A comparative proteomics analysis of the egg secretions of three major schistosome species

Jack P. Carson[a], **Mark W. Robinson**[a], **Michael H. Hsieh**[b], **James Cody**[c], **Loc Le**[d], **Hong You**[e], **Donald P. McManus**[e], **Geoffrey N. Gobert**[a,*]

[a]School of Biological Sciences, Queen's University Belfast, Belfast, United Kingdom

[b]Division of Urology, Children's National Hospital, George Washington University School of Medicine and Health Sciences, Washington DC, USA

[c]Vigene Biosciences, Rockville, MD, USA

[d]A-TEK, Tysons, VA, USA

[e]Molecular Parasitology Laboratory, Immunology Department, QIMR Berghofer Medical Research Institute, Brisbane, Australia

Abstract

Morbidity associated with hepatic and urogenital schistosomiasis stems primarily from the host immune response directed against schistosome eggs. When eggs become entrapped in host tissues, the development of fibrotic plaques drives downstream pathology. These events occur due to the antigenic nature of egg excretory/secretory products (ESPs). Both *Schistosoma mansoni* and *S. japonicum* ESPs have been shown to interact with several cell populations in the host liver including hepatocytes, macrophages, and hepatic stellate cells, with both immunomodulatory and pathological consequences. Several protein components of the ESPs of *S. mansoni* and *S. japonicum* eggs have been characterised; however, studies into the collective contents of schistosome egg ESPs are lacking. Utilising shotgun mass spectrometry and an array of *in silico* analyses, we identified 266, 90 and 50 proteins within the *S. mansoni, S. japonicum* and *S. haematobium* egg secretomes respectively. We identified numerous proteins with already established immunomodulatory activities, vaccine candidates and vesicle markers. Relatively few common orthologues within the ESPs were identified by BLAST, indicating that the three egg

secretomes differ in content significantly. Having a clearer understanding of these components may lead to the identification of new proteins with uncharacterised immunomodulatory potential or pathological relevance. This will enhance our understanding of host-parasite interactions, particularly those occurring during chronic schistosomiasis, and pave the way towards novel therapeutics and vaccines.

Keywords

Proteomics; Excretory/secretory protein; *Schistosoma mansoni*; *Schistosoma japonicum*; *Schistosoma haematobium*; Egg

1. Introduction

Schistosomiasis is a disease caused by infection with parasitic blood flukes of the genus *Schistosoma*. It affects over 200 million people worldwide, primarily in sub-Saharan Africa, Asia and South America, and causes as many as 200,000 deaths per year [1]. Three species of schistosomes are primarily responsible for human cases; *Schistosoma mansoni*, *S. japonicum* and *S. haematobium* [1]. Human infection occurs when water-borne larval cercariae penetrate the skin of the mammalian host; following penetration, cercariae transform into immature schistosomula which enter the circulation and migrate towards the mesenteric veins of the intestines (*S. mansoni* and *S. japonicum*) or the venous plexus of the bladder (*S. haematobium*) [2]. During this migratory phase the schistosomula mature into dioecious adult worms which, upon reaching the terminal point of their migration, pair up to produce eggs which are then shed in the faeces or urine [2].

The egg burden produced by mature worm pairs varies significantly between the three schistosome species. Female *S. japonicum* worms are the most fecund, laying ~1,000–2,500 eggs per day per pair in an established murine infection, with 30–50 % being successfully shed in the faeces [3]. By comparison, *S. mansoni* females lay approximately 350 eggs per day in murine models, with 30 % being successfully shed [3], while *S. haematobium* females produce around 100–200 eggs per day in human infections [4]. Eggs that fail to shed become swept up in the circulation and deposited within the host tissues, either the liver sinusoids (*S. mansoni* and *S. japonicum*) [5], or the bladder, cervical, and vaginal wall mucosa (*S. haematobium*) [6].

The chronic pathology associated with hepatoenteric and urogenital schistosomiasis arises due to a shift from a moderate host type 1 helper (Th1)-mediated inflammatory immune response against the migrating schistosomes to a strong type 2 helper (Th2)-mediated granulomatous response against schistosome eggs [5]. This change occurs alongside the commencement of egg laying and is brought about by components of the soluble egg antigens (SEA) released by eggs acting on host immune cells to alter the balance of cytokines and chemokines towards a Th2 phenotype [5]. This shift is orchestrated to assist the migrating egg in successfully shedding from the host; by reducing local inflammation and acquiring a layer of host cells in the form of a granuloma, the eggs will encounter fewer obstacles to transmission and can better pass through the lamina propria and intestinal and pelvic organ walls [7]. The modulation away from a Th1-mediated response also ensures

host survival. Studies in murine models have shown that preventing the Th1-to-Th2 shift and enforcing a dominant Th1 response throughout the chronic stages of schistosomiasis leads to substantial host death due to enhanced egg-related immunopathology, including larger granuloma and increased inflammation in response to increasing egg burden [8]. Certain SEA components released by *S. mansoni* eggs have been shown to be hepatotoxic, causing cell death in hepatocytes [9], driving the formation of granuloma around the eggs. This last mechanism benefits the host by containing harmful antigens and protecting the liver tissue from necrosis. Eggs that become entrapped in host tissues will eventually be destroyed within the granuloma; however, the resolution of these granuloma following egg death leaves behind fibrotic plaques. These plaques accumulate throughout the course of chronic infection and eventually lead to impaired liver function and downstream schistosomiasis pathology.

Several components of schistosome egg ESPs have previously been characterised, and the most extensively characterised of these proteins include IPSE/α−1, omega-1, and major egg antigen P40. IPSE/α−1 is a hepatotoxic glycoprotein released by *S. mansoni* and *S. haematobium* eggs. In *S. mansoni* infections, IPSE/α−1 inhibits neutrophil migration to the site of the granuloma and assists in driving the host immune shift from a Th1 to a Th2 mediated response that follows egg laying [10,11]. Omega-1 is another hepatotoxic glycoprotein released by *S. mansoni* eggs that functions as a T2 ribonuclease (RNase). Omega-1 is internalised by dendritic cells (DCs), where it degrades DC ribosomal and messenger RNA (rRNA, mRNA) to reduce the expression of pro-Th1 mediators [12]. Ribonucleases released by *S. japonicum* eggs have similar functions [13,14]. P40 is a component of both *S. mansoni* and *S. japonicum* egg ESPs. Unlike IPSE/α−1 and omega-1, P40 has been linked with anti-fibrotic roles; *Sm*-P40 stimulation of murine lymphocytes induces interleukin (IL)-2 and interferon-gamma (IFN-γ) expression, both of which are considered Th1 cytokines, while *Sj*-P40 has been linked with decreasing hepatic stellate cell (HSC) activity and contribution to fibrosis by inducing apoptosis and senescence in these stellate cells [15–18]. Together, these proteins act to reduce local inflammation at the site of the granuloma and promote the regulated development of a fibrotic phenotype that assists egg survival and transmission.

Identifying the full range of (immunomodulatory) protein components that comprise schistosome egg ESPs is advantageous, as it could further advance knowledge of the host-parasite dynamic that is established during chronic schistosomiasis. The egg stage secretomes for *S. mansoni*, *S. japonicum* and *S. haematobium* have been reported previously [19–21]. However, this is the first comparative study that aims to identify common, or species-specific, antigens within the egg secretions of the three species in an effort to link these findings to differential host pathology. While most of these previously published secretomes used a gel-based MS approach, we have utilised shotgun proteomics and found differing ESP profiles in the 3 secretomes.

2.   Materials and methods

2.1.   Animal work

All animal ethics were approved by the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute (Brisbane, Australia) and the Biomedical Research Institute (BRI; Rockville, Maryland, United States of America).

Female four-week-old Swiss Webster mice were percutaneously infected with 150–200 *S. mansoni* cercariae (Puerto Rico strain) via tail exposure or with 20–30 *S. japonicum* cercariae (Chinese Anhui strain) via abdominal exposure for 45 min. Mice were sacrificed after 6–7 weeks post infection. Five to six-week-old, male and female Golden Syrian LVG hamsters were percutaneously infected with 350 *S. haematobium* cercariae (Egyptian strain) via abdominal exposure for 30 min. Hamsters were sacrificed after 14–16 weeks post infection.

2.2.   Egg isolation and culture

S. mansoni and *S. japonicum* eggs were isolated from perfused murine livers by repeated homogenisation and sieving of the liver tissue using a Waring blender and several sieves with pore sizes ranging from 420 μm to 45 μm. The homogenate was maintained in cold 1.2 % (w/v) NaCl throughout to prevent egg hatching. Mature eggs were enriched by gentle swirling in a petri dish, with the mature eggs collecting in the centre of the dish. These mature eggs were concentrated using a 40 μm cell strainer. *S. haematobium* eggs were obtained from perfused hamster livers and intestines using the same method.

Approximately 500,000 mature eggs of each schistosome species were cultured separately in 10 ml of RPMI-1640 media (ThermoFisher, Waltham, USA) supplemented with 300 units/mL penicillin, 300 μg/mL streptomycin and 500 μg/mL gentamycin antibiotics (ThermoFisher), in the absence of phenol red or any serum supplements. Eggs were maintained at 37 °C and 5% CO_2 for 72 h, after which the culture medium was collected, eggs pelleted and stored at −80 °C. Egg viability was assessed using an egg hatching assay [20] and preparations were only retained with 85 % minimum hatch rate. The egg culture media were then concentrated down using 3 kDa MW cut-off centrifugal filter units (Merck, Kenilworth, USA) to a final concentration of approximately 15 μg/mL each, as determined by the bicinchoninic acid (BCA) assay (ThermoFisher). Approximately 15 μg of total protein from each sample were submitted for MS/MS analysis.

