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Research Paper

Proteomic identification of larval excretory/secretory diagnostic biomarkers for early hyperinfection in strongyloidiasis

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The aim of the current study was to identify early specific diagnostic proteins from the components of *Strongyloides ratti* larval Excretory/Secretory (E/S) products. Products from the treated (Prednisolone) and non-treated (control) groups were analysed using two dimensional (2D) gel electrophoresis, LC-MS/MS and MALDI-TOF/TOF Mass Spectrophotometry. A total of 10 different protein biomarkers were detected as overexpressed from the ES treated products with molecular weights ranging from 30 - 90 kDa and isoelectric points (IP) range from 4 - 9. Eight (8) overexpressed protein biomarkers were also identified from the infected and prednisolone-treated rat sera. Relative quantitative real-time PCR (qPCR) was performed to compare the expression level of the biomarker genes between the treated and non-treated groups in order to validate the related expressed biomarkers. Nine (9) related genes were observed to show significant high expression levels of the identified biomarkers, whereas one (1) gene (*Arg*) recorded down regulation in its expression. The identified proteins in this study might be of specific importance as diagnostic biomarkers for early hyperinfection syndrome in strongyloidiasis.

Key words: Proteomics, diagnostic biomarkers, hyperinfection, strongyloidiasis

INTRODUCTION

Many studies have reported strongyloidiasis in travelers and immigrants from tropical and sub-tropical countries (Croker *et al*., 2010), mainly affecting school aged children, people living in unhygienic habitats and of low socio-economic status (Lim *et al*., 2004), immunocompromised patients resulting from cancer, HIV,

HTLV-1, immunosuppressive treatments (Ramanathan and Nutman, 2008; Machado *et al*., 2011) and after organ transplant (Khieu et al., 2013). The present worldwide estimation of Strongyloidiasis cannot be precise and accurate, because most of the patients are asymptomatic; hence, the infection can sometimes be misdiagnosed or hard to detect due to lack of sensitive diagnostic methods as well as the frequent unsatisfactory epidemiological mapping (Johansen et al., 2010; Montes et al., 2010). Consequently, strongyloidiasis is still regarded as one of the most unattended and neglected soil-transmitted infections causing major health problem with crucial public health importance (Azira et al., 2013; Levenhagen and Costa-Cruz, 2014).

Diagnosis of strongyloidiasis usually relies on detection and identification of rhabditiform larvae in the stool using a number of conventional techniques (Koczka *et al*., 2012). However, in most uncomplicated cases of strongyloidiasis, the intestinal parasites load is often very low and the emergence of rhabditiform larvae is minimal. As such, many light (chronic) infections usually go unnoticed leading to underestimated prevalences in endemic areas. Serologic tests are very useful, but their specificity is variable (Requena-Mendez *et al*., 2013). As clinical observation and early response to strongyloidiasis depends on rapid diagnosis and detection, molecules (biomarkers) that can be sensitively and specifically measured are typically of potential diagnostic importance. The efficacy of these biomarkers lies in their potential to provide early recognition, as to establish highly specific diagnosis, determine accurate prognosis and monitor disease succession. This is to prevent suffering of patients and to reverse the fatal course of the life-threatening hyperinfection syndrome.

Proteomic analysis is a widely used technique for identification of proteins and their functions. Moreover, proteomics have been used to discover new proteins as biomarker candidates in the early detection of diseases (Iff, 2007). One of the targets of proteomics for biomarker identification in strongyloidiasis is that excretory/secretory products (ES products) from the parasitic nematodes are thought to be essential for its establishment and their lifestyle (Moreno, 2011). Excretory/Secretory products (ESP) of nematodes include proteases, protease inhibitors, lipids, glycans, allergen homologues, and glycolytic enzymes which used to invade their mammalian hosts and penetrate the defense tissue barriers (Dzik, 2006). They are also associated with evasion or modulation of the host immune response (Perally *et al*., 2008).

The objective of the present study was to identify potential biomarkers from the excretory/secretory (ES) products of the infective larvae of *S. ratti*, as well as from serum samples of infected rats treated with immunosuppressive drugs, so that these biomarkers may be of use as potential tools in the diagnosis of early hyperinfection in strongyloidiasis.

