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*Full Length Research Paper*

# Gene expression profiling of selected *Strongyloides ratti* genes expressed in immunocompromised rat

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***Strongyloides stercoralis*** **is a soil transmitted helminth (STH) that can be fatal in hyperinfection cases in the immunocompromised patients. As a result of the short-comings associated with diagnosis, many chronic infections usually go unnoticed. This necessitates the urgent need to develop innovative methods for rapid diagnosis through detection of molecules (biomarkers) that can be sensitively and specifically measured. This study was aimed to investigate the expressions of some specific diagnostic genes from the components of *S. ratti* larval excretory/secretory products that are associated with early hyperinfection syndrome in an animal model. Ten (10) genes from samples of experimentally induced immunosuppression (prednisolone) and from non-treated (control) group were analyzed for relative changes in gene expression by real-time PCR. The results indicated that the RT-qPCR method has successfully monitored the transcriptional development of the selected genes. Using the relative quantification, the expression of 10 genes in two *S. ratti* samples were investigated. The results have shown that *Acon, Put, Gal 1, Gal 2, Gal 3, Gal 4, Gal 5, Lec,* and *Trop* genes were up-regulated in treated samples, while Arginine Kinase (*Arg*) gene was down-regulated. The cause of this change in expression of *Arg* remains to be determined. It is concluded that the success of the *S. ratti* genes profiling analysis may provide a new insight of the functional genes that play role during the early stage of hyperinfection. Thus, it may be a useful indicator for clinicians in order to predict the clinical outcome of disease in the immunocompromised patients.**

**Keywords:** Gene expression, profile, *Strongyloides ratti*, immunocompromised rat

## INTRODUCTION

Comparative proteomics research is fundamental to the detection of potential protein markers of pathogen prognosis (Kumar *et al.*, 2012). Proteomics techniques analyses proteins, the end product of transcription and translation of genetic material. The field of transcriptomics is concerned with the intermediate product, RNA. The analysis and measurement of messenger RNA (mRNA) levels within a system can provide information about diverse cellular processes earlier than would be possible by proteomics. One of the

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methods of measuring mRNA expression is by quantitative polymerase chain reaction (qPCR). This technique is highly accurate, but more suited to smaller numbers of sequences of interest (Vermont, 2012).

Real-time qPCR is a great tool and the most widely applied technique for the analysis and quantification of mRNA. Relative expression is widely used where the expression of a target gene is normalized by an unregulated reference gene (Pfaffl *et al.*, 2002). The real-time assay is a reaction of specific PCR amplification and real-time fluorescence monitoring. There is a correlation between the amounts of the target DNA or complementary DNA (cDNA) as a template and the PCR product during the cycles of the exponential amplification stage. The more copies of these templates are present at the beginning of a reaction, the fewer PCR cycles are required for material detection. Fluorescence data appear in graphical form and can then be collected by connecting a computer to the real-time machine (Pabinger *et al.*, 2014).The sensitive but less specific dye, SYBR Green is a simple method for revealing nucleic acids with real-time PCR to detect the product accumulation of double-stranded DNA or cDNA. The melting curve analysis is implemented in the green reaction to detect the melting temperature, which is used to determine the amplification products based on their length of amplification product and G+C content (Espy *et al.*, 2006).

Designing gene specific primers and choosing the target of DNA/cDNA are very important for the improvement of the real-time PCR test. Accurate results can only be obtained using primers which amplify a single product. The primer must be selected with low potency to prevent substructures, including cross-hybridization with itself and other oligonucleotides. The type and quality of polymerase enzyme used may also play an important role, by reducing the generation of fragments of sequences that are not specific, as these enzymes do not work until reaching a maximum critical temperature; therefore, hot start PCR is the preferred option (Burpo, 2001; Lorenz, 2012). The main purpose of this study is to analyze and validate the gene expression of identified biomarkers from the excretory/secretory (ES) products of the infective larvae of *S. ratti* at transcriptomic level using quantitative real time PCR.

## MATERIALS AND METHODS

### RNA Extraction

To avoid any RNase contamination during RNA extraction the RNA work station, gloves and pipettes were thoroughly sprayed with RNaseZAP Solution (Sigma-Aldrich, USA). RNase/ DNase free pipette tips and tubes (Eppendorf, Germany) were used all the time. One hundred thousand freshly harvested filariform larvae were thoroughly washed in DEPC-treated water (HiMedia, India) several times, and then the larvae were pelleted in a bottom of 15 mL conical tube by centrifugation at 5,000 x g. Tissue of larvae was ruptured with the aid of rapid freezing in liquid nitrogen and grinding in a glass mortar and pestle. RNA extraction was performed by using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Finally, the RNA was eluted with 30-50 µL of RNase-free water.

### DNase treatment

The presence of DNA contamination in the RNA preparations was removed using 5 µL of RNase-free DNase I and 5µL of gDNA Wipeout buffer (Qiagen, Germany) at 37 °C for 10 min and at 42 °C for 3 mins, respectively. Then, to inactivate the DNase I enzyme, 5 µL of 25 mM EDTA was added to the mixture and incubated at 65°C for 10 min. Purified RNA was aliquoted and stored at -80°C until required.

