spot No.	Protein name	Species	NCBI Accession No.	Protein score	PI	Daltons	Trend
33	Actin, putative	*S. japonicum*	gi|238661234	230	5.30	41706.8	↓
34	Branched-chain amino acid aminotransferase	*S. mansoni*	gi|238663016	145	8.56	42818.8	↓
35	Lactate dehydrogenase A	*S. japonicum*	gi|226471614	275	8.45	36103.9	↓
36	Heterogeneous nuclear ribonucleoprotein A2 homolog 1	*S. japonicum*	gi|226473314	163	8.63	33823.0	↓
37	Hypothetical protein RNA-binding region RNP-1	*S. japonicum*	gi|29841467	256	8.83	26766.6	↓
38	Alpha tubulin, putative	*S. mansoni*	gi|238665538	580	4.92	48380.7	↓
39	Calreticulin	*S. japonicum*	gi|226473210	187	4.59	45832.3	↓
40	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	*S. japonicum*	gi|226487050	885	5.21	55762.9	↓
41	Heat shock protein 60	*S. japonicum*	gi|257215736	337	6.68	50571.6	↓
42	26S protease regulatory subunit 6a, putative	*S. japonicum*	gi|238660820	333	5.61	41048.5	↓
43	Actin	*S. mansoni*	gi|256079407	399	5.30	41704.8	↓
44	Actin 5C	*S. japonicum*	gi|226472932	224	5.66	40099.1	↓
45	Troponin T	*S. japonicum*	gi|226478018	73	6.04	38401.4	↓
46	Major egg antigen (P40)	*S. japonicum*	gi|226475110	266	6.79	28337.1	↓
47	Major egg antigen (P40)	*S. japonicum*	gi|226477156	290	6.28	39155.4	↓
48	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|226477756	141	6.76	31964.2	↓
49	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|226477740	204	7.60	31991.2	↓
50	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|1916689	233	8.11	36551.6	↓
51	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|257209371	61	7.59	33347.0	↓
52	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|226477756	61	7.60	31964.2	↓
53	Phosphatase 2A inhibitor I2PP2A	*S. japonicum*	gi|226477576	142	5.05	18716.3	↓
54	Putative tubulin, beta, 2	*S. japonicum*	gi|226471718	394	5.29	37017.1	↓
55	Major egg antigen (p40)	*S. japonicum*	gi|226477150	286	6.40	39214.5	↓
56	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|226477756	141	6.76	31964.2	↓
57	Glyceraldehyde 3-phosphate dehydrogenase	*S. japonicum*	gi|226477740	204	7.60	31991.2	↓
58	Ester hydrolase C11orf54 homolog	*S. japonicum*	gi|226481407	174	7.19	21866.0	↓
59	Heat shock protein HSP60, putative	*S. mansoni*	gi|238657488	91	6.35	60932.7	-
60	Conserved hypothetical protein	*S. japonicum*	gi|226483787	105	4.36	20908.8	-
61	Putative tubulin, beta, 2	*S. japonicum*	gi|226471718	115	5.29	37017.1	-
62	Myosin regulatory light chain 2, smooth muscle minor isoform (G1)	*S. japonicum*	gi|226469350	123	5.23	20742.2	-
63	Hypothetical protein	*S. japonicum*	gi|226473034	291	8.56	24987.8	-
64	20 kDa calcium-binding protein (Antigen SM20)	*S. japonicum*	gi|226489228	122	4.34	17868.8	↓
65	Heat shock protein 70, putative	*S. mansoni*	gi|238667420	332	5.36	68960.1	-
66	Heat shock protein 70, putative	*S. mansoni*	gi|238667421	338	5.42	69785.6	-
67	Putative UDP-galactose-4-epimerase	*S. japonicum*	gi|226487124	63	7.55	72326.7	-

During the complex life stages of schistosomes, the egg is the key agent responsible for both the transmission and pathogenesis of schistosomiasis. Immunopathology in schistosomiasis is due to granulomatous inflammation around parasite eggs in the host liver and intestines which may result in scarring, portal hypertension, haemorrhage and death. The treatment and control of schistosomiasis relies virtually on a single drug, PZQ. However, chemotherapy with PZQ causes a series of problems for serological diagnosis of schistosomiasis (Pearce and MacDonald, 2002; Mutapi *et al*., 1998; Martins-Leite *et al*., 2008). Characterization of the SEA immunome may provide clues for potential diagnosis markers and for broader understanding of the egg's interaction with the mammalian host. In this study, a combination of 2-dimensional electrophoresis and Western blotting was employed to profile *S. japonicum* SEAs with pooled human serum of pre and post treatment by PZQ.