MATERIALS AND METHODS

Source of parasite and experimental animals

Strongyloides ratti was originally isolated from wild rats, (*Rattus norvegicus*) and was maintained in the laboratory by serial passages in Wistar rats. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia (UPM), Malaysia. The infection was established in two groups of Wistar rats (4 weeks old) following subcutaneous injection on the dorsum of the neck with 2, 000 infective larvae. One group of the rats was given oral prednisolone at 4.4 mg/kg for 10 days in order to induce experimental immunosuppression, with the untreated group maintained as control. Larvae from both groups were collected using modified fecal filtration technique (MFFT) as previously described (Mahmuda *et al*., 2017), which were then prepared for comparative proteomic analysis.

Preparation of excretory/secretory (ES) protein

The ES proteins were prepared by first sterilizing the surface of the infective larvae by incubation in 0.15% NaOCl for five minutes at room temperature. The larvae were then thoroughly washed several times in sterile phosphate buffered saline (1 X PBS) and then with serum-free (RPMI-1640) media supplemented with 100 IU penicillin/mL and 100 μg/mL streptomycin. Fifty-thousand larvae per milliliter were incubated in the same medium at 37°C in 5% CO_2 for 28 days following substitution of the old media with new media after every 7 days. After the incubation period, the medium containing the ES proteins was collected into 15 mL conical tubes, and the larvae were allowed to settle for 20 min. The supernatant containing the ES products was collected and filtered through a 0.22 μm membrane using an Acrodisc syringe filter (PALL Life Science, UK).The ES products were concentrated and freeze-dried (Telstar Cryodos, Spain). The protein concentration was determined by the Bradford assay method. Then, complete mini protease inhibitor (1 X) cocktail (Roche diagnostic, Mannheim, Germany) was added to the protein solution, and aliquots were stored at –80°C until required for SDS PAGE and 2D gel electrophoresis.

Blood collection and serum preparation

Five milliliters of intravenous blood were collected into an anti-coagulant free vacutainer blood collection tube. The tube was incubated at room temperature for 30 min for serum collection. The serum was then separated by centrifuging the coagulated blood sample at 2,000 rpm for 15 min. Serum was then collected into clean labeled

micro-centrifuge tubes and stored at -30°C until required for further analysis.

Sonication of serum proteins

Sonication of proteins was performed in order to reduce the complexity of the crude serum sample using physical methods to distort complex inter-and intra-protein interactions and improve the quality of separation. As the biological fluids like serum are extremely complex and have a wide variety of proteins, which can hinder the separation in 1D and 2D gel electrophoresis. Briefly, 200 µL of serum was pipetted in a clean micro-centrifuge tube. The serum was then diluted five times using phosphate buffered saline (1 x PBS), pH 7.4, the content was mixed thoroughly by vortexing for 20 s. The sample was then transferred into a clean 15 ml centrifuge tube and sonicated (Misonix 2020, USA) for 10 cycles of 5 s at 20 % amplitude (pulse) with a 1 min interval between each cycle with the tube permanently immersed in ice.

Depletion of highly abundant proteins

Removal of abundant serum proteins will help to obtain effective protein separation. This was carried out by using modified albumin removal method with trichloroacetic acid (TCA)/acetone as previously described (Chen et al., 2005). Briefly, A 20 mL sample of the serum was precipitated by the rapid addition of four volumes of ice-cold acetone containing 10 % w/v TCA and was immediately mixed by gentle vortexing. The mixture was then incubated at -20°C for 90 min and centrifuged at 15, 000 rpm, at 4°C, for 20 min. The supernatant was removed and collected by pipetting. One millilitre of ice-cold acetone was added to wash the precipitate. The sample was incubated on ice for 15 min and centrifuged again at 15, 000 rpm, at 4°C, for 20 min. The acetone-containing supernatant was removed and the precipitate was lyophilised. One millilitre of ice-cold acetone was added to the 10% TCA/acetone- containing supernatant to completely precipitate the proteins in the supernatant.

Determination of protein concentration

The protein concentration was measured using the Bradford assay (Bio-Rad, USA). Briefly, one volume of dye reagent concentrate was mixed with four volumes of de-ionized water. Serial dilutions of bovine serum albumin (BSA) ranging from 0. 2 - 2. 0 mg/mL were prepared and used as protein standards at 0.3 mg/mL intervals. For protein measurement, 1 mL of the premixed dye reagent was loaded in a disposable plastic cuvette (Eppendorf, Germany), 20 µL of each BSA standards and unknown samples (20 µL of water was used for blank cuvette) were added to the cuvette and mixed by inversion. Five minutes later, the absorbance reading and protein concentration were measured at wavelength of 595 nm by using the Eppendorf Biophotometer Plus (Eppendorf, Germany). Unknown protein samples were prepared in duplicate.