### Measurement of RNA concentration, purity, and integrity

Isolated RNA was quantified and the purity was evaluated using the absorbance ratio A260/A280 as an indicator of protein contamination and A260/A230 absorbance ratio as an indicator of phenol, polysaccharide, and/or chaotropic salt contamination. The values in the range of 1.9 to 2.1 were considered pure using NanoDrop® ND-1000 UV-VIS spectrophotometer (ThermoFisher 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). Only RNA samples with an integrity number (RIN) greater than 8.0 were used.

### cDNA Synthesis

Single stranded cDNA was synthesized from the purified RNA using RevertAid first strand cDNA synthesis kit (Fermentas Life Science, USA) with random hexamer primer according to the manufacturer's instructions. Briefly, 1 µL of total RNA (200 ng/µL) was mixed with 1µL of random hexamer primer (0.2 µg/ µL) and RNase-free water up to 12 µL, mixed gently and incubated at 65 °C for 5 min to remove any secondary structure. After chilling on ice for 5 min and centrifugation, the following components were added in the indicated order; 4 µL of 5x reaction buffer, 1 µL of Ribolock RNase inhibitor (20 u/µL), 2 µL of 10 mM dNTP Mix, and 1 µL of RevertAid M-MuLV Reverse transcriptase (200 u/µL). The mixture was incubated at 25°C for 5 min followed by 60 min at 42°C. The reverse transcriptase reaction was terminated

by heating at 70°C for 5 min. To measure the concentration, the cDNA template was assigned a concentration unit relative to the original concentration of RNA loaded in the Reverse transcriptase reaction. cDNA was synthesized from 200 ng of total RNA from both samples. The synthesized cDNAs were then diluted 1:50 dilution factor with DNase-free water as a final concentration. The samples to be used to generate the standard curve of efficiency test were not diluted at all. The synthesized cDNA template was stored at -20 °C until required for RT-PCR.

**Verifying the Integrity of cDNA**

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. The intact cDNAs were aliquoted and stored at -20°C until required.

**Primer Design and their Specificity for RT-qPCR**

Sequences of target DNAs were selected and primers were designed from the database of the National Center for Biotechnology Information (www.ncbi.nih.gow). The primers were submitted to BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to check their specificity (determine if they would align to any other nematode sequences). Gene specific primer sets were ordered from Integrated DNA Technologies (IDT, Singapore) (Table 1). The stock primers were supplied in lyophilised form and dissolved in Nuclease-Free Milli-Q water (Merck Millipore, USA) to prepare a stock concentration of 100 µM. The working dilution was 10 µM and the primers were stored at -20°C in small reaction aliquots. Prior to quantitative PCR (qPCR) analysis, primers were tested in conventional PCR reaction with the same settings as were used in qPCR using cDNA templates. PCR was performed using GoTaq green master mix (Promega, Madison, USA) in thermocycler (Biometra, Germany). PCR products were separated on 1.5% Agarose gel in TBE buffers containing GelRed stain (Biotium, USA) and visualized under UV light. Gel electrophoresis was performed to check the presence of single product and absence of primer dimers.

**Optimization of annealing temperature**

Gradient PCR was used to determine the optimum annealing temperature. The PCR products were analyzed by 1.5 % Agarose gel electrophoresis, and the temperature that yielded the strongest band of the right size was chosen. Moreover, all positive specimens were evaluated by electrophoresis on 1% Agarose gels. Gels were run for one hour at 100 volts, stained with GelRed, and visualized using ultraviolet light.

**Validation of efficiency of real-time primers**

In order to determine the efficiency of the designed primers, gene specific primers and those of selected endogenous genes were calibrated using 3-5 dilution series of total cDNA as a template and efficiencies calculated using standard curve generated by a real time thermal cycler (Mastercycler ep Realplex, Eppendorf, Germany). The reactions were performed in three biological replicates, each in three technical replicates. From these reactions, a standard curve was generated for each primer. In addition, melting curves were also performed by the software on the same qPCR machine. Primers were excluded from further experiments if they gave more than one single band or if they form primer dimers.

**Endogenous Control for Relative Quantitation**

Two housekeeping genes, *Sr*-GAPDH and 28S rRNA were evaluated as reference gene candidates. Forward and reverse primers of the endogenous genes were ordered as earlier mentioned above for the specific genes. Their stability was determined by BestKeeper software(http://www.wzw.tum.de/gene-uantification/bestkeeper.html) (Pfaffl *et al.*, 2004) in order to achieve accurate and reliable gene quantification.

**Semi-quantitative real time PCR**

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 10 µL 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 reaction conditions were as follows: 95°C for 2 minutes (polymerase activation) followed by 40 cycles of 95°C for 5 seconds (denaturation), 60-65°C for 10 seconds (annealing) and 72°C for 20 seconds (extension). Collection of data, by detection of the SYBR dye, occurred at the final phase of each cycle; this enabled a crossing threshold (Ct) value to be determined for each well. This value shows at how many PCR cycles the linear phase of the PCR reaction crossed a predetermined threshold. Real-time PCR runs were performed in triplicate for each gene under test. The baseline and threshold for each of the three runs for each gene were set identically and the Ct values recalculated in order to compare between the run data to be made.