2.3.   Mass spectrometry analysis

Three technical replicates of *S. mansoni*, *S. japonicum* and *S. haematobium* egg secretions were analysed by LC–MS/MS. Samples were digested with 100 ng/μl sequencing grade trypsin (Sigma-Aldrich, St. Louis, USA) at 37 °C overnight. The samples were then dried in a vacuum centrifuge and reconstituted with 10 μl of 0.1 % trifluoroacetic acid before analysis. Five μl of the resulting suspension were delivered to an analytical column (*S. mansoni*; Acquity UPLC Peptide BEH C18 nano column, 130A, 1.7 um, 75 umx200 mm, *S. japonicum/S. haematobium*; Eksigen C18-CL NanoLC Column, 3 μm; 75 μm x15 cm) equilibrated in 5% acetonitrile/0.1 % formic acid (FA). Elution was carried out with a linear

gradient of 5–35 % buffer B in buffer A for 30 min (buffer A: 0.1 % FA; buffer B: acetonitrile, 0.1 % FA) at a flow rate of 300 nl/min. Peptides were analysed in an Orbitrap Fusion mass spectrometer (ThermoFisher) (*S. mansoni*) or a nanoESI QqTOF mass spectrometer (5600 TripleTOF, ABSCIEX- Warrington, UK) (*S. japonicum/S. haematobium*) operating in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from was performed (*S. mansoni*; 380–1500 *m/z*, *S. japonicum/S. haematobium*; 350–1250 *m/z*), followed by 0.05-s product ion scans from 100 to 1500 *m/z* on the 50 most intense 2–5 charged ions. Peak list files were generated by Proteome discoverer v2.2 (ThermoFisher) (*S. mansoni*) or Protein Pilot v4.5 (Applied Biosystems, Foster City, USA) (*S. japonicum/S. haematobium*) using default parameters.

2.4. Database searching

MS/MS samples were analysed using Sequest HT on Proteome discoverer v2.2 (ThermoFisher) (*S. mansoni*) or MaxQuant v1.6.6 (*S. japonicum/S. haematobium*) set up to search databases comprised of proteins identified within the *S. mansoni*, *S. japonicum* and *S. haematobium* genomes (obtained from WormBase Parasite; *S. mansoni* WBPS10, accession PRJEA36577, 11774 entries; *S. japonicum* WBPS10, accession PRJEA34885, 12738 entries; *S. haematobium* WBPS10, accession PRJNA78265, 11140 entries) assuming strict trypsin digestion with 2 missed cleavages permitted. Sequest HT was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20.0 ppm. MaxQuant was searched with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethyl of cysteine was specified in MaxQuant as a fixed modification. Gln->pyro-Glu of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, dioxidation of methionine and acetyl of the N-terminus were specified in MaxQuant as variable modifications. Searches were also carried out against proteome databases of the host animals *Mus musculus* (Uniprot ID: UP000000589, 55,462 entries for *S. mansoni* and *S. japonicum*) and *Mesocricetus auratus* (Uniprot ID: UP000189706, 31,693 entries for *S. haematobium*) using the same settings. Detected host proteins that share resemblance with schistosome proteins are reported (Supplementary Tables 1–3).

2.5. Criteria for protein identification

Proteome discoverer v2.2 (ThermoFisher) (*S. mansoni*) or Scaffold (version Scaffold_4.8.9, Proteome Software Inc., Portland, USA) (*S. japonicum/S. haematobium*) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.

2.6. Bioinformatic analysis

Identified proteins were assigned functional annotation through Blast2GO [22] software against the entire non-redundant (nr) NCBI database using default settings, supplemented with additional annotation from Annotation Expander (ANNEX) and gene ontology (GO) terms obtained from InterPro databases. Gene ontology annotations were also obtained for all proteins recorded in the NCBI database for each of the three species. Comparisons were

made between secretome lists by utilising BLASTp (<u>B</u>asic <u>L</u>ocal <u>A</u>lignment <u>S</u>earch <u>T</u>ool protein); any protein that had an 80 % or greater sequence identity with a protein from another species was considered an orthologue of that protein (e-value 10^{-5}). Functional domains within selected proteins were identified using the InterPro database through UniProt. Alignments of these domains were carried out using BLASTp through NCBI using default settings. The SecretomeP v2.0 algorithm [23] was used to predict the method of secretion for each protein. Proteins that were assigned a neural network (NN) value >0.5 were assumed to be secreted. Secretory proteins that were predicted to possess a signal peptide were assumed to be secreted via classical pathways, while those which did not possess a signal peptide were assumed to be secreted via non-classical pathways. Those with a NN value   0.5 were assumed to be non-secretory.

3.   Results

3.1.   S. mansoni egg secretome

LC–MS/MS analysis identified 266 proteins within the *S. mansoni* egg secretome, including 36 uncharacterised proteins, and we confirmed the presence of IPSE/α−1 (IL-4-inducing protein), omega-1 and P40 (HSP20) (Table 1, Supplementary Table 1). Numerous additional proteins with immunomodulatory properties were noted, including glutathione S-transferase, phosphoenolpyruvate carboxykinase (*Sm*PEPCK), *Sm*VAL9, and peroxiredoxin (Prx1). Several other *S. mansoni* ESPs identified also share similarity with immunomodulatory proteins from other parasitic species, including calreticulin, released by *Haemonchus contortus* and *Necator americanus*, and serpin, produced by *Brugia malayi* [24–26].

We also observed the presence of several putative vaccine candidates, including *Sm*14FABP (fatty acid-binding protein) [27,28], peptidylglycine alpha hydroxylating mono-oxygenase (*Sm*PHM) [29], 14-3-3 protein [30], *Sm*21.7 [31,32] and triosephosphate isomerase [33]. Many ubiquitous protein components involved in energy production or metabolism (fructose-bisphosphate aldolase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase), DNA/RNA synthesis (uridine phosphorylase, hypoxanthine phosphoribosyltransferase), amino acid synthesis (ornithine-oxo-acid transaminase), nutrition (*Sm*22.6, cathepsins B and D, leucine aminopeptidase) and detoxification (superoxide dismutase, thioredoxin) were also identified. Furthermore, proteins such as phosphopyruvate hydratase (enolase), heat shock protein 70 (HSP70), elongation factor 1-α/γ [34–36], thimet oligopeptidase [35], ferritin, as well as several histones and structural proteins (actin, tubulin, filamin, spectrin-β), which are characteristic of vesicle components [37,38], were noted. The presence of these latter proteins suggests that a portion of the proteins identified are packaged and released within extracellular vesicles (EVs) in vivo.

We compared the 266 proteins identified in our study with those described by others in both the secretome of adult male *S. mansoni* worms and the previously published egg stage secretome. The adult male *S. mansoni* secretome has been previously reported to comprise 111 proteins [39], and of these we found 70 proteins that had   80 % sequence identity with our data set (Supplementary Table 1). This result suggests that there are significant similarities in ESPs released in these two life cycle stages. The previously published *S.*

mansoni egg secretome comprised 188 proteins [19], and of these we found 122 proteins sharing 80 % sequence identity with proteins in our own dataset (Supplementary Table 1).

3.2. S. japonicum egg secretome

Ninety *S. japonicum* proteins were identified within the egg secretome, including 28 uncharacterised proteins (Table 2, Supplementary Table 2). We confirmed the presence of P40, in addition to 3 ribonucleases (T2, Oy and X25) that share sequence identity with the *S. mansoni* protein omega-1. *Sj*E16.7 (16 kDa calcium-binding protein) and *Sj*97 (paramyosin) were also present. In addition, glutathione S-transferase, serpin and calreticulin, are three proteins previously shown to have immunoregulatory activity in multiple helminths (see Section 3.1 above).

Many ubiquitous protein components involved in energy production (fructose-bisphosphate aldolase, malate dehydrogenase, nucleoside diphosphate kinase), DNA synthesis (purine nucleoside phosphorylase, transaldolase), nutrition (aminopeptidase) and detoxification (superoxide dismutase, tryparedoxin peroxidase) were also identified. Again, we noted the presence of characteristic vesicle components including enolase, HSP70, elongation factor 1-α, histones, ferritin, and structural proteins (actin, tubulin) indicating that our data includes proteins packaged within EVs released from *S. japonicum* eggs.

The 90 newly identified proteins were then compared with previously described datasets of the secretome of adult *S. japonicum* worms and the previously published egg stage secretome. The adult *S. japonicum* secretome consists of 101 proteins [40], and of these only 27 proteins had 80 % sequence identity with proteins in our own dataset (Supplementary Table 2), suggesting that there are significantly different ESPs being produced between the two life cycle stages. It is noteworthy, however, that only 23 proteins from the previously published *S. japonicum* egg stage secretome, comprising 95 proteins [20], shared 80 % sequence identity with proteins in our dataset (Supplementary Table 2).

3.3. S. haematobium egg secretome

Fifty proteins were identified within the egg secretome of *S. haematobium*, including 7 uncharacterised proteins (Table 3, Supplementary Table 3). We noted the presence of *Sh*-IPSE/α−1, which shares sequence identity with *Sm*-IPSE/α−1. The components of *S. haematobium* egg ESPs are poorly characterised compared to those of *S. mansoni* or *S. japonicum*, which makes assigning their potential functions problematical. However, we did confirm the presence of proteins such as *Sh*E16 (16 kDa calcium-binding protein) and neuroserpin, which are similar to other proteins which have documented immunoregulatory roles in both *S. japonicum* ESPs, and in other helminths as considered above.