Protein precipitation for SDS PAGE

Extracted proteins were precipitated for SDS PAGE using acetone precipitation method. High quality acetone solution (80 %) was prepared ahead of time and stored at -20°C. Four volumes of chilled acetone stock were added to one volume of a protein sample in 2 mL tube and were vortexed. The tube was incubated overnight at -20°C. Protein sample was then spun at high speed (>12, 000 rpm) at 4°C for 20 min. The supernatant was gently discarded. The pellet was washed with 1 mL cold Milli Q water and thoroughly broken up by sonication. After washing step, the suspension was centrifuged, supernatant was discarded as before and dried pellets frozen at - 80°C until re-suspension step.

Protein precipitation for 2D gel electrophoresis

The ES proteins were cleaned and precipitated using the 2D Clean-up Kit (Bio-Rad, USA). Briefly, extracted proteins (1–500 µg) were transferred in a final volume of 100 µL into a 1.5 ml micro centrifuge tube. A volume of 300 µL precipitating agent was added to the protein sample and mix well by vortexing, while incubated on ice for 15 min. Wash reagent of 300 µL was added to the suspension which was mixed well by vortexing and centrifuged at maximum speed (> 12,000 rpm) for 5 min. The supernatant was removed and discarded using a pipette without disturbing the pellet. The content was again centrifuged for a second time for 15–30 s to collect any residual liquid. The remaining supernatant was carefully removed using a pipette. Wash reagent of 40 µL was added into the tube containing the pellet, and was centrifuged at maximum speed (> 12,000 rpm) for 5 min after which the wash reagent was removed and discarded with a pipette. Ultrapure water (25 µL) was then added onto the pellet, and the tube was vortexed for 20 s. One millilitre of pre-chilled wash reagent and 5 µL of wash additives were added to the tube and was vortexed for 1 min. The tube was then incubated at -20°C for 30 min, vortexed afterwards for 30 s after every 10 min during the incubation period. After the incubation, the tube was centrifuged at top speed for 5 min to form a tight pellet; the supernatant was removed and discarded. The tube was centrifuged again briefly (15–30 s) to remove and discard any remaining wash reagent. The pellet was air-dried at room temperature for not more than 5 min. The pellet was re-suspended by adding an

appropriate volume of 2-D rehydration buffer.

SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of the extracted proteins was prepared as previously described (Laemmli, 1970). The protein samples were mixed with 4 x SDS sample buffer (62.5mM Tris, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5% (v/v) mercaptoethanol, and trace of bromophenol blue) and heated at 75°C for 15 min. Fifteen microlitres of ES and serum proteins and 5µl of pre-stained molecular weight protein ladders (FirstBase, Singapore) were loaded in acrylamide gel wells. The protein samples were electrophoretically separated through 5 % acrylamide stacking gel and 12% acrylamide separating gel at 25 mA, 175 Volts for 60 min. After running the gels were gently washed with ultrapure water (MilliQ) three times each and were then stained with Silver stain plus (Bio-Rad, USA) as described by the manufacturer or using Bio-Safe Coomassie Stain (Bio-Rad, USA) by overnight incubation of gels with gentle agitation. After staining, the gels were washed with distilled water or de-staining solution (10% Glacial acetic acid; 20% Methanol; ddH2O), scanned and photographed using Densitometer GS-800 Mode Imager (Bio-Rad, USA).

First-dimensional (1D) protein separation

The 2-D electrophoresis was performed by suspending the pelleted protein in rehydration buffer (7M urea, 2M thiourea, 4 % CHAPS, 65mM DTT, Destreak reagent, 0.2 % IPG buffer (pH 3-11), and trace of bromophenol blue, containing 50 µg of the protein sample in a total volume of 125 μL was centrifuged at 12,000 g for 10 min at room temperature. The supernatant was loaded onto 7 cm pH 3-11 immobilised pH gradient Drystrip (GE healthcare, Sweden) overnight in re-swelling tray and separated by isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad, USA) at 20^{o}C as follows: S1: 250V, 50 min; S2: 4000V, 120 min; S3: 4000V, 10000Vh; S4: 500V, 5 h; (using a limit of 50 μA/strip). After focusing period, the Drystrip were equilibrated sequentially; first in equilibration buffer I (6M urea, 0.05M Tris-HCl, pH 8.8, 2% SDS, and 20% glycerol) containing 2% dithiothreitol (DTT), then in second equilibration buffer containing 2.5% iodoacetamide.