**Analysis of real-time data**

The raw Ct values for the 10 target genes and the *Sr*-

**Table 1:** Real Time PCR primer sets of larval Excretory/Secretory genes

| Primer | Sequence 5-3 | Size | Reference |
|---|---|---|---|
| **Acon** | ACACACGCTTTTGTTGCTTCA<br>CATCTCCACTTGGTGGGTCAA | 136 | Present study |
| **Put** | TGGGGAAACAGTATCGTCCAT<br>GCTGCTCTTGCTACCTGTGA | 115 | Present study |
| **Arg** | ATCAAAGGTCAAAGCGGTCT<br>GGATAGTAGGTGCCTCCCAA | 66 | Present study |
| **gal 1** | CAAGCTGGAGAATGGGGTAATGAGG<br>ATCACAACGATGAGCAAAAGTGCAG | 150 | Younis, 2011 |
| **gal 2** | GGAATGCCTGAAAAAAAAGGTAAACG<br>CTCTCTCTTCATTACCCCATTCACC | 148 | Younis, 2011 |
| **gal 3** | TGAGCATCGTGTACCACTTTC<br>ATAAGACTTTTTCCAGGAACTAACC | 150 | Younis, 2011 |
| **gal 4** | GGCTGTCGTTCGCAATAACC<br>TGAGCAAAAGTGCAGAAGCG | 162 | Present study |
| **gal 5** | TTGAAACTCCATATACTGCTCTTGC<br>AGCTGATTCACCATATCTAATTGAGAC | 152 | Younis, 2011 |
| **Lec** | ACCCCTGTTCCATTACCAACAA<br>TTTGGGATAGCCTGGGGAGG | 117 | Present study |
| **Trop** | TGTCGTCGGAAATAACCTGA<br>TGGTACGAATCTGCTCTTCG | 89 | Present study |
| **GAPDH** | GTACCACTAACTGTTTAGCTCC<br>GCACCTCTTCCATCTCTCC | 154 | Younis, 2011 |

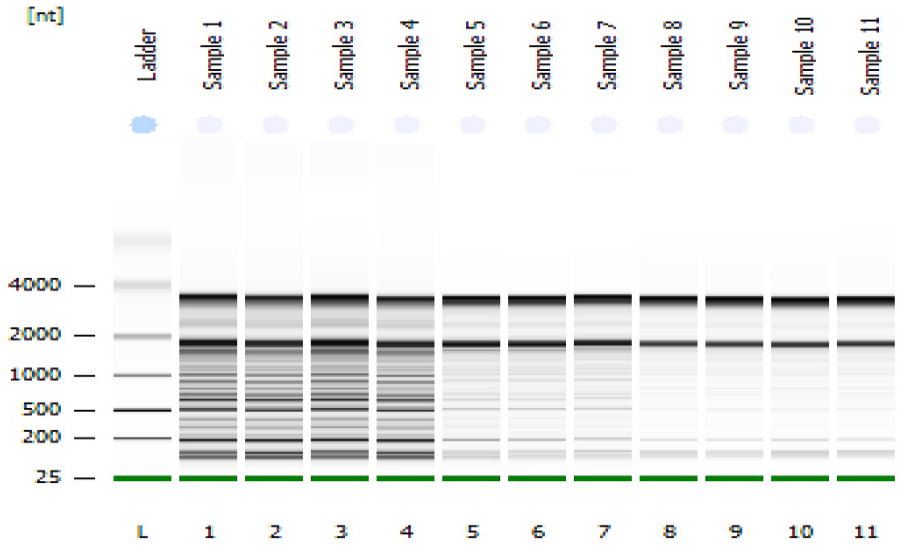



**Figure 1:** Virtual gel images of eleven RNA samples isolated from treated and non-treated filariform larvae of *Strongyloides ratti*.

GAPDH (endogenous control) gene for non-treated (control) and treated (prednisolone) samples 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. The regulation mean factor in expression of a particular gene reflected the cDNA level between two different samples (non-treated and treated).

### Visualization of PCR products

Five microliter (5µL) of the PCR reaction was loaded onto a 1% Agarose gel containing 1X TBE buffer (Ready to use, Vivantis, UK) and GelRed (Biotium, USA) to visualize qPCR products. Electrophoresis was carried out at 70-100V for 60 min to allow separation of any qPCR products according to their band sizes. The gel was visualized under UV light and the size of the bands was