Several ubiquitous proteins involved in energy production (fructose-bisphosphate aldolase, alpha-galactosidase), DNA synthesis (purine nucleoside phosphorylase) and detoxification (superoxide dismutase) were once again identified, as were several proteins associated with vesicle packaging, including enolase, 78 kDa glucose-regulated protein (HSP70), histones, structural components (actin, filamin, fibrocystin, spectrin) and ferritin. Taken together, this indicates that *S. haematobium* EV-related protein components are also included in this dataset.

The *S. haematobium* egg secretome has previously been described as comprising 138 proteins [21]. Despite the difference in the number of proteins, we compared the two datasets and found that, of the 50 proteins we identified, 23 shared 80 % sequence identity with proteins in the previously published dataset (Supplementary Table 3).

3.4. Comparison of the three egg secretomes

BLAST was used to compare the proteins making up the three egg secretomes according to sequence identity given the difficulty in comparing proteins from the different schistosome species (different naming conventions, accession numbers etc). Proteins that shared 80 % sequence identity with an e-value cut off of 10^{-5} were considered orthologues (Fig. 1, Tables 4 and 5). When several proteins matched against the same prospective orthologue, the match with the highest % sequence identity was accepted.

Twelve (12) proteins were common between *S. mansoni, S. japonicum* and *S. haematobium* egg ESPs. These included several proteins documented as vaccine candidates in one or more species (fatty acid-binding protein, fructose-bisphosphate aldolase), proteins generally associated with either vesicle markers or cargo proteins (histones H2B and H4, enolase, actin, HSP70), signalling (thioredoxin, calmodulin) and antioxidant (redoxin) proteins.

S. mansoni and *S. japonicum* egg ESPs shared 18 proteins in common, while *S. mansoni* and *S. haematobium* eggs had 15 proteins in common. Again, a large proportion of these proteins are either vaccine targets (superoxide dismutase), vesicle markers (ferritin, elongation factor 1-α, structural proteins) or various ubiquitous elements, particularly involved in energy production/metabolism and DNA/RNA synthesis (glucose-6-phosphate isomerase, alpha-galactosidase, malate dehydrogenase, nucleoside diphosphate kinase). It should be noted that *S. mansoni* eggs produce two isoforms of ferritin and protein disulphide-isomerase, both of which share very low sequence identity with their counterparts; hence both proteins are listed as orthologous with *S. japonicum* and *S. haematobium* proteins separately and not grouped together.

S. japonicum and *S. haematobium* eggs shared only 4 proteins in common: 2 histones, actin and polyubiquitin C. These are extremely ubiquitous proteins that are again likely released as part of vesicle content.

3.5. Assignment of Gene Ontology (GO) terms

Gene ontology (GO) terms were assigned to all the identified schistosome proteins according to their function (Molecular Function or MF) or role (Biological Process or BP), as seen in Table 6. GO terms were also assigned to all recorded proteins for each species available in the NCBI database (Table 6).

GO annotation revealed that most proteins within the schistosome egg secretomes are involved in either binding or catalytic activities (MF) and take part in mostly cellular or metabolic processes (BP). Thus, many of the proteins enriched are enzymes; energy-producing enzymes are particularly abundant, including fructose-bisphosphate aldolase, malate dehydrogenase and alpha-galactosidase. Enzymes involved in DNA/RNA synthesis (purine nucleoside phosphorylase, uridine phosphorylase), amino acid synthesis (ornithine-

oxo-acid transaminase) and various proteases (thimet oligopeptidase, aminopeptidase and calpain) were also identified. Antioxidant activity (MF) was consistently enriched across the three schistosome secretomes and appeared to be upregulated in the egg stage in comparison with the schisto-NCBI annotations. Potential antioxidant activity could be attributed to proteins such as superoxide dismutase, tryparedoxin peroxidase, glutathione S-transferase and various peroxidase enzymes. Conversely, transporter activity (MF) and localization (BP) appeared to be downregulated in comparison with schisto-NCBI annotations.

3.6. SecretomeP analysis for signal peptides

Secretory proteins generally contain an N-terminal signal peptide sequence, unless they are secreted by non-classical means such as being packaged within vesicles. Given that we expected the proteins described here to be secretory in nature due to their presence in ESP, the algorithm SecretomeP v2.0 [23] was used to analyse the protein sequences and to determine the likelihood of classical (involving a signal peptide) or non-classical (alternative pathways) secretion based on the detection of a signal peptide and a neural network (NN) value assigned by the algorithm.

Of the 266 proteins identified in *S. mansoni* ESP, 151 were assigned a NN value of >0.5 (56 %) and thus were assumed to be secretory (Supplementary Table 1). Forty-nine (49) of these proteins had predicted signal peptides (18 %), while 102 did not (38 %). One protein, titin, was too large for the SecretomeP algorithm to analyse; however, a select analysis of the N-terminal half of the protein indicated that it is not secreted. Of the 90 proteins identified in *S. japonicum* ESP, 56 were assigned a NN value of >0.5 (62 %) and therefore assumed to be secretory (Supplementary Table 2). Eighteen (18) of these proteins had predicted signal peptides (20 %), while 38 did not (42 %). Of the 50 proteins identified in *S. haematobium* ESP, 26 were assigned a NN value of >0.5 (52 %) and thus are assumed to be secretory (Supplementary Table 3). Eight of these proteins had predicted signal peptides (16 %), while 18 did not (36 %).

4.   Discussion

In 2007, Cass et al. published the first mass spectrometry-based proteomic analysis of a schistosome egg secretome, describing 188 *S. mansoni* egg ESPs [19]. Since then, mass spectrometry has been used in combination with one- (1-DE) and two-dimensional gel electrophoresis (2-DE), or OFFGEL electrophoresis, to further characterize *S. mansoni*, *S. japonicum* and *S. haematobium* egg secretomes. Efforts were made by Curwen et al. and Mathieson and Wilson to decipher the *S. mansoni* egg secretome as part of studies aimed at characterising the proteomes of multiple *S. mansoni* life cycle stages [41,42]. In both these latter studies, the number of proteins found was significantly lower than that of Cass et al.'s original description; both studies utilized a 2-DE/MS approach, but Curwen et al. identified only 32 unique proteins whereas Mathieson and Wilson described a mixture of isoforms and variants of only 4 distinct proteins. Mathieson and Wilson attributed the disparity to differences in the methods used to obtain the *S. mansoni* ESP, claiming that Cass's method of incubating eggs overnight in a cold salt solution likely resulted in the egg membrane rupturing, contaminating the ESP mixture with the cytosolic contents of dead or dying eggs.

However, differences in the detection method used (shotgun MS vs MS of 2D-PAGE protein spots) and the databases interrogated with MS/MS data could also, at least in part, explain the observed disparity.

The *S. japonicum* egg secretome of 95 proteins was recently described using a 1-DE/MS approach [20], while the *S. haematobium* egg secretome, investigated using OFFGEL/MS, comprises 138 proteins [21]. In contrast to most of the earlier studies, we analysed the schistosome egg ESPs directly in solution without prior separation. Our reasoning was that, with 1-DE or 2-DE/MS, very large molecular weight proteins may not enter the gel (and will be missed from the subsequent MS analysis), whereas very small proteins/peptides will be too small to be retained. Furthermore, MS analysis may also miss proteins expressed at low levels due to incomplete recovery of tryptic peptides following in-gel digestion. Accordingly, we detected 266, 90 and 50 proteins within the egg secretomes of *S. mansoni*, *S. japonicum* and *S. haematobium*, respectively. We consider these proteins are indeed reflective of the live egg secretome, as in vitro egg hatch assays showed that >85 % of our egg preparations were viable. Thus, we consider that the risk of ESP contamination with cytosolic proteins from dead or dying eggs was low.

Despite finding significantly more proteins in the *S. mansoni* egg secretome compared to Cass et al.'s original work [19], we identified only 122 proteins that shared   80 % sequence identity between the two datasets. These discrepancies were anticipated, and we attribute them to the dated nature of the database used in the earlier study. Similarly, despite identifying a similar number of proteins in the *S. japonicum* egg secretome compared to De Marco Verissimo's study [20], we found only 23 proteins that shared   80 % sequence identity. This is likely due to a variety of reasons, including the method of mass spectrometry utilised and the precise settings applied to the analysis. Differences in the protein database used for peptide searching would also largely impact the final protein list generated. The large discrepancy in the number of proteins we detected in *S. haematobium* ESP compared with that previously described is likely due to the difference in MS methods used. Sotillo et al. [21] used OFFGEL electrophoresis to separate crude *S. haematobium* ESP into 24 distinct fractions which were each subjected to MS analysis. This procedure is reported to be more sensitive than traditional proteomics approaches used for the analysis of helminth secretions [43].

4.1. Immunomodulation by the schistosome egg

As schistosome egg laying begins, the interactions that occur between host and parasite change rapidly. Juvenile and adult schistosomes adapt to hide, or otherwise avoid inciting a major response from the host immune system indefinitely [2]. Schistosome eggs have no or limited capacity for such evasion but instead rely on quickly escaping from the host and hatching to facilitate transmission to the intermediate snail host. Following egg laying, the host immune response undergoes a shift from a low level Th1 inflammatory response to an aggressive Th2 response [5]. This is considered to be brought about by immunomodulatory components within the crude egg ESP acting on host immune cells. This shift prevents host death due to systemic inflammatory shock [8], and creates conditions that promote egg survival, host exit and successful disease transmission [7].