A total of 84 proteins spots recognized by human infection sera were subjected to MS analysis and 37 different characterized proteins were successfully identified, four of which have no functional annotation. Moreover of these, the reactivity of 49 protein spots (30 characterized proteins) to sera was reduced after 20 weeks by chemotherapy with PZQ whereas only five spots (3 different characterized proteins) showed increases in the intensity of recognition after treatment.

Of the 37 different immunogenic proteins identified, proteins related to catabolic activity were the most abundant, forming the largest functional category in SEA. This is probably because eggs are rich in carbohydrates and polysaccharide antigens and tend to stimulate an IgM response (Woolhouse and Hagan, 1999). Consequently, numerous enzymes might be involved in PZQ chemotherapy. For instance, one of the most abundant proteins with catabolic activity, glyceraldehyde-3 phosphate dehydrogenase (GAPDH; spots 48, 49, 50, 51,

Table 3: Summary of identified proteins, categorized by molecular function

Function category	Protein identity
Chaperone	Heat shock protein 60
	Heat shock protein 70
	Major egg antigen (P40)
Motor protein	Actin
	Tubulin
	Troponin T
	Myosin heavy chain
	Myosin regulatory light chain 2, smooth muscle minor isoform (G1)
Catabolic activity	Enolase
	Aconitate hydratase
	Arginine kinase
	Phosphoglycerate kinase 1
	Phosphoenolpyruvate carboxykinase, putative
	Glyceraldehyde 3-phosphate dehydrogenase
	Dihydropyrimidinase-like 2
	ATP synthase alpha subunit mitochondrial, putative
	Lactate dehydrogenase A
	Uracil-DNA glycosylase, putative
	Branched-chain amino acid aminotransferase
	Tubulin tyrosine ligase related
	Ester hydrolase C11orf 54 homolog
	Endoplasmin
	Putative UDP-galactose-4-epimerase
Bingding	Calreticulin
	20 kDa calcium-binding protein (Antigen SM20)
	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
	KH-type splicing regulatory protein (FUSE binding protein 2)
Other	Dentin sialophosphoprotein precursor
	Rootletin (Ciliary rootlet coiled-coil protein), putative
	Phosphatase 2A inhibitor I2PP2A
	26S protease regulatory subunit 6a, putative
	Heterogeneous nuclear ribonucleoprotein A2 homolog 1
	SJCHGC04997 protein
	SJCHGC03722 protein
	Hypotherical protein (spots 11, 25 and 26)

56 and 57) which was considered as a putative vaccine candidate against *S. japonicum* showed a correlation of antibody titer with resistance to reinfection (Waine *et al*., 1993; Argiro *et al*., 2000). Its abundant expression across developmental stages of *S. mansoni* (Curwen *et al*., 2004) indicated that GAPDH is associated with the growth and development of schistosomes. Furthermore, MS/MS analysis identified a glycolytic and gluconeogenic enzyme i.e., enolase which is also found in excretory/secretory proteins of schistosome adult worms with high expression (Liu *et al*., 2009a). Many studies have shown that enolase can be found on the surface of several eukaryotes and prokaryotes including schistosomes in which it acts as a plasminogen binding receptor (Ramajo-Hernandez *et al*., 2007; Seweryn *et al*., 2007). It is well known that energy acquisition by schistosomes relies mainly on the glycolytic pathway and enolase is a key enzyme in this pathway. Enolase exhibited only poor protection against *S. japonicum* schistosomulum infection (Yang *et al*., 2010) but assessment of its potential as a diagnostic target has yet to be reported.