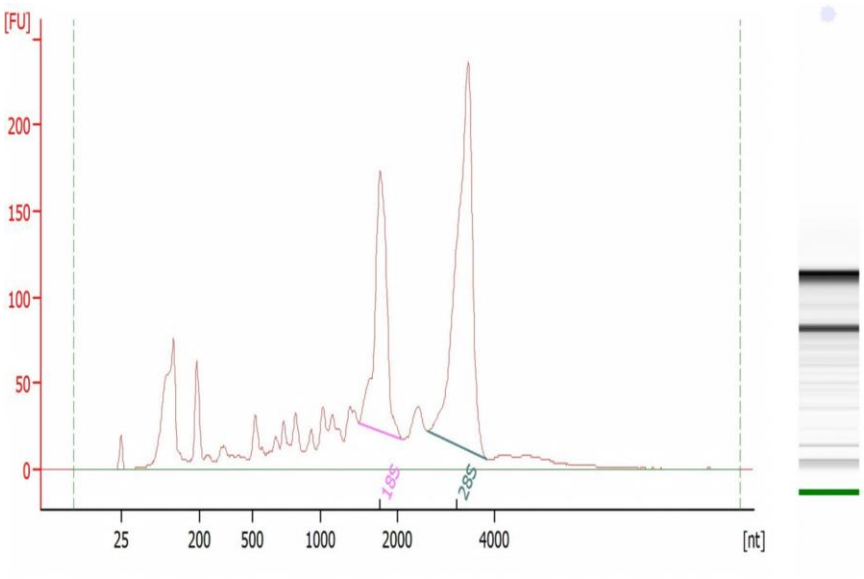
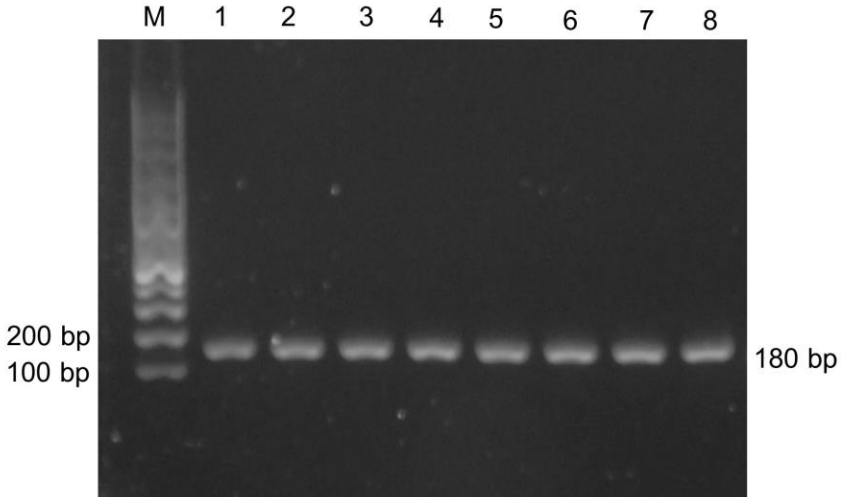



**Figure 2:** Bioanalyzer analysis of RNA sample (RIN: 8.40) selected for RT-qPCR. Two peaks (left) and virtual gel image shows the predominant two bands, 18S and 28S rRNAs (right).




F**igure 3:** The integrity of the constructed cDNA using agarose gel electrophoresis. M: DNA Ladder; 1-8 test samples

detected against DNA molecular weight marker VC100 bp (Ready to use, Vivantis, UK).

### Statistical analysis

A standalone tool, REST 2009 software was used for gene expression analysis of data from qPCR experiments. The analysis or quantitation of relative gene expression used reference gene expression to normalize the expression levels of genes of interest (GOI) in treated and control samples.

## RESULTS

### RNA concentration, purity, and integrity

The concentration and purity of the extracted RNA was checked using a NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, USA) and the result of the A260/A280 ratio was approximately close to 2, indicating pure RNA (free of DNA and protein). The integrity was initially checked using Agarose gel electrophoresis which observed two clear bands