To bring about this host immune shift, components within the crude egg ESP act on host cells to alter the balance of cytokine and chemokine expression. These modulations are promoted by inducing the release of pro-fibrotic mediators, while dampening the release of proinflammatory agents from host cells. This stratagem can be most clearly seen in *S. mansoni* egg ESP, as these secretions have undergone the most detailed functional characterisation. IPSE/α−1, omega-1 and major egg antigen P40 are among the most significant modulatory elements deployed by *S. mansoni* eggs [9,44]. IPSE/α−1 is a hepatotoxic glycoprotein that inhibits neutrophil migration to the site of a granuloma and induces basophils to degranulate and release IL-4 and IL-13. IL-4 is a known mediator of the Th2 response and IL-13 is a key component of fibrosis, facilitating collagen production [10,11]. Omega-1 is another hepatotoxic glycoprotein that functions as a T2 ribonuclease (RNase). Omega-1 is bound and internalised by dendritic cells (DCs) in which it utilises its RNase activity to degrade DC ribosomal and messenger RNA (rRNA, mRNA) to influence DC function. DCs exposed to omega-1 have been shown to express reduced levels of IL-12, a chemokine involved with developing Th1 cells [12]. In contrast, P40 has been linked with anti-fibrotic and regulatory roles. P40 stimulation of murine lymphocytes induces IL-2 and IFN-γ expression, both of which are considered Th1 cytokines [18]. Taken together, these three proteins work in tandem towards driving the controlled and measured shift from a Th1 to a Th2 dominant host immune response.

Similar to *S. mansoni*, *S. japonicum* eggs secrete several ribonucleases (T2, Oy and X25) that share sequence identity with omega-1 (31 %, 52 % and 35 % respectively) and its own variant of P40 (66 % sequence identity with *Sm*-P40). In murine models, ribonuclease T2 has been shown to stimulate a Th2 host immune response by affecting DC maturation, inducing macrophages to produce anti-inflammatory IL-10 and upregulating serum levels of IL-4 [14], while *Sj*-P40 inhibits the progression of fibrosis by inhibiting HSC activation and promoting HSC apoptosis and senescence [15–17].

The ESPs of *S. haematobium* are not extensively characterised; however the eggs are known to secrete a variant of IPSE/α−1 [45] which shares 67 % sequence identity with the *Sm* counterpart, and although it is not known if it functions in a similar manner, it has been shown to be internalised by HTB-9 bladder cells, where it migrates to the nucleus, intimating a possible role in controlling host cell gene expression [45]. A significant clinical consequence of chronic infection with *S. haematobium* is the development of urogenital squamous cell carcinoma [6,46–48]. The role of secreted proteins released by *S. haematobium* eggs in carcinogenesis has not been clearly formulated. However, IPSE/α−1 is a major component of *S. haematobium* secretions and has been shown to induce cell proliferation and angiogenesis [21, 48], implying a role for this protein in urogenital carcinogenesis.

All of these proteins were found in our datasets, but schistosome egg ESPs contain additional immune-regulatory elements that may also be involved in assisting the driving, regulation, and maintenance of the dominant chronic Th2 host immune response.

In our *S. mansoni* protein dataset we identified several glutathione S-transferase (GST) enzymes. Members of the GST family found in *S. mansoni* have been shown to assist in

priming a host Th2 phenotype in murine models. Treatment with recombinant *Sm*GST28 induced the upregulation of the Th2 cytokines IL-4, IL-5 and IL-13, and reduced the expression of the Th1 cytokines TNF and IL-1β, thereby assisting the development of the Th2 host response [49]. We also found *Sm*PEPCK, a protein capable of inducing proliferation of murine CD4 + T cells and priming them to release significant levels of INF-γ, IL-4 and IL-5 to promote a mixed Th1/Th2 response [50,51]. Also detected was *Sm*VAL9, which modulates host extracellular matrix (ECM) remodelling in mice by altering the expression profile of various matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) genes. This ultimately promotes the degradation of host ECM, likely with the aim of assisting egg translocation into the intestinal lumen [52]. Furthermore, we also detected peroxiredoxin (Prx1), a protein which assists in priming a Th2-based immune response by inducing alternatively activated macrophages which, in turn, upregulate the expression of the Th-2 cytokines IL-4, IL-5 and IL-13 in naïve CD4+ cells [53]. Moreover, calreticulin and serpin were found which share similarity to known immunoregulators secreted by other helminths. Calreticulin, also released by *Haemonchus contortus* and *Necator americanus*, has been shown to inhibit the complement cascade by interacting with the complement protein 1q (C1q) [24,25]. Serpin, also produced by *Brugia malayi*, inhibits neutrophil-associated cathepsin G and elastase [26] to facilitate immune-evasion.

In our *S. japonicum* protein dataset we noted the presence of *Sj*E16.7, a potent neutrophil attractant and inducer of inflammation [54], and *Sj*97 (paramyosin), capable of inhibiting the complement pathway and binding to the Fc region of human immunoglobulins [55,56]. *Sj*E16.7 likely acts in tandem with *Sj*-P40 to balance and regulate the developing Th2 immune response, while *Sj*97 both promotes resistance to complement-mediated killing in immature and adult worms by binding complement-associated proteins, and also facilitates immune-evasion by providing the worm with a protective coating of host antigens.

We detected *Sh*E16 In the *S. haematobium* egg secretome. It is not known if *Sh*E16 possesses the same capacity to attract neutrophils and induce inflammation as its *S. japonicum* counterpart; however, both proteins do share strong (70 %) sequence identity. Unfortunately, the function of many of the components present in *S. haematobium* egg ESPs have not been thoroughly characterised; thus, we are unable to elaborate on the specific utility of many of these detected proteins. The only other potentially immunoregulatory protein we identified is neuroserpin, which shares sequence identity with both *S. mansoni* and *S. japonicum* serpins (79 % and 62 % respectively).

Our data indicate that *S. mansoni* egg ESPs contains a broader range of immune-regulatory elements than either *S. japonicum* or *S. haematobium*. Most of these proteins are involved in inciting a host Th2 immune response as part of the Th1 to Th2 shift that follows egg laying; however, several proteins that promote mixed or Th1 phenotypes (e.g. *Sm*PEPCK, *Sj*E16.7) were also identified. These proteins are likely responsible for modulating the intensity of the developing Th2 response, allowing the egg a degree of variable control over the host response. The relatively few immunomodulatory proteins detectable in *S. japonicum* could be considered a contributing factor to the marked disease severity this species causes compared with a *S. mansoni* infection [1]. Generally, this is attributed to the larger number

of eggs produced per day by a mature adult female *S. japonicum* worm, resulting in greater hepatic inflammation and fibrosis. However, the apparent lack of a diverse range of immunomodulatory proteins present in the *S. japonicum* ESP could indicate that the *S. japonicum* egg is unable to modulate the host immune system as effectively as that of an *S. mansoni* egg. This would result in a less efficient Th1 to Th2 shift and a more prolonged chronic inflammatory phenotype, partly resulting in more pronounced pathology.

4.2. Shared proteins between all three species

As mentioned above, given the difficulty of accurately comparing proteins across different species, BLAST was used to compare protein sequence identity. Proteins that showed 80 % identity were considered orthologues.

It was not expected that the egg-stage of the three schistosome species would share many pathologically relevant proteins in common. Unsurprisingly, only 12 proteins were found to be orthologous amongst all three species. Most of these proteins are associated with the release of EVs, either as structural components of the vesicle or as cargo, and included histones (H2B and H4), enolase, actin and HSP70 [37,38]. Several redox proteins (thioredoxin, redoxin peroxidases) were also found to be common. At some point during transmission, regardless of species, the schistosome egg will likely become encapsulated within a granuloma. At this time, the immune cells within the granuloma will release reactive oxygen species (ROS) to destroy the egg through oxidative stress. In response to this, schistosome eggs are known to secrete antioxidant proteins capable of scavenging ROS, thereby providing protection [57].

The high degree of commonality in the pathology induced by *S. mansoni* and *S. japonicum* eggs, and the relatively low overall number of proteins found in *S. haematobium* egg ESPs, is reflected in the patterns of assumed orthologues shared between the three species. *S. mansoni* and *S. japonicum* have the most proteins in common, with *S. japonicum* and *S. haematobium* the least. *S. mansoni* shares 18 proteins in common with *S. japonicum*, and 15 proteins in common with *S. haematobium*. Again, these proteins are primarily ubiquitous enzymes involved in energy production/metabolism and DNA/RNA synthesis (glucose-6-phosphate isomerase, nucleoside diphosphate kinase etc.) or are vesicle markers (ferritin, elongation factor 1-α, structural proteins). Conversely, *S. japonicum* and *S. haematobium* eggs share only 4 proteins in common, with 3 (histones H2A and H3, and actin) associated with vesicles.

To explore protein identity further, we examined the functional domains of some of the more extensively characterised proteins. *Sm*- and *Sh*-IPSE/α−1 share an interleukin-4 inducing immunoglobulin-binding domain (InterPro: IPR041305). Sequence alignment showed that this domain shares 69 % sequence identity between *Sm*- and *Sh*-IPSE/α−1, a level of identity similar to that shared by the full-length proteins (67 %). Omega-1 (*Sm*) and ribonuclease T2 (*Sj*) have 31 % sequence identity and are known to share similar activity [12,14]. Both proteins also contain a ribonuclease T2-like domain (InterPro: IPR001568), and alignment shows this domain also shares 31 % sequence identity between the two proteins. In contrast, *Sm*-P40 and *Sj*-P40 both contain two alpha crystallin/hsp20 domains (InterPro: IPR002068). While the full-length proteins share 66 % sequence identity, the first

sHSP domain in each protein is 81 % similar and the second is 74 % similar. These findings indicate that some egg ESPs may possess functional domains that are 80 % similar with those released by another schistosome species, despite a lower level of sequence identity across the whole protein which, according to our criteria, prevents their classification as orthologues.