Second-dimensional (2D) gel electrophoresis

The second dimension was performed on 12 % SDS-PAGE using a Mini Protean cell (Bio-Rad, USA). Proteins were separated for 60 min, 175V, at 20 mA and then at 25 mA until the dye front reached the bottom of the gel. Three replicates of 2D were run for each sample. After 2D gel electrophoresis, proteins were stained with Silver Stain Plus or Bio-Safe Coomassie Stain (Bio-Rad, USA) for proteomic analysis.

Gel excision and tryptic digestion

Three gels were prepared for the studied samples and scanned into a computer using a G-800 Densitometer Scanner (Bio-Rad, USA) in transmission mode. The SDS PAGE gel images were transferred to Image Lab software (Bio-Rad, USA) and 2D gel images were transferred to PDQuest software (Bio-Rad, USA) for direct analysis. The stained proteins were compared to determine the differences in protein profiles between the gel images. Data of 2D gel electrophoresis were normalized through the local regression model which is recommended by PDQuest software. Student's *t*-test (95% confidence) was performed in the statistical analysis to detect any significant difference in spot intensity ($p < 0.05$). The significance of protein changes in the 2D profiles was considered at two fold change of protein intensity. The protein bands and spots were excised from the gels for digestion. The excised pieces were destained for 20 min in 30 mM potassium ferricyanide/100 mMsodium thiosulfate and washed in Milli-Q water until gels were visibly clear. The bands and spots were kept in 0.2 M NH_4HCO_3 for 20 min before lyophilisation. All the lyophilised samples were digested overnight at 37°C with 12.5 ng/mL trypsin in 25 mmol/L NH_4HCO_3. The peptides were extracted three times with 60 % ACN/0.1 % trifluoroacetic acid (TFA). The extracts were pooled and dried completely by centrifugal lyophilisation. After the in-gel digestion, gel bands and spots were sent to Proteomics International Co. Ltd. (Nedlands, Western Australia) for MALDI-TOF/ TOF and LC/MS-MS analysis.

RNA Extraction, cDNA synthesis, integrity and validation

RNA extraction from the washed, cleaned and treated larval homogenate was performed by using RN easy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purity of the RNA was evaluated using the absorbance ratio A260/A280 and NanoDrop® ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The integrity of the extracted RNAs was assessed using agarose gel electrophoresis and further confirmed by measuring the 28S:18S ribosomal ratio on an RNA picoChip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Single stranded cDNA was synthesized from the purified RNA using Revert Aid first strand cDNA synthesis kit (Fermentas Life Science,

USA) with random hexamer primer according to the manufacturer's instructions. Constructed cDNAs to be used in real-time PCR were tested for their validation and integrity by conventional PCR using 28S housekeeping gene assay before use in qPCR for the amplification of selected target genes. Validation of the real time primers was done using 3-5 dilution series of the total cDNA. Sequences of the target DNAs were selected and primers were designed from the database of NCBI. They were blasted for specificity check and were ordered from the Integrated DNA Technologies (IDT Singapore).

Semi-quantitative real time PCR (RT-qPCR) and Analysis of real-time data

All real-time PCR runs were performed on Mastercycler ep Realplex (Eppendorf, Germany) using SensiFAST™ SYBR® No-ROX Kit (Bioline Reagents Ltd, USA). A 20 µl final reaction mix contains 10µl of 1x master mix, 1µl of each primer pair (400 nM), 5 µl cDNA templates, and Nuclease-Free Milli-Q water (Merck Millipore, USA) up to 20 µl. A blank tube (qPCR reaction mix without cDNA template) was used as a negative control to indicate absence of environmental contamination. The raw Ct values for the 10 target genes and the *Sr*-GAPDH (endogenous control) gene for non-treated (control) and treated samples with corticosteroid were exported from the Mastercycler ep Realplex analysis program into relative expression software tool REST© (Qiagen Group, Microsoft® Corporation, Germany) for gene expression profile analysis.

Bioinformatics analysis

Peptide mass fingerprinting (PMF) and MS/MS data were obtained using the MASCOT search engine (Matrix Science, UK). The online software COMPUTE PI/MW (http://www.expasy.org/tools/pi tool.html) tool was used to predict the molecular weight (MW) and isoelectric point (PI) of each protein.