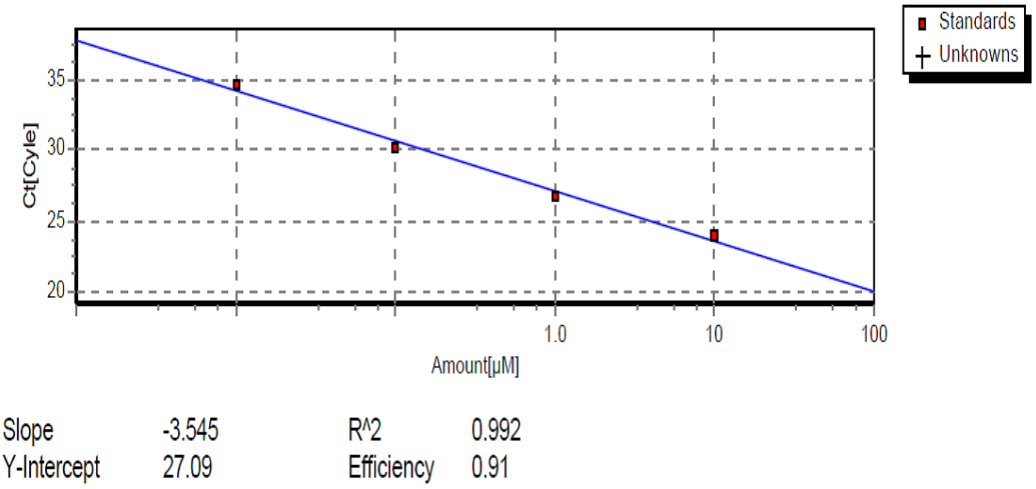



**Figure 4:** Standard curve of the primer efficiency test of *Sr*-GAPDH housekeeping gene generated by RT-qPCR.

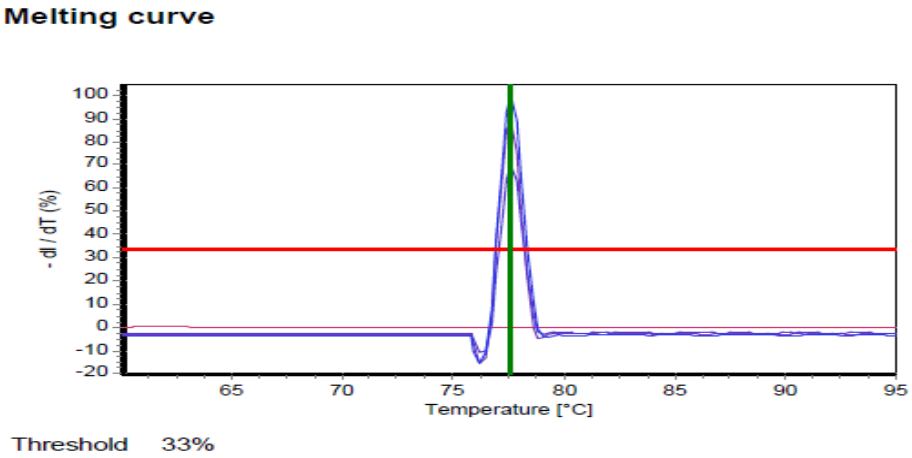



**Figure 5:** Melting curve of *Sr*-GAPDH housekeeping gene primer generated by RT-qPCR.

**Table 2:** Standard curve analysis (Slope, Reaction Efficiency, Correlation coefficient, and Y-intercept) for each of the target genes.

| Gene | Slope | Reaction Efficiency | Correlation coefficient (R2) | Y-intercept |
|---|---|---|---|---|
| ***GAPDH*** | -3.545 | 0.91 | 0.992 | 27.09 |
| ***Acon*** | -3.252 | 1.03 | 1.000 | 24.10 |
| ***Put*** | -3.194 | 1.06 | 0.994 | 26.34 |
| ***Arg*** | -3.510 | 0.93 | 0.995 | 27.66 |
| ***Gal 1*** | -3.241 | 1.04 | 1.000 | 27.77 |
| ***Gal 2*** | -3.200 | 1.05 | 0.999 | 27.31 |
| ***Gal 3*** | -3.235 | 1.04 | 0.994 | 25.16 |
| ***Gal 4*** | -3.113 | 1.1 | 0.999 | 23.77 |
| ***Gal 5*** | -3.232 | 1.04 | 0.975 | 25.12 |
| ***Lec*** | -3.159 | 1.07 | 0.964 | 24.84 |
| ***Trop*** | -3.084 | 1.1 | 0.998 | 24.15 |

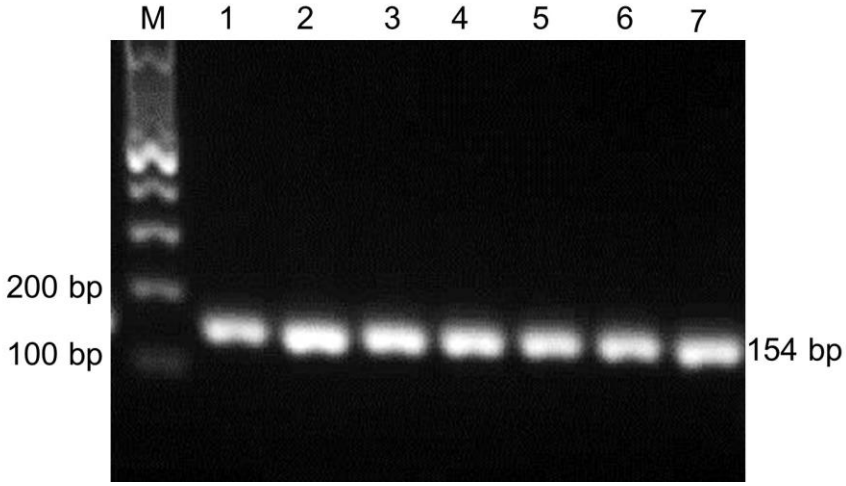



**Figure 6**: Agarose gel electrophoresis representing a 154 bp qPCR product of *Sr*-GAPDH reference gene. M: DNA Ladder; 1-7 test samples

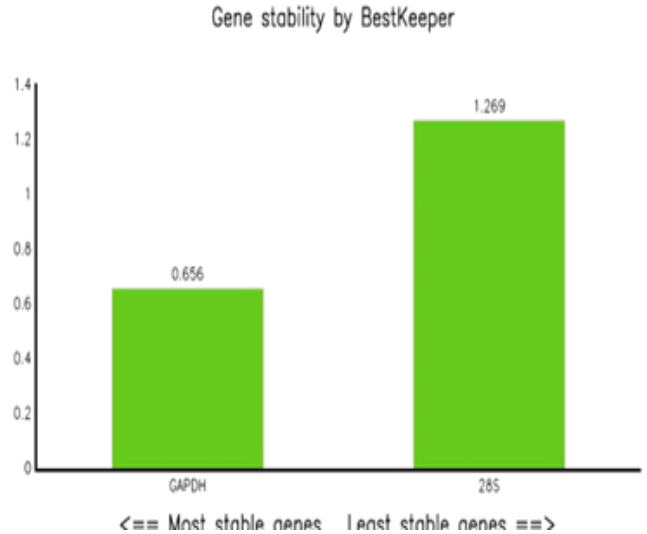



**Figure 7:** Expression stability of two candidate reference housekeeping genes, *GAPDH* and 28S rRNA generated by BestKeeper software**.**

indicating 18S and 28S (Figure 1). The integrity was then confirmed by Bioanalyzer (Agilent Technologies, USA) (Figure 2).