4.3. Gene ontology analysis

The most highly enriched annotations for all three secretomes were binding and catalytic activity (MF), and cellular and metabolic processes (BP). This reflects the high proportion of ubiquitous protein elements involved in energy production, DNA regulation, amino acid synthesis, detoxification and other processes present in the egg secretomes. Antioxidant activity (MF) was also enriched and, in comparison with annotations from all schistosome proteins recorded in the NCBI database, was upregulated in each of the three schistosome egg ESPs. Several redox proteins were identified in the egg secretomes which highlights how the schistosome egg releases proteins to combat oxidative stress during prolonged periods under assault by the host immune system whilst trapped within the granuloma [57]. Conversely, transporter activity (MF) and localization (BP) appeared to be downregulated in the egg stage when compared with all schisto-NCBI annotations. This agrees with our finding that approximately 40 % of proteins across all three secretomes were predicted to be secreted non-classically, i.e. via membrane budding/vesicle cargo and would therefore not be associated with transporter proteins or localization sequences.

4.4. Identification of secretory proteins

Several methods of protein secretion are employed by a typical cell, with the two main classes being classical and non-classical secretion [58]. Classical secretion involves the packaging of a protein into membrane-bound vesicles and subsequent transport to the cell surface for export. In this case, an N-terminal signal peptide sequence marks the protein for such transport. Non-classical secretion involves the protein moving directly to the cell surface without the aid of a signal peptide sequence, where it is released via budding from, or fusing with, the membrane [58,59].

SecretomeP analysis revealed that approximately 20 % of proteins within each of the three schistosome egg secretomes are secreted via the classical pathway, while around 40 % are secreted non-classically and the remaining 40 % are not predicted to be secreted at all. One might expect, as we are investigating excretory/secretory proteins specifically, that it is unusual to have such a high proportion of proteins predicted to be non-secretory. However, the previously published *S. mansoni* and *S. japonicum* egg stage secretomes [19,20] also reported very similar ratios of classically, non-classically and non-secreted proteins. The likely explanation is that the SecretomeP algorithm is trained specifically on mammalian and bacterial protein sequences, and therefore some degree of misinterpretation would be expected.

Our finding that most proteins across the ESPs of all three schistosomes are secreted via non-classical means indicates that secretion of EVs is a major mechanism for release of proteins from the eggs. Indeed, proteins commonly associated with EVs [37,38], either

structurally or as cargo components, were detected within all three ESP lists, including enolase, histones and structural proteins (actin, filamin, tubulin etc.). The presence of these proteins within our data set suggests that it reflects both the soluble and EV-associated proteins present in schistosome ESPs. The release of proteins within EVs is of interest because the vesicle itself offers protection to its cargo proteins from proteolytic digestion and facilitates a simple method of targeted delivery to a recipient cell via internalisation. By contrast, classically secreted proteins are vulnerable to digestion and would therefore have a small local sphere of influence [60]. For parasite EVs this would be an appropriate method of delivering immunomodulatory proteins [61].

It is difficult to state emphatically whether the mode of secretion of the proteins is relevant to their function, but it must be considered that for a protein to function it must reach its target, and the non-classical method of secretion within EVs may provide the best chance of this occurring. The distance between source and target must also be considered; classically secreted proteins likely have a more significant effect within the immediate local environment of their release whereas proteins within EVs are more likely to engage with targets at a systemic level.

5. Conclusions

The commencement of egg laying during a schistosome infection marks a period of abrupt change to the host-parasite dynamic. Immature and adult worms need to evade the host immune system to ensure their survival, while the egg demonstrates an ability to manipulate it in a manner that promotes host/egg survival and increases the chance of successful egg transmission. This is brought about by immunomodulatory proteins secreted by the egg. Our findings indicate that, despite the similarities in host and pathogenicity, the egg secretomes of *S. mansoni, S. japonicum* and *S. haematobium* differ significantly and may be linked to parasite niche and the severity of host pathology. Relatively few proteins displayed sufficient similarity to be considered orthologues by our criteria, and many that did reflect ubiquitous protein elements with no clear role in host-parasite interaction. We confirmed the presence of several well characterised immunomodulatory proteins, but the function of many proteins within the three secretomes described here remains undefined. Further characterisation of these proteins is necessary to identify potential mediators of host-parasite interactions as they may provide new target candidates for vaccines against schistosomiasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

[1]. Lewis F, Karunaratne LB, Lewis FA, Freitas TC, Liang Y, Schistosomiasis, Curr. Protoc. Immunol (2001). Unit 19.1. Chapter 19.

[2]. Colley DG, Bustinduy AL, Secor WE, King CH, Human schistosomiasis, Lancet (Lond. Engl.) 383 (2014) 2253–2264.

[3]. Cheever EA, Macedonia JG, Mosimann JE, Cheever AW, Kinetics of Egg Production and Egg Excretion by *Schistosoma mansoni* and *S. japonicum* in Mice Infected with a Single Pair of Worms, Am. J. Trop. Med. Hyg 50 (1994) 281–295. [PubMed: 8147487]

[4]. Deelder AM, Krijger FW, Kremsner PG, Van Etten L, Day-to-Day variation of egg output and schistosome circulating antigens in urine of *Schistosoma haematobium*-Infected school children from Gabon and follow-up after chemotherapy, Am. J. Trop. Med. Hyg 57 (1997) 337–341. [PubMed: 9311646]

[5]. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn TA, Immunopathology of schistosomiasis, Immunol. Cell Biol 85 (2007) 148–154. [PubMed: 17160074]

[6]. Barsoum RS, Urinary schistosomiasis: review, J. Adv. Res 4 (2013) 453–459. [PubMed: 25685452]

[7]. Schwartz C, Fallon PG, Schistosoma "Eggs-Iting" the host: granuloma formation and egg excretion, Front. Immunol 9 (2018) 2492. [PubMed: 30459767]

[8]. Rutitzky LI, Hernandez HJ, Stadecker MJ, Th1-polarizing immunization with egg antigens correlates with severe exacerbation of immunopathology and death in schistosome infection, Proc. Natl. Acad. Sci. U. S. A 98 (2001) 13243–13248. [PubMed: 11606762]

[9]. Dunne DW, Jones FM, Doenhoff MJ, The purification, characterization, serological activity and hepatotoxic properties of two cationic glycoproteins (alpha 1 and omega 1) from *Schistosoma mansoni* eggs, Parasitology 103 (Pt 2) (1991) 225–236. [PubMed: 1745548]

[10]. Smith P, Fallon RE, Mangan NE, Walsh CM, Saraiva M, Sayers JR, McKenzie ANJ, Alcami A, Fallon PG, *Schistosoma mansoni* secretes a chemokine binding protein with anti-inflammatory activity, J. Exp. Med 202 (2005) 1319–1325. [PubMed: 16301741]

[11]. Schramm G, Gronow A, Knobloch J, Wippersteg V, Grevelding CG, Galle J, Fuller H, Stanley RG, Chiodini PL, Haas H, Doenhoff MJ, IPSE/alpha-1: a major immunogenic component secreted from *Schistosoma mansoni* eggs, Mol. Biochem. Parasitol 147 (2006) 9–19. [PubMed: 16480783]

[12]. Everts B, Perona-Wright G, Smits HH, Hokke CH, van der Ham AJ, Fitzsimmons CM, Doenhoff MJ, van der Bosch J, Mohrs K, Haas H, Mohrs M, Yazdanbakhsh M, Schramm G, Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses, J. Exp. Med 206 (2009) 1673–1680. [PubMed: 19635864]

[13]. Wang J, Peng W, Feng J, Zhu D, Chen J, Sun X, Lyu L, Ju S, Duan Y, Recombinant T2 RNase protein of *Schistosoma japonicum* inhibits expression of a-SMA in LX-2 cells, Parasitol. Res 115 (2016) 4055–4060. [PubMed: 27384176]

[14]. Ke X-D, Shen S, Song L-J, Yu C-X, Kikuchi M, Hirayama K, Gao H, Wang J, Yin X, Yao Y, Liu Q, Zhou W, Characterization of *Schistosoma japonicum* CP1412 protein as a novel member of the ribonuclease T2 molecule family with immune regulatory function, Parasit. Vectors 10 (2017) 89. [PubMed: 28212670]

[15]. Sun X, Zhang L, Wang J, Chen J, Zhu D, Shen P, He X, Pan J, Peng W, Duan Y, *Schistosoma japonicum* protein SjP40 inhibits TGF-β1-induced activation of hepatic stellate cells, Parasitol. Res 114 (2015) 4251–4257. [PubMed: 26268567]

[16]. Chen J, Xu T, Zhu D, Wang J, Huang C, Lyu L, Hu B, Sun W, Duan Y, Egg antigen p40 of *Schistosoma japonicum* promotes senescence in activated hepatic stellate cells by activation of the STAT3/p53/p21 pathway, Cell Death Dis 7 (2016) e2315. [PubMed: 27468691]

[17]. Chen L, Zhou Q, Liu E, Zhang J, Duan L, Zhu D, Chen J, Duan Y, rSjp40 inhibits activated hepatic stellate cells by promoting nuclear translocation of YB1 and inducing BMP-7/Smad1/5/8 pathway, Parasit. Vectors 12 (2019) 279. [PubMed: 31151477]

[18]. Cai Y, Langley JG, Smith DI, Boros DL, A cloned major *Schistosoma mansoni* egg antigen with homologies to small heat shock proteins elicits Th1 responsiveness, Infect. Immun 64 (1996) 1750–1755. [PubMed: 8613387]

[19]. Cass CL, Johnson JR, Califf LL, Xu T, Hernandez HJ, Stadecker MJ, Yates JR, Williams DL, Proteomic analysis of *Schistosoma mansoni* egg secretions, Mol. Biochem. Parasitol 155 (2007) 84–93. [PubMed: 17644200]

[20]. De Marco Verissimo C, Potriquet J, You H, McManus DP, Mulvenna J, Jones MK, Qualitative and quantitative proteomic analyses of *Schistosoma japonicum* eggs and egg-derived secretory-excretory proteins, Parasit. Vectors 12 (2019) 173. [PubMed: 30992086]

[21]. Sotillo J, Pearson MS, Becker L, Mekonnen GG, Amoahid AS, van Dam G, Corstjens PLAM, Murray J, Mduluza T, Mutapi F, Loukas A, In-depth proteomic characterization of *schistosoma haematobium*: towards the development of new tools for elimination, PLoS Negl. Trop. Dis 13 (2019).