The chaperone group such as Heat Shock Proteins (HSP) was another abundantly expressed group of immunogenic proteins in *S. japonicum* SEA. Both HSP60 and HSP70 were identified in this investigation. Serological evaluation of HSP60 demonstrated that the sensitivity of this antigen was >90% with ELISA. HSP70 is considered the predominant HSP family and plays a key regulatory role in parasite development and pathogenesis. A DNA vaccine of SjGST combined with SjMLP/HSP70 could induce a 31.31% reduction of the worm burden and a 58.59% reduction of the egg burden in the intestinal tissue of immunized mice (He *et al*., 2010). Another typical immunogen chaperone protein is a major egg antigen, P40. The pathogenesis of *S. mansoni* infection depends largely on host T-cell mediated immune responses such as the granulomatous response to tissue deposited eggs and subsequent fibrosis (Pearce and MacDonald, 2002). The major egg antigens have a valuable role in desensitizing the CD4+ Th cells that mediate granuloma formation which may prevent or ameliorate clinical signs of schistosomiasis (Abouel-Nour *et al*., 2006). There were significant differences after Smp40 stimulation in IL-5 and 10 levels compared with the control group. There is no information available on the changes in reactivity of this antigen following chemotherapy before this study. Among the recognized proteins, motor and binding proteins are the other two groups of immunogens. The former group might be involved in the formation and development of eggs. It is generally believed that Ca^{2+} channels are the molecular targets of PZQ action (Greenberg, 2005). Interestingly, the latter group comprises components of Ca^{2+} ion signaling pathways such as calreticulin and calcium binding protein. In addition, these immunogens also include some *S. japonicum* egg-specific proteins such as troponin T and their biological characterization needs to be further explored.

Researchers found that some relatively heavily stained gel spots failed to be recognized by serum samples which is consistent with the results for *S. haematobium* Soluble Worm Antigens (SWA) (Mutapi *et al*., 2005). Mature schistosomes have evolved highly with effective mechanisms for evading the consequences of the cellular and humoral immune responses that they provoke (Pearce and Sher, 1987; Pearce and MacDonald, 2002). Perhaps these unrecognized SEA components also play a vital role in host immune evasion or modulation and hence deserve attention and further evaluation.

Comparative analysis on the serological recognition of SEA revealed that chemotherapy with PZQ suppressed the reactivity of most of SEA spots to human sera. On the

contrary, PZQ could increase the intensity of recognition for some SWA spots after treatment (Mutapi *et al*., 2005). As a single drug for the treatment of schistosomiasis, it is well established that PZQ exerts different actions on mature or immature adult worms and eggs: PZQ kills adult worms (Andrews, 1985) and mature eggs (Richards *et al*., 1989) but does not significantly affect immature worms (Sabah *et al*., 1986). PZQ can damage the tegument of adult worms and exposes more parasite antigens to the host immune system compared with natural infection (Mutapi, 2001). The changes in serological recognition of SEA might be directly ascribed to the reduction of egg burden because of PZQ induced worm death. Moreover, although PZQ has no effect on *S. japonicum* eggs trapped in tissues, previous research showed that PZQ can restrain schistosomal ovum granulomas (Xu *et al*., 2007). Therefore, researchers presume that the decreases in serological recognition of SEA were probably related to the change of ovum granuloma after treatment. PZQ treatment resulted in marked changes in *S. haematobium* specific antibody responses directed against SEA which persisted from 18 weeks to at least 36 weeks following chemotherapy (Mutapi *et al*., 1998). This reminds us that extension of the sera samples collection time after chemotherapy may be able to reflect the dynamic changes of the immunogenic antigens researchers identified.

It is well known that antibodies of high affinity are more efficient than lower affinity antibodies in a wide range of biological reactions. Antibodies are traditionally considered to engage antigens in a mono specific bivalent manner which enhances apparent affinity by decreasing the rate of dissociation from the ligand. Heteroligation could improve antibody affinity when homotypic bivalent binding was not possible during the immune response to human immunodeficiency virus (Mouquet *et al*., 2010). Patients who developed AIDS either lost or failed to develop high affinity antibodies early in the infection. The antibody affinity values declined with time in some AIDS patients who remained asymptomatic (Chargelegue *et al*., 1995). Chemotherapy with PZQ caused changes in the amount of antigens directly into the bloodstream, thus stimulating different antigen presenting cells which resulted in different interactions between the antigens and the immune system when compared with natural infection (Mutapi, 2001). During this process, the alteration of SEA reactivity to sera after treatment may be associated with the change in the affinity of specific antibodies against SEA.

CONCLUSION

The present study suggested that chemotherapy with PZQ mainly affects the intensity of serological recognition of *S. japonicum* SEA. The immunomic proteins that were identified may facilitate a better understanding of the egg-induced pathogenesis of schistosomiasis and host-parasite interplay and may provide potential targets for the diagnosis and evaluation of treatment for the disease as well.

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