Clear and sharp peaks which indicated the two dominant RNA fragments 18S (lower band) and 28S (upper band) rRNAs were observed, RINs of 8.30 - 8.40 (RIN range 1.00 - 10.00) were also recorded from the Bioanalyzer report of some of the tested samples which were appropriate for any downstream applications, including RT-qPCR. The samples representing treated and non-treated RNA were chosen for the next experiments (Figure 3). Samples which contained degraded RNA (RINs 3.00-5.70) and partly degraded RNA (RINs 5.80 -7.00) were excluded from further analysis.

**Integrity of cDNA**

In this present study, 200 ng of RNA was loaded into a 20 µL RT reaction. Thus, the designated concentration of the resultant cDNA was 10 ng/µL which means that 1uL of the sample consist of the cDNA constructed from 10 ng of RNA. The integrity of synthesized cDNAs was evaluated using the conventional PCR assay method. A 180 bp amplification band was obtained from all the RNA samples (Figure 3).

**Table 3:** Relative gene expression values of treated and non-treated samples

| Gene | Type | Reaction Efficiency | Expression | 95% C.I. | P(H1) | Result |
|---|---|---|---|---|---|---|
| ***GAPDH*** | REF | 0.91 | 1.000 | | | |
| ***Acon*** | TRG | 1.03 | 13.456 | 10.603 - 17.112 | 0.000 | UP |
| ***Put*** | TRG | 1.06 | 10.163 | 7.786 - 13.272 | 0.000 | UP |
| ***Arg*** | TRG | 0.93 | 0.017 | 0.009 - 0.032 | 0.000 | DOWN |
| ***Gal 1*** | TRG | 1.04 | 13.692 | 9.923 - 18.946 | 0.000 | UP |
| ***Gal 2*** | TRG | 1.05 | 19.633 | 13.982 - 27.802 | 0.000 | UP |
| ***Gal 3*** | TRG | 1.04 | 4.627 | 3.531 - 6.067 | 0.000 | UP |
| ***Gal 4*** | TRG | 1.1 | 8.001 | 3.240 - 20.254 | 0.000 | UP |
| ***Gal 5*** | TRG | 1.04 | 3.261 | 1.054 - 10.109 | 0.000 | UP |
| ***Lec*** | TRG | 1.07 | 6.256 | 4.604 - 8.502 | 0.000 | UP |
| ***Trop*** | TRG | 1.1 | 29.862 | 9.208 - 98.286 | 0.000 | UP |

TRG: Target REF: Reference

Boxplot

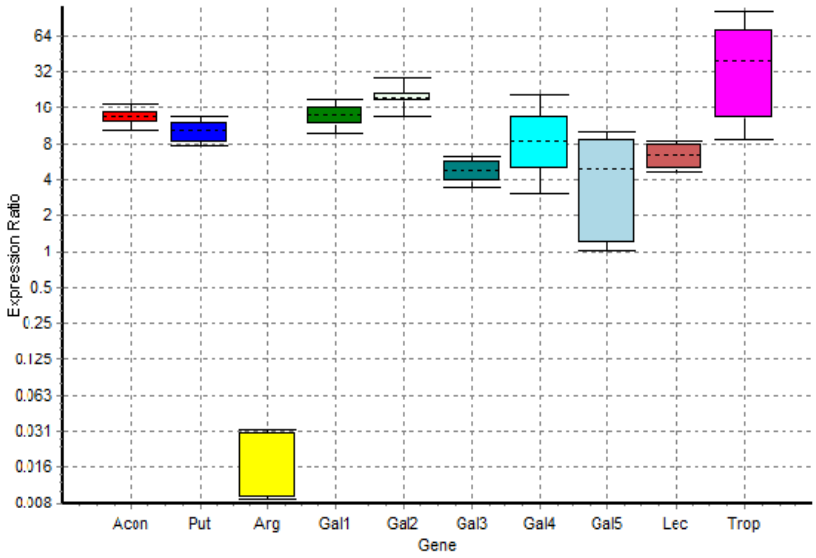



**Figure 8:** Relative gene expression folds. The dotted lines represent the median gene expression. The whiskers represent the upper and lower observations.

## Primer efficiency and specificity for RT-qPCR

Primer sets designed to target the respective genes in the current study were used for RT-qPCR and real time software of Mastercycler ep Realplex (Eppendorf, Germany) and gave approximately -3.3 and 1 for curve slope and correlation coefficient respectively (Figure 4). The melting curve analysis of each primer pair revealed a single peak indicating their specificity for the respective genes and absence of primer dimer (Figure 5). The details of the standard curve analysis, which contains reaction efficiency and related slope, correlation coefficient, and Y-intercept for each of the genes is as shown in Table 2.