RESULTS

Protein profiles of ES products from *strongyloides ratti* larvae

The ES products of *S. ratti* filariform larvae from both treated and non-treated samples were separated by SDS PAGE (Figure 1) and 2D gel electrophoresis (Figure 3) to compare the expressed proteins between the samples. The profiles of the ES proteins from these preparations were well represented by 2D analysis covering pH 3-11. The 1D and 2D protein profiles constructed and visualized by staining the gels with Silver Stain Plus (Bio-Rad, USA) demonstrated reasonably comparable numbers of bands and spots in both samples (Figures 2, 3). The PIs of these spots varied from 4 to 9, and their MWs ranged from 30 to 90 kDa. Most of the protein spots were detected in the range (pH 3-11) and migrated at 30-90 kDa.

Protein variability was evaluated by analysis of comparative proteomics of the ES proteins from the control and prednisolone-treated samples. Image lab and PDQuest software analyses were employed to analyze 1D and 2D gel maps of these preparations. To cover all bands and spots, gels were prepared in triplicate, and master gels were constructed from these gel triplicates. The master gel of the treated sample was then analyzed and compared with that of the control sample.

The comparison showed that there were unique protein bands and spots within the ES products of the corticosteroid-treated filariform larvae. Bands and spots of the differentially expressed proteins from 1D and 2D gels were selected for MALDI-TOF/TOF and LC-MS/MS analysis. Ten (10) proteins were analyzed and identified using these techniques for peptide mass fingerprinting (PMF). The identified proteins are listed in (Table 1).

Profiles of serum proteins from *Strongyloides ratti* infected rats

Serum samples from rats treated with prednisolone and non-treated (control) samples were prepared and analyzed using SDS PAGE (Figures 4, 5) and 2D gel electrophoresis (Figure 6) in order to assess the expression of protein profiles from these samples. Software analysis was conducted in the same manner as with ES proteins to analyze 1D and 2D gel maps of these preparations. Ten (10) protein spots were found to be differentially expressed. Therefore, these spots were cut, prepared and sent for MALDI-TOF/TOF and LC-MS/MS analysis. Using these techniques, eight (8) different proteins were identified from the spots (Table 2).

DISCUSSION

Recently, the development of several rapid and sensitive proteomics techniques such as the two-dimensional (2D) gel electrophoresis and Mass spectrometry (MS) has increased the possibility of identifying the protein content of helminth parasites, including ES products (Bennuru et al., 2009; Mulvenna et al., 2009; Robinson et al., 2009). The protein pattern of ES products in this current study demonstrated the presence of unique proteins related to the hyperinfection stage. These proteins may be vital to the parasite's survival, immune evasion, and immunopathogenesis.

This study has reported a technique that allows the identification of many proteins present within the ES

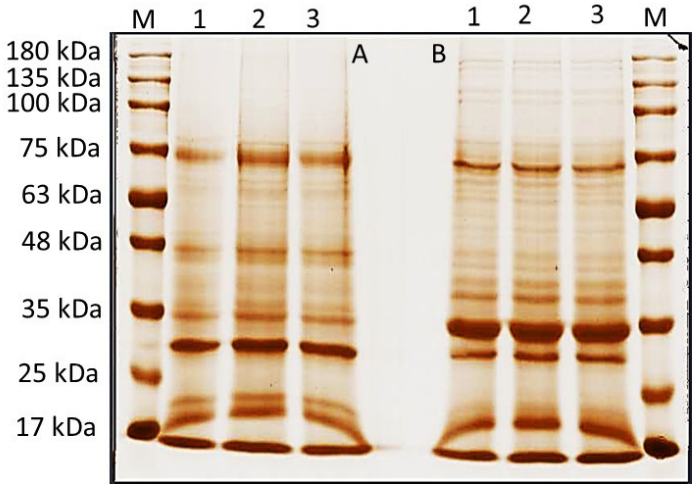

Figure 1. SDS PAGE analysis of *Strongyloides ratti* larval ES proteins of non-treated (A) and treated samples (B) with a corresponding protein ladder (M).