[22]. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M, Blast2GO: A universal annotation and visualization tool in functional genomics research. Application note, Bioinformatics 21 (2005) 3674–3676. [PubMed: 16081474]

[23]. Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S, Feature-based prediction of non-classical and leaderless protein secretion, Protein Eng. Des. Sel 17 (2004) 349–356. [PubMed: 15115854]

[24]. Naresha S, Suryawanshi A, Agarwal M, Singh BP, Joshi P, Mapping the complement C1q binding site in *Haemonchus contortus* calreticulin, Mol. Biochem. Parasitol 166 (2009) 42–46. [PubMed: 19428671]

[25]. Kasper G, Brown A, Eberl M, Vallar L, Kieffer N, Berry C, Girdwood K, Eggleton P, Quinnell R, Pritchard DI, A calreticulin-like molecule from the human hookworm *Necator americanus* interacts with C1q and the cytoplasmic signalling domains of some integrins, Parasite Immunol 23 (2001) 141–152. [PubMed: 11240905]

[26]. Zang X, Yazdanbakhsh M, Jiang H, Kanost MR, Maizels RM, A novel serpin expressed by blood-borne microfilariae of the parasitic nematode *Brugia malayi* inhibits human neutrophil serine proteinases, Blood 94 (1999) 1418–1428. [PubMed: 10438730]

[27]. Aly I, ELnain G, Hamad RS, Kilany M, Ghramh HA, Alshehri A, Dajem SM, Ibrahim EH, DNA vaccination using recombinant *Schistosoma mansoni* fatty acid binding protein (smFABP) gene, Exp. Parasitol 194 (2018) 53–59. [PubMed: 30266572]

[28]. Al-Sherbiny M, Osman A, Barakat R, El Morshedy H, Bergquist R, Olds R, *In vitro* cellular and humoral responses to *Schistosoma mansoni* vaccine candidate antigens, Acta Trop 88 (2003) 117–130. [PubMed: 14516923]

[29]. Mair GR, Niciu MJ, Stewart MT, Brennan G, Omar H, Halton DW, Mains R, Eipper BA, Maule AG, Day TA, A functionally atypical amidating enzyme from the human parasite *Schistosoma mansoni*, FASEB J 18 (2004) 114–121. [PubMed: 14718392]

[30]. Schechtman D, Tarrab-Hazdai R, Arnon R, The 14–3-3 protein as a vaccine candidate against schistosomiasis, Parasite Immunol 23 (2001) 213–217. [PubMed: 11298298]

[31]. Rezende CMF, Coitinho JB, Costa M, Silva MR, Giusta M, Oliveira-Prado R, Corrêa-Oliveira R, Nagem R, Goes AM, Biochemical analysis and identification of linear B-cell epitopes from recombinant Sm21.7 antigen from *Schistosoma mansoni*, Mol. Immunol 101 (2018) 29–37. [PubMed: 29857222]

[32]. Francis P, Bickle Q, Cloning of a 21.7-kDa vaccine-dominant antigen gene of *Schistosoma mansoni* reveals an EF hand-like motif, Mol. Biochem. Parasitol 50 (1992) 215–224. [PubMed: 1371327]

[33]. Reynolds SR, Dahl CE, Harn DA, T and B epitope determination and analysis of multiple antigenic peptides for the *Schistosoma mansoni* experimental vaccine triose-phosphate isomerase, J. Immunol 152 (1994) 193–200. [PubMed: 7504709]

[34]. Sotillo J, Pearson M, Potriquet J, Becker L, Pickering D, Mulvenna J, Loukas A, Extracellular vesicles secreted by *Schistosoma mansoni* contain protein vaccine candidates, Int. J. Parasitol 46 (2016) 1–5. [PubMed: 26460238]

[35]. Samoil V, Dagenais M, Ganapathy V, Aldridge J, Glebov A, Jardim A, Ribeiro P, Vesicle-based secretion in schistosomes: analysis of protein and microRNA (miRNA) content of exosome-like vesicles derived from Schistosoma mansoni, Sci. Rep 8 (2018) 3286. [PubMed: 29459722]

[36]. Demarta-Gatsi C, Rivkin A, Di Bartolo V, Peronet R, Ding S, Commere PH, Guillonneau F, Bellalou J, Brûé S, Abou Karam P, Cohen SR, Lagache T, Janse CJ, Regev-Rudzki N, Mécheri S, Histamine releasing factor and elongation factor 1 alpha secreted via malaria parasites extracellular vesicles promote immune evasion by inhibiting specific T cell responses, Cell. Microbiol 21 (2019) e13021. [PubMed: 30835870]

[37]. Wu Z, Wang L, Li J, Wang L, Wu Z, Sun X, Extracellular vesicle-mediated communication within host-parasite interactions, Front. Immunol 10 (2019).

[38]. Marcilla A, Trelis M, Cortés A, Sotillo J, Cantalapiedra F, Minguez MT, Valero ML, Sánchez del Pino MM, Muñz-Antoli C, Toledo R, Bernal D, Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells, PLoS One 7 (2012) e45974. [PubMed: 23029346]

[39]. Floudas A, Cluxton CD, Fahel J, Khan AR, Saunders SP, Amu S, Alcami A, Fallon PG, Composition of the *Schistosoma mansoni* worm secretome: identification of immune modulatory cyclophilin A, PLoS Negl. Trop. Dis 11 (2017) e0006012. [PubMed: 29073139]

[40]. Liu F, Cui S-J, Hu W, Feng Z, Wang Z-Q, Han Z-G, Excretory/secretory proteome of the adult developmental stage of human blood fluke, *Schistosoma japonicum*, Mol. Cell Proteom 8 (2009) 1236–1251.

[41]. Curwen RS, Ashton PD, Johnston DA, Wilson RA, The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages, Mol. Biochem. Parasitol 138 (2004) 57–66. [PubMed: 15500916]

[42]. Mathieson W, Wilson RA, A comparative proteomic study of the undeveloped and developed *Schistosoma mansoni* egg and its contents: the miracidium, hatch fluid and secretions, Int. J. Parasitol 40 (2010) 617–628. [PubMed: 19917288]

[43]. Mulvenna J, Hamilton B, Nagaraj SH, Smyth D, Loukas A, Gorman JJ, Proteomics analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, *Ancylostoma caninum*, Mol. Cell Proteom 8 (2009) 109–121.

[44]. Stadecker MJ, Hernandez HJ, The immune response and immunopathology in infection with *Schistosoma mansoni*: a key role of major egg antigen Sm-p40, Parasite Immunol 20 (1998) 217–221. [PubMed: 9651922]

[45]. Pennington LF, Alouffi A, Mbanefo EC, Ray D, Heery DM, Jardetzky TS, Hsieh MH, Falcone FH, H-IPSE is a pathogen secreted host nucleus-infiltrating protein (infiltrin) expressed exclusively by the Schistosoma haematobium egg stage, Infect. Immun 85 (2017).

[46]. IARC working group on the evaluation of carcinogenic risks to humans, biological agents. Volume 100 B. A review of human carcinogens, IARC Monogr. Eval. Carcinog. Risks Hum 100 (2012) 1–441.

[47]. Brindley PJ, Loukas A, Helminth infection–induced malignancy, PLoS Pathog 13 (2017).

[48]. Ishida K, Hsieh MH, Understanding urogenital schistosomiasis-related bladder cancer: an update, Front. Med 5 (2018).