## Endogenous genes

The expression stability of the two endogenous reference genes (Figure 6), *Sr*-GAPDH and 28S rRNA was evaluated using BestKeeper software. GAPDH showed a constant and a stable expression profile over 28S rRNA and had the higher *M* value (Figure 7). Therefore, *Sr*-GAPDH was included as an internal control to normalize qPCR data.

## Semi-quantitative real time PCR

Ten genes from the samples treated with corticosteroid and non-treated (control) were analyzed for relative

changes in gene expression by qPCR. Evaluations of real time PCR expression profiles demonstrated that *Acon*, *Put*, *Gal1, Gal2, Gal3, Gal4, Gal5*, *Lec*, and *Trop* genes were up-regulated in the treated sample in comparison to the non-treated ones (control) by a mean factors ranged from 3.26 for the *Gal*5 gene to 29.86 for the *Trop* gene. In contrast to all the above genes, the Arginine Kinase (*Arg*) gene was down-regulated in the treated sample in comparison to the control by a mean factor of 0.017 (Table 3; Figure 8).

## DISCUSSION

Proteomics technology was successfully implemented in the discovery stage of biomarker detection, recognizing several proteins that differentiates the healthy condition from the disease (Gao *et al.*, 2005; Kang *et al.*, 2005). Nevertheless, this stage should to be followed up by other steps for validating these markers and establishing their efficacy (Good *et al.*, 2007), using one of the currently available techniques that measures the change in mRNA transcription of gene-encoded biomarkers. Quantitative real-time PCR is one of the most commonly used techniques for quantitatively measuring DNA/cDNA in various samples and is considered a useful tool in biotechnology, molecular medicine, microbiology and diagnostics. RT-qPCR combines reverse transcription with qPCR and is currently the method of choice for amplification and detection of low levels of mRNA gene expression in any biological matrixes. The expression levels in many different samples for a limited number of genes can be measured using this application (Bustin, 2000; Bustin *et al.*, 2009; Higuchi *et al.*, 1993).

RNA quality assessment is a fundamental step in RT-qPCR and RNA integrity must be checked in every preparation. This can be done by inspecting the two distinct transcriptional products (bands) from 28S and 18S RNA, which are the predominant RNAs. The conventional method of performing this inspection is gel electrophoresis. However, this method is time consuming and requires high amounts of RNA. Measurement of spectrometric OD is another method which can be carried out to measure the quantity and check the purity of extracted RNA at different wavelengths (Fleige and Pfaffl, 2006). These conventional methods are, however, not sensitive enough to detect RNA degradation. The Agilent 2100 Bioanalyzer PicoChip (Agilent Technologies, USA) has gained increased attention for RNA quality and quantity assessments (Imbeaud *et al.*, 2005). RNA samples extracted in this study and assessed using the two conventional methods above, had their integrity confirmed using the Bioanalyzer PicoChip kit and achieved high RNA quality (RINs) for both treated and non-treated samples which were then used to synthesize cDNA as a template for the qPCR experiment. Changes in mRNA transcription levels are critical in many biological processes and RT-qPCR has been frequently applied to measure the effects of various compounds or experimental conditions as to how these compounds, or altered conditions, affect organisms and cells at the molecular level and to what extent a certain gene is expressed at a certain time (Bustin, 2000).

Evaluation of the amplification efficiency is a crucial step and reliable indicator in quantification procedures of real-time gene expression. The amount of PCR product is doubled with each cycle in case of 100% amplification efficiency. This is not always the case and different PCR reactions can have different amplification efficiencies that should therefore be calculated for each PCR assay (amplicon specific efficiency) (Kubista *et al.*, 2006). Fluorescence measurement is monitored during each cycle, and the slope in the plot of fluorescence intensity versus cycle number should be equal to the logarithm of the efficiency. The value of the standard curve slope is used to determine the exponential amplification, PCR efficiency, and the regression and correlation coefficients (expressed as R2) (Higuchi *et al.*, 1993). Numerous factors may affect the amplification efficiency such as inhibitors in the sample, interference of the secondary structure, the design of primers, and how well the PCR conditions are optimized (Kubista *et al.*, 2006). Differences in amplification efficiency can lead to under- or over-estimation of the number of copies in the sample, due to the relationship between amplification efficiency and the expected target copy number. Dropping of approximately 80% of product amount can be a result of a 10% amplification efficiency reduction (Tichopad *et al.*, 2003).

The use of a reference gene (in the current study, *Sr*-GAPDH) in the qPCR experimental procedure was for normalization of the tested gene results, taking into account any probable cDNA level variation, thereby eliminating the variation errors of the tested samples. Using reference genes for normalization relies on the fact that a constant expression level should be achieved from the ideal reference gene in all tissues or cell types, at all developmental stages and not be affected by any experimental treatment. This means that the reference gene expression should remain stable while the expression of the gene of interest could be up-regulated or down-regulated. It is necessary to validate and confirm that the reference gene is stably expressed in each experiment sample before they can be used for normalization (Bustin, 2002; Stürzenbaum and Kille, 2001).