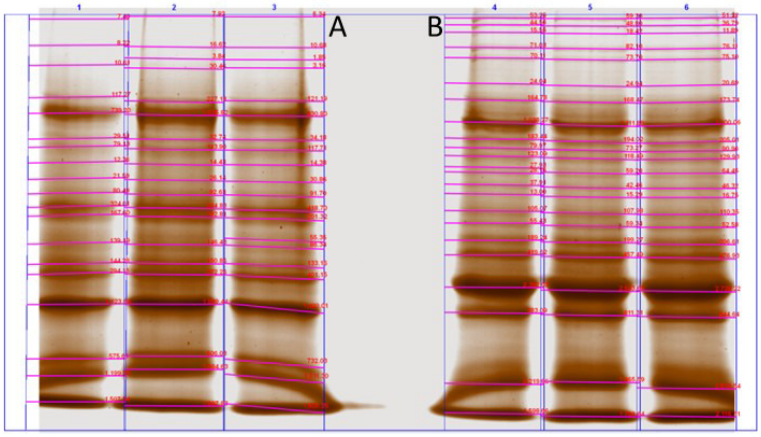

Figure 2. SDS PAGE analysis of *Strongyloides ratti* larval ES proteins of non-treated (A) and treated samples (B) using Image Lab Software.

product of the infective stage of *S. ratti* and serum proteome of rats with chronic disease or hyperinfection syndrome. The other part of this proteomic study has documented many changes present in the rat's serum proteome with corticosteroid treatment. Eight potential candidate biomarkers were identified, with the probability of having utmost diagnostic significance.

C-type lectins (C-TLs) are a family of carbohydrate-binding proteins involved in diverse processes including vertebrate immune cell signaling and trafficking and activation of innate immunity in both vertebrates and invertebrates. Helminth C-TLs sharing sequence and structural similarity with mammalian immune cell lectins have recently been detected from nematode parasites, suggesting clear proteins roles in the host–parasite relationship, particularly in immune evasion (Loucas and Maizels, 2000).

Two C-TL encoding cDNAs have been identified recently from expressed sequence tags (ESTs) of the human hookworm *Necator americanus* and the rodent gastrointestinal parasite *Nippostrongylus brasiliensis*. Each of these proteins showed sequence similarities to host lectins, raising interesting questions about the role they might play in modulating inflammation in nematode infections (Craig *et al*., 2006; Hewiston *et al*., 2008). ESTs encoding C-TL-like proteins have more recently been identified from *Ascaris suum*, hookworm *Ancylostoma ceylanicum*, and sheep nematode

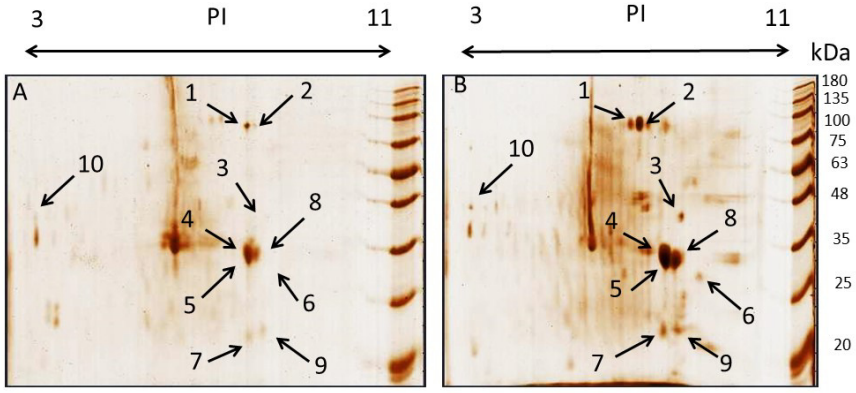

Figure 3. Two dimensional (2D) gel electrophoresis analysis of *Strongyloides ratti* larval ES proteins of non-treated (A) and treated samples (B) with a corresponding protein ladder (kDa).

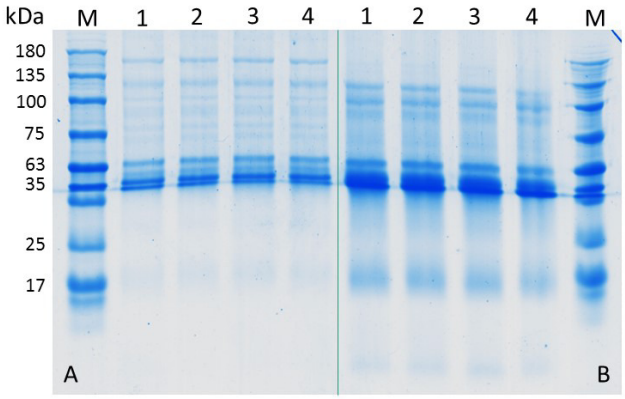

Figure 4. SDS PAGE of serum proteins from *Strongyloides* infected rats of both non-treated (A) and treated samples (B) and a corresponding protein ladder (M).