[49]. Driss V, El Nady M, Delbeke M, Rousseaux C, Dubuquoy C, Sarazin A, Gatault S, Dendooven A, Riveau G, Colombel JF, Desreumaux P, Dubuquoy L, Capron M, The schistosome glutathione S-transferase P28GST, a unique helminth protein, prevents intestinal inflammation in experimental colitis through a Th2-type response with mucosal eosinophils, Mucosal Immunol 9 (2016) 322–335. [PubMed: 26174763]

[50]. Asahi H, Osman A, Cook RM, LoVerde PT, Stadecker MJ, *Schistosoma mansoni* phosphoenolpyruvate carboxykinase, a novel egg antigen: immunological properties of the recombinant protein and identification of a T-cell epitope, Infect. Immun 68 (2000) 3385–3393. [PubMed: 10816489]

[51]. Asahi H, Hernandez HJ, Stadecker MJ, A novel 62-kilodalton egg antigen from *Schistosoma mansoni* induces a potent CD4+ T helper cell response in the C57BL/6 mouse, Infect. Immun 67 (1999) 1729–1735. [PubMed: 10085011]

[52]. Yoshino TP, Brown M, Wu X-J, Jackson CJ, Ocadiz-Ruiz R, Chalmers IW, Kolb M, Hokke CH, Hoffmann KF, Excreted/secreted Schistosoma mansoni venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression, Int. J. Parasitol 44 (2014) 551–563. [PubMed: 24859313]

[53]. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL, Dalton JP, Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages, FASEB J 22 (2008) 4022–4032. [PubMed: 18708590]

[54]. Wu C, Chen Q, Fang Y, Wu J, Han Y, Wang Y, Yang Y, Chu M, Feng Y, Tan L, Guo X, Hu W, Wang Z, Schistosoma japonicum egg specific protein SjE16.7 recruits neutrophils and induces inflammatory hepatic granuloma initiation, PLoS Negl. Trop. Dis 8 (2014) e2703. [PubMed: 24551263]

[55]. Deng J, Gold D, LoVerde PT, Fishelson Z, Inhibition of the complement membrane attack complex by *Schistosoma mansoni* paramyosin, Infect. Immun 71 (2003) 6402–6410. [PubMed: 14573661]

[56]. Loukas A, Jones MK, King LT, Brindley PJ, McManus DP, Receptor for Fc on the surfaces of schistosomes, Infect. Immun 69 (2001) 3646–3651. [PubMed: 11349025]

[57]. Alger HM, Sayed AA, Stadecker MJ, Williams DL, Molecular and enzymatic characterisation of *Schistosoma mansoni* thioredoxin, Int. J. Parasitol 32 (2002) 1285–1292. [PubMed: 12204228]

[58]. Kim J, Gee HY, Lee MG, Unconventional protein secretion – new insights into the pathogenesis and therapeutic targets of human diseases, J. Cell. Sci 131 (2018) jcs213686. [PubMed: 29941450]

[59]. lie Ståhl A, Johansson K, Mossberg M, Kahn R, Karpman D, Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases, Pediatr. Nephrol 34 (2019) 11–30. [PubMed: 29181712]

[60]. Akuma P, Okagu OD, Udenigwe CC, Naturally occurring exosome vesicles as potential delivery vehicle for bioactive compounds, Front. Sustain. Food Syst 3 (2019) 23.

[61]. de la Torre-Escudero E, Gerlach JQ, Bennett APS, Cwiklinski K, Jewhurst HL, Huson KM, Joshi L, Kilcoyne M, O'Neill S, Dalton JP, Robinson MW, Surface molecules of extracellular vesicles secreted by the helminth pathogen Fasciola hepatica direct their internalisation by host cells, PLoS Negl. Trop. Dis 13 (2019).

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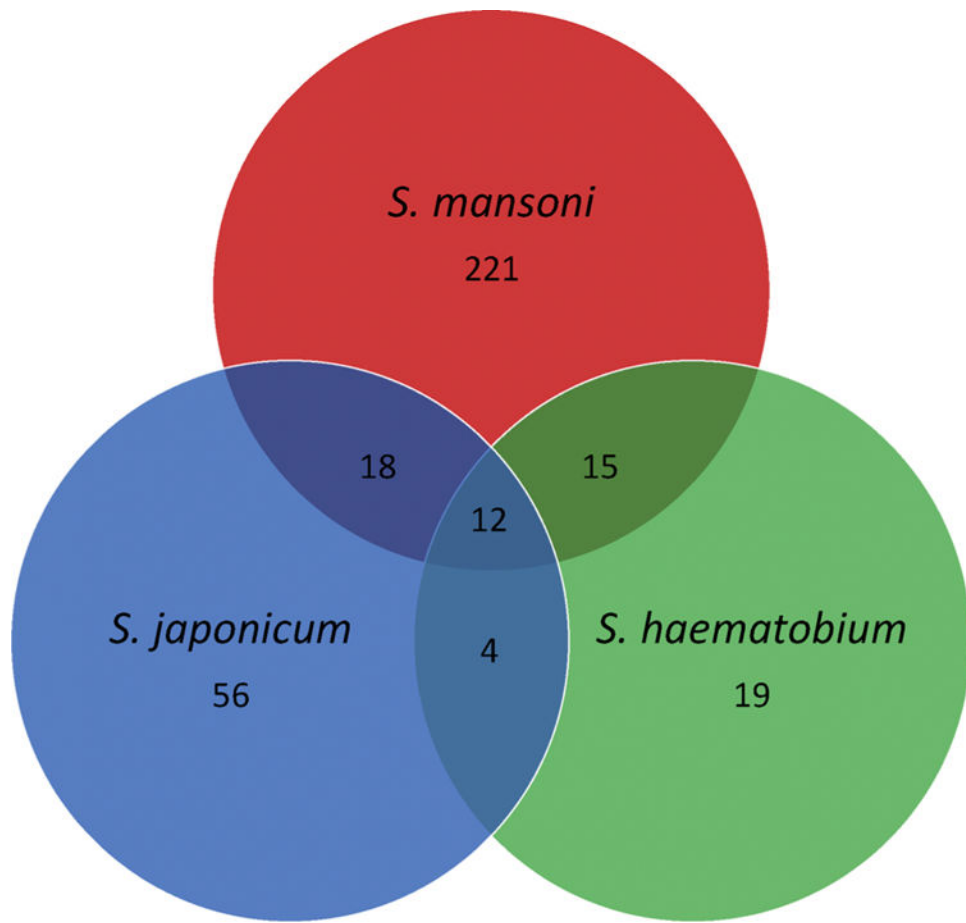
Fig. 1.
The number of shared egg stage ESPs between *S. mansoni*, *S. japonicum* and *S. haematobium*.

Table 1

Top 20 most abundant protein components of *S. mansoni* egg ESPs. The method of protein secretion as predicted by SecretomeP is listed; classically secreted (Y), non-classically secreted (A) or non-secreted (N). Proteins are ranked in order of the highest total unique matched peptides taken from three technical replicates.

Accession Number	Protein ID	# Unique peptides	% Coverage	SecretomeP
G4V5U5	Putative macroglobulin/complement	49	26.7	N
G4V9B9	Putative heat shock protein	27	40.0	N
G4VPH5	Glucose-6-phosphate isomerase	20	48.0	A
G4LW91	Thimet oligopeptidase	19	31.7	N
G4V721	Putative phosphoenolpyruvate carboxykinase	18	40.7	N
G4LYU6	Serpin, putative	18	52.7	A
G4V855	Aminopeptidase PILS	17	21.0	A
G4VFC2	Protein disulfide-isomerase	16	37.3	Y
G4VJT9	Fructose-bisphosphate aldolase	16	57.7	N
G4V9U9	Putative major vault protein	16	25.3	N
G4V8L4	Putative heat shock protein 70	15	33.7	N
G4V8C5	Putative alpha-glucosidase	15	20.3	A
C4Q5I7	Calreticulin autoantigen homolog, putative	13	34.7	Y
G4M057	WD-repeat protein, putative	12	26.3	A
G4LYC3	Protein disulfide-isomerase	12	30.0	Y
G4VG40	Dipeptidyl peptidase 3	12	21.3	A
P09792	Glutathione S-transferase class-mu 28 kDa isozyme	12	51.3	N
G4LVU8	Filamin	11	7.3	N
G4VQ41	Low-density lipoprotein receptor	11	14.7	Y
G4V8X7	Putative heat shock protein-HSP20	11	36.7	A

Table 2

Top 20 most abundant protein components of *S. japonicum* egg ESPs. The method of protein secretion as predicted by SecretomeP is listed; classically secreted (Y), non-classically secreted (A) or non-secreted (N). Proteins are ranked in order of the highest total unique matched peptides taken from three technical replicates.

Accession Number	Protein ID	# Unique Peptides	% Coverage	SecretomeP
Q5DET3	Histone H4	19	71.8	N
Q5DFZ8	Fructose-bisphosphate aldolase	16	55.8	N
Q86G46	Polyubiquitin	16	32.1	N
C1L4Z7	Actin 5C	13	33.2	N
Q5DHF8	Histone H3	12	64.5	A
C1LFC4	Aminopeptidase	10	18.2	A
C1L9B2	Calreticulin	8	19.8	Y
Q5DDU9	Histone H2B	7	38.0	N
C1LEJ4	Histone H2A	7	46.4	A
Q86EU4	Cytochrome c proximal	7	50.8	A
C1LQJ9	Uncharacterized protein	6	23.3	A
C1LQT3	Thioredoxin	6	53.5	A
Q5D8W0	SJCHGC06710 protein	6	40.8	Y
P33676	Enolase	6	16.0	A
Q5D9T1	Malate dehydrogenase	6	27.1	A
C1LJG7	NADH:ubiquinone oxidoreductase	5	22.0	A
C1LYI9	Calcium-binding EF-hand protein	5	58.7	A
C1LNR0	Protein disulfide-isomerase	5	14.5	Y
C1LV44	Tryparedoxin peroxidase	5	28.1	A
C1LNY3	Superoxide dismutase [Cu-Zn]	5	62.6	A

Table 3

Top 20 most abundant protein components of *S. haematobium* egg ESPs. The method of protein secretion as predicted by SecretomeP is listed; classically secreted (Y), non-classically secreted (A) or non-secreted (N). Proteins are ranked in order of the highest total unique matched peptides taken from three technical replicates.