Over the years, most commonly used reference genes have included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal genes, e.g. 18S and 28S rRNAs, hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin and elongation factor 1-α (EF1-α) (Stürzenbaum and Kille, 2001; Thellin *et al.*, 1999). More and more studies have indicated that expression of these reference genes is

indeed affected by different tissues/cell types or experimental conditions (Radonić *et al.*, 2004; Thellin *et al.*, 1999). The use of unstable reference genes for normalization may greatly influence the gene expression results by increasing the noise of the assay, thereby small expression changes of the target gene might not be detected (Huggett *et al.*, 2005). *Sr*-GAPDH was used for the first time as a housekeeping reference gene as previously reported (Younis *et al*., 2011). At the time of carrying out this experiment, this gene was chosen as the control gene as it appeared to be more stably expressed between different *S. ratti* genes compared to 28S rDNA, which can be observed in the recorded Ct values from the semi-quantitative PCR results and BestKeeper software, supporting the previous studies that used *Sr*-GAPDH as a housekeeping gene which showed a stable and constant expression level in all parasitic and free-living life cycle stages of *S. ratti* (Younis *et al*., 2011).

In this study, RT-qPCR was used as an assay for the relative quantification of expression of several genes from control and those treated with corticosteroid. For data analysis, REST-2009 software was used. This software tool uses statistical whisker-box plots in which the area of the box includes 50% of all observations, spotted lines represent the median of the samples and the whiskers represent the outer 50% of the observations (Pfaffl, 2006). The purpose of using this software tool was to detect the changes in target gene expressions standardized by a non-regulated reference gene, and to check and assess any significant differences if present in gene expression profile between *S. ratti* filariform larvae samples exposed to corticosteroid and samples without treatment as controls. However, little is known about the relative quantification of the expression of these genes at transcriptomic level and all available information is not related to hyperinfection syndrome.

Galectin acts an essential function in host-parasite relationship and represents one of the host immunomodulation associated molecules (Wang *et al.*, 2014; Mello *et al.*, 2015; Yuan *et al.*, 2015). This study may provide a better understanding of the role of this parasitic galectin within the host immune system and hyperinfection in strongyloidiasis. The up-regulation of all galectins in the filariform larvae treated with corticosteroid was noteworthy in this research.

The expression of galectins has been studied by transcriptome analysis and characterized as reliable biomarkers of several diseases, especially in the progression of cancer diseases. The expression of *galectin*-3 was reported different in gastric carcinoma patients in comparison to individuals with normal tissue. The gene expression level was significantly lower in the normal tissue in comparison to the primary tumour in 55% of the cases with well-differentiated tubular carcinoma ( Lotan *et al.*, 1994; Balan *et al*., 2010). Yoshimura *et al.*, (2003) studied the levels of *galectin*-3 mRNA using qRT-PCR in cell lines of lung cancer and in tumour tissue isolated by thoracoscopic biopsy. This gene down-regulated three times lower in normal epithelial cells compared to the examined population with the non-small-cell lung cancers. The gene expression of two galectins (*galectin*-1 and *galectin*-3) was also examined. *Galectin*-1 mRNA levels were highly increased in 38 human bladder transitional carcinomas of different histological grades and clinical stages compared to five normal urothelium samples or low-grade tumors (Balan *et al.*, 2010).

Parasitic galectins have showed 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 the study of Wang *et al.* (2014), *rHco-gal-m* strongly bound to the monocytes and T cells of goat. A cascade of transmembrane 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, 2009). In that 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.

MHC-II molecules are expressed on the antigen-presenting cells (APCs) surface for extracellular antigens presenting and creating the adaptive immune response (Kaufmann and Schaible, 2005). APCs activation lead to increase in the expression of MHC-II (Chamuleau *et al.*, 2006). Barrionuevo *et al.*, (2007) mentioned that the expression of constitutive and inducible MHC-II can be inhibited by galectin in human monocytes. Interference of galectin with MHC-II-dependent antigen presentation was also reported. Wang *et al.*, (2014) also noted that *Haemonchus contortus* galectin, *rHco- gal-m* has ability to inhibit the expression of MHC-II in monocytes in certain doses. This could be due to the deactivation of monocyte elicited by high amounts of interleukin-10 (IL-10) (Gordon and Martinez, 2010). However, the real mechanisms still need further investigation.

## CONCLUSION

This study represents the first attempt to employ large-scale transcriptomic analysis to identify the relative expression of gene-encoded biomarkers identified from ES products of *S. ratti* in the immunocompromised rat. The results indicated that the RT-qPCR method successfully monitored the transcriptional development of the ten selected genes. The results have also shown that prednisolone treatment have stimulated the expression of galectins, as they are overexpressed in the treated

samples compared to those in the control group. Comparatively, some genes were not affected by the treatment. Further studies are essential to detect the effects of prednisolone treatment on *Arg* gene expression and possibly to investigate the effects of some other immunosuppressive drugs used in *Strongyloides*-infected individuals.

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