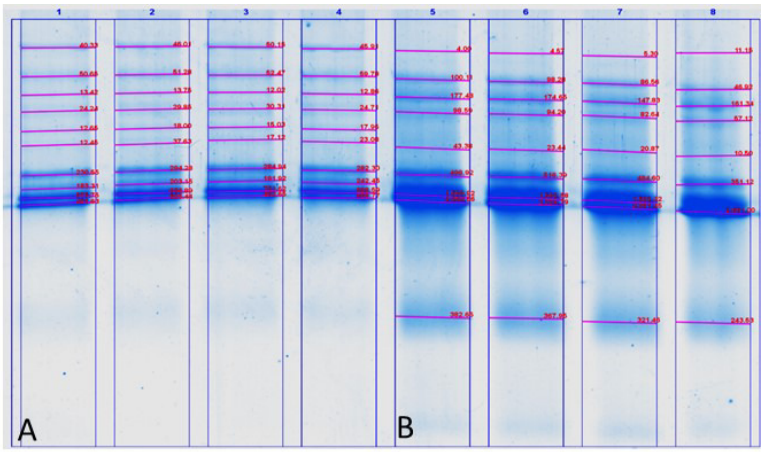

Figure 5. SDS PAGE of serum proteins from *Strongyloides ratti* infected rats of both non-treated (A) and treated samples (B) using Image Lab Software.

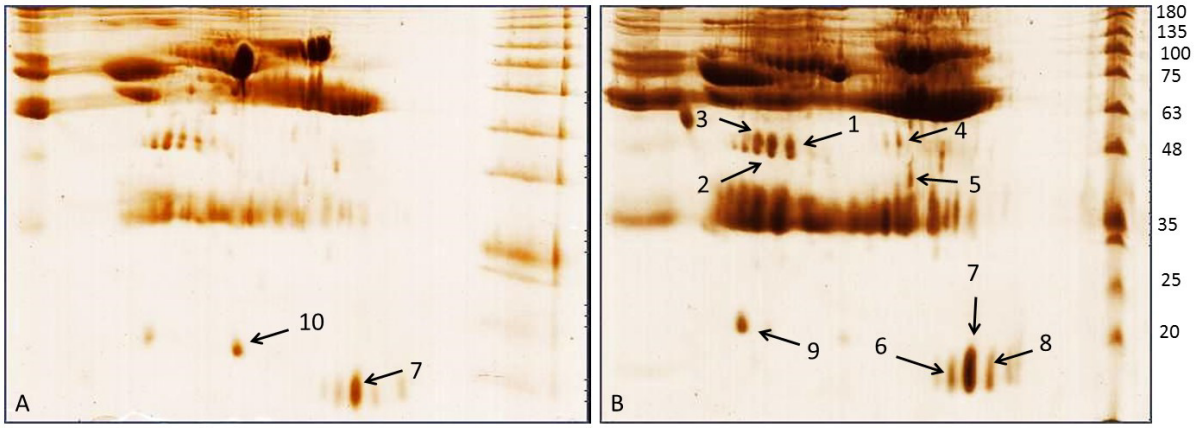

Figure 6. Two dimensional (2D) gel electrophoretic analysis of rat serum proteins from *Strongyloides ratti* infected rats for both non- treated (A) and treated samples (B) with a corresponding protein ladder.

Table 2. Differentially expressed proteins from sera of infected and treated rats as identified by MALDI-TOF/TOF analysis.

Spot	(Uniprot) Accession No.	Protein ID	Theoretical MW/PI	Score	No. of Matched peptides
1*	Q63041	Alpha-1-macroglobulin	167019/ 6.46	802	45
2*	Q63041	Alpha-1-macroglobulin	167019/ 6.46	77	2
3	Q6PAH0	Apolipoprotein	35741/ 5.23	896	38
4	P12346	Serotransferrin	76346/ 7.14	285	17
5*	Q63041	Alpha-1-macroglobulin	167019/ 6.46	316	28
6	P02091	Hemoglobin subunit beta-1	15969/ 7.88	641	29
7	P01946	Hemoglobin subunit alpha-1/2	15319/ 7.82	1476	78
8	P11517	Hemoglobin subunit beta-2	15972/ 8.91	386	18
9	P06866	Haptoglobin n=2	38539/ 6.10	1617	66
10	P02767	Transthyretin n=2	15710/ 5.77	1093	45

* Similar protein biomarker

Haemonchus contortus, adding to the growing number of nematode C-TL genes that share sequence similarity with mammalian immune cell lectins (Mulvenna *et al*., 2009).