Accession Number	Protein ID	# Unique Peptides	% Coverage	SecretomeP
A0A094ZWN9	Histone H4	11	61.5	N
A0A095B131	Polyubiquitin-C	8	30.2	N
A0A094ZDN2	Thioredoxin peroxidase 2	8	43.6	A
A0A095AIQ5	Cytochrome c	8	55.6	N
A0A095AKM1	Fructose-bisphosphate aldolase	8	27.4	N
A0A094ZX17	GLIPR1-like protein 1	7	39.6	Y
A0A095A2M4	Thioredoxin	7	61.6	A
A0A095ANZ3	Histone H3	5	33.3	A
A0A095A3Q8	Alpha-galactosidase	5	19.2	N
A5A6F5	14 kDa fatty acid-binding protein	5	32.3	A
A0A095A0L7	Superoxide dismutase [Cu-Zn]	4	37.5	A
A0A095A463	Uncharacterized protein	3	13.5	N
A0A095B4U1	Histone H2B	3	27.9	N
A0A094ZS57	16 kDa calcium-binding protein	3	25.2	A
A0A094ZZE3	Basement membrane proteoglycan	3	4.8	N
A0A095CF43	Alpha-galactosidase	3	12.5	A
A0A094ZS46	Spectrin alpha chain	3	1.0	N
A0A094ZVR4	Peptidase inhibitor 16	3	24.5	Y
A0A095CGL8	78 kDa glucose-regulated protein	3	6.8	Y
A0A094ZD40	Actin-2	2	13.8	A

Table 4

Twelve (12) predicted orthologues shared between *S. mansoni* (*Sm*), *S. japonicum* (*Sj*) and *S. haematobium* (*Sh*) egg excretory/secretory products.

S. mansoni		S. japonicum		S. haematobium		% identity		
Acc. No.	Protein ID	Acc. No.	Protein ID	Acc. No.	Protein ID	Sm-Sj	Sj-Sh	Sm-Sh
C4QBN1	Histone H4	Q5DET3	Histone H4	A0A094ZWN9	Histone H4	100	100	100
G4VLW2	Putative actin-1	C1L4Z7	Actin 5C	A0A094ZD40	Actin-2	98	97	96
G4VIT9	Fructose-bisphosphate aldolase	Q5DFZ8	Fructose-bisphosphate aldolase	A0A095AKM1	Fructose-bisphosphate aldolase	96	97	98
G4V6E9	Histone H2B	Q5DDU9	Histone H2B	A0A095B4U1	Histone H2B	95	100	95
G4VT44	Putative calmodulin	Q5DA21	Calmodulin 3b	A0A094ZPX2	Calmodulin	90	97	98
G4M131	Fatty acid binding protein	O45035	Fatty acid-binding protein	A5A6F5	14 kDa fatty acid-binding protein	86	86	99
Q15EU2	Cytochrome c-like protein	Q86EU4	Cytochrome c proximal	A0A095AIQ5	Cytochrome c	90	88	93
G4VE60	Thioredoxin, Trx M	Q5DA40	SJCHGC03107 protein	A0A095CEH3	Thioredoxin, mitochondrial	89	84	94
G4VQ58	Phosphopyruvate hydratase	P33676	Enolase	A0A095AJN4	Enolase	85	85	93
G4VKD9	Peroxiredoxin, Prx1	C1LV44	Tryparedoxin peroxidase	A0A094ZDN2	Thioredoxin peroxidase 2	82	85	93
G4V910	Putative heat shock protein 70	O45038	HSP70	A0A095CGL8	78 kDa glucose-regulated protein	83	81	95
G4M0D0	Superoxide dismutase [Cu-Zn]	C1LNY3	Superoxide dismutase [Cu-Zn]	A0A095A0L7	Superoxide dismutase [Cu-Zn]	81	80	95

Table 5

Predicted orthologues between paired comparisons of *S. mansoni*, *S. japonicum* and *S. haematobium* egg excretory/secretory products.

Acc. No.	Protein ID	Acc. No.	Protein ID	% identity
S. mansoni		**S. japonicum**		
G4VG19	Phosphoglycerate kinase	B3W661	Phosphoglycerate kinase	100
G4VHK8	Tubulin beta chain	C1LVD4	Tubulin beta chain	99
G4VMF9	Histone H2A	Q5DE19	Histone H2A	99
G4V865	Tubulin alpha chain	Q5D9M8	Tubulin alpha chain	98
G4VBJ0	Malate dehydrogenase	Q5D9T1	Malate dehydrogenase	94
G4VPH5	Glucose-6-phosphate isomerase	C1LK30	Glucose-6-phosphate isomerase	93
G4LYC3	Protein disulfide-isomerase	Q5C0A7	Uncharacterized protein	92
G4V721	Putative phosphoenolpyruvate carboxykinase	Q5DGF3	SJCHGC06900 protein	91
G4VCN8	Dihydrolipoyl dehydrogenase	C1LIA4	Dihydrolipoyl dehydrogenase	91
Q9Y0D3	Peroxiredoxin, Prx2	C1LMD2	Thioredoxin peroxidase	90
G4VAD2	Elongation factor 1-alpha	C1LWQ6	Elongation factor 1-alpha	89
G4VRK1	Putative glyoxalase II	Q86DW6	Hydroxyacylglutathione hydrolase	89
G4VJY9	Nucleoside diphosphate kinase	Q86EJ4	Nucleoside diphosphate kinase	88
G4M0P7	Cell division control protein 48	Q5D9C5	SJCHGC09453 protein	88
G4VBG6	Ferritin	C1L7G5	Ferritin	87
G4VFQ0	Peptidyl-prolyl cis-trans isomerase	Q5D8J4	Peptidyl-prolyl cis-trans isomerase	85
G4V9B9	Putative heat shock protein	Q5D947	SJCHGC00820 protein	84
G4VH95	Putative acyl-CoA-binding protein	C1LJT2	Diazepam-binding inhibitor	84
S. mansoni		**S. haematobium**		
G4LVU8	Filamin	A0A094ZGB5	Filamin-A	95
G4LXS0	Aldo-keto reductase, putative	A0A095AEB2	Alcohol dehydrogenase [NADP(+)] A	95
G4VAG2	Calpain	A0A094ZH52	Calpain	95
G4VDR4	Family C56 non-peptidase homologue	A0A095C8I8	Protein DJ-1	93
G4VMD1	Venom allergen-like (VAL) 26 protein	A0A094ZX17	GLIPR1-like protein 1	90
Q8T9N5	Thioredoxin	A0A095A2M4	Thioredoxin	90
G4VSQ5	Superoxide dismutase	A0A095BT16	Superoxide dismutase	88
G4VP82	Purine nucleoside phosphorylase	A0A094ZR11	Purine nucleoside phosphorylase	88

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Acc. No.	Protein ID	Acc. No.	Protein ID	% identity
G4VA23	Putative 22.6 kDa tegument-associated antigen	A0A095C6E4	Tegument antigen	88
G4VDQ3	Uncharacterized protein	A0A094ZWF6	Uncharacterized protein	87
G4M0I0	Low-density lipoprotein receptor	A0A094ZZE3	Basement membrane heparan sulfate proteoglycan	86
G4LUZ7	Ferritin	A0A094ZYX2	Ferritin	85
G4VNW4	Putative fibrocystin 1	A0A094ZQC8	Fibrocystin-L	85
G4VFC2	Protein disulfide-isomerase	A0A095A463	Uncharacterized protein	83
G4VLD6	Alpha-galactosidase	A0A095CF43	Alpha-galactosidase	83
S. japonicum		*S. haematobium*		
Q86G46	Polyubiquitin	A0A095B131	Polyubiquitin-C	100
C1LEJ4	Histone H2A	A0A094ZQV5	Histone H2A	99
Q5DHF8	Histone H3	A0A095ANZ3	Histone H3	98
Q5C3I8	SJCHGC05636 protein	A0A094ZS46	Spectrin alpha chain	88

Table 6

Gene Ontology (GO) term annotations and their % representation for *S. mansoni* (*Sm*), *S. japonicum* (*Sj*) and *S. haematobium* (*Sh*) excreted/secreted products (ESPs) in our dataset compared with the complete NCBI protein groups. GO terms that are higher in ESP compared to NCBI groups are shaded

Molecular Function (MF)		Sm ESP	Sm NCBI	Sj ESP	Sj NCBI	Sh ESP	Sh NCBI
antioxidant activity	GO:0016209	2.68	0.86	3.91	0.61	6.98	0.22
binding	GO:0005488	46.34	52.08	50	46.9	56.98	55.97
catalytic activity	GO:0003824	47.32	35.86	40.63	37.47	31.4	36.28
molecular function regulator	GO:0098772	1.71	1.42	2.34	1.38	0	1.85
structural molecule activity	GO:0005198	0.98	1.62	1.56	7.76	1.16	2.1
transporter activity	GO:0005215	0.73	6.31	1.56	5.88	2.33	5.39
Biological Process (BP)							
biological regulation	GO:0065007	5.11	10.03	8.05	6.87	10.33	11.35
carbon utilization	GO:0015976	1	0.12	0.77	0.26	0.33	0.11
cellular component organization	GO:0071840	1.56	4.18	3.45	6.95	4.67	4.51
cellular process	GO:0009987	37.67	35.07	39.46	35.06	31.67	34.69
detoxification	GO:0098754	0.89	0.3	1.15	0.22	1.67	0.1
developmental process	GO:0032502	2	1.11	0.77	0.96	3	1.15
immune system process	GO:0002376	0.33	0.16	0.38	0.31	0.67	0.14
localization	GO:0051179	2.22	7.35	1.92	9.73	3.67	7.05
metabolic process	GO:0008152	41.22	30.38	35.25	33.79	23.33	29.15
multicellular organismal process	GO:0032501	1.44	1.12	1.53	1.02	3.67	1.26
response to stimulus	GO:0050896	4.11	5.16	5.36	2.81	9.33	5.49
signalling	GO:0023052	0.67	3.58	1.15	1.6	3	4.32