The findings from this study have further highlighted the use of RT-qPCR as an assay for the relative quantification of expression of several genes from two samples (Corticosteroid-treated and non-treated). The effects of corticosteroid (prednisolone) treatment on the filariform larval stages were assessed by expression analysis of the studied genes. The expression level of nine (9) genes in the non-treated group was extremely low compared to that in the treated group. In contrast, one gene (*Arg*) was found to be down-regulated in the treated samples. Significant regulation mean factors of (0.017-29.86) were recorded in the study of all tested genes while comparing the two sample types.

The ultimate future goal would be to combine serum biomarkers with information gained from cytology, histology, immunophenotyping and molecular diagnostics of hyperinfection in strongyloidiasis in order to give an accurate prognosis for individuals who are on immunosuppressive therapy who might also have chronic strongyloidiasis. The two dimensional gel electrophoresis (2D) technique could be applied to assess other immunosuppressive drugs and under other conditions, in order to gain further knowledge on the prognosis of strongyloidiasis and possibly discover other potential candidate biomarkers. The expression of galectins has been previously studied by transcriptome analysis and characterized as reliable biomarkers of several diseases, especially in the progression of cancer diseases (Yoshimura *et al*., 2003; Balan *et al*., 2010).

One of the significant finding from this present study is that the up-regulation of galectin gene expression. The observations made in this study may equally provide a better understanding of the role of this parasitic galectin within the host immune system and hyperinfection in strongyloidiasis. Other studies (Mello *et al*., 2015; Wang *et al*., 2014; Yuan *et al*., 2015) have also reported that galectin acts an essential function in host-parasite relationship and represents one of the host immunomodulation associated molecules.

The up-regulation of all galectins in the filariform larvae treated with corticosteroid was noteworthy in this research. Importantly, galectin concentration can be measured in host serum and might be considered as a biomarker for hyperinfection in strongyloidiasis through the interaction between serum glycoproteins and different galectins. A well characterized glycoproteins mixture provided by serum can be exploited to test the function of galectin for natural ligand binding.

Parasitic galectins have shown an increase in gene expression during the infection and have consequently been taken into consideration as key players in parasite host interactions (Wu *et al*., 2008). In a related study (Wang *et al*., 2014), rHco-gal-m was documented to bind strongly to the monocytes and T cells of goat.

A cascade of trans-membrane signaling events in different biological processes may be triggered by this binding such as host immune cells activation and homeostasis (Vasta, 2009).

The proteomic analysis and the subsequent screening of ES products from *S. ratti* and *S. stercoralis* EST databases led to the identification of seven different galectins (Soblik et al., 2011).

In the same study, galectins represented an abundant protein family being secreted by *S. ratti* iL3, parasitic female and free-living stages. All of these galectins were only reported with chronic strongyloidiasis and there was no indication to relate these proteins to hyperinfection syndrome.

The present study demonstrates the feasibility of using RT-qPCR expression analysis as a technique to monitor relative gene expression profiles in *S. ratti* filariform larvae.

The adaptation of the RT-qPCR method has proved to be successful and the method was successfully applied for measuring changes in transcription of several genes in *S. ratti* larvae between the two samples. The study indicates that corticosteroid treatment may have stimulated the expression of some important gene, including five galectins in the larvae, without the expression of *Arg*.

The lower expression level observed in this gene whose protein was abundant in protein profile may indicate that the *Arg* gene may have mutated.

In summary, this study provided the first report for application of proteomics technology in hyperinfection in strongyloidiasis and the foundation for future studies that will be considered to specifically evaluate the variations of *Strongyloides* proteins during chronic and hyperinfection cases.

The proteomics-based detection in strongyloidiasis employed in this present study need to be supported by other studies to discover more phases and markers. Sensitivity and specificity of the characterized biomarkers should be further tested to establish sufficient validation before their efficacy can be evaluated using different diagnostic techniques.

Conclusion

The findings in the present study have observed up-regulation of galectin gene expression during hyperinfection stage and the existence of their encoded proteins in 1D and 2D protein profile of excretory-secretory products (identified by MS). This clearly suggests that these proteins are greatly secreted from the infective larvae during host immunosuppression and may play a crucial parasitic role in the autoinfection or hyperinfection processes in disease conditions. Further studies are required to evidently explain the role of the validated proteins in the parasitic process.

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