

Identification of upregulated immune-related genes in *Vibrio harveyi* challenged *Penaeus monodon* postlarvae

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Abstract

A subtracted cDNA library was constructed and analyzed to elucidate the response of *Penaeus monodon* postlarvae challenged with *Vibrio harveyi*. As many as 960 randomly selected cDNA fragments generated through suppression subtractive hybridization were single pass sequenced. Fortyfive genes and 20 hypothetical proteins were identified, a few being first reports from shrimps. The most abundant immune relevant genes were ferritin, hemocyanin, and TCTP (translationally controlled tumor protein) indicating their up-regulation as also confirmed through qPCR. Post-infection qPCR analyses confirmed 2.04, 2.09, 3.28, 5.49, 6.47, and 11.63 fold rise respectively in ferritin, penaeidin, MnSOD, lysozyme, TCTP, and hemocyanin genes. These genes may be involved in the regulation of the host defense against *V. harveyi*.

Keywords: Shrimp diseases, *Penaeus monodon*; *Vibrio harveyi*; EST; cDNA library; SSH; qPCR

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1. Introduction

Shrimp aquaculture continues to face the disease threats including those caused by pathogenic bacteria that gain entry into the hatchery systems through water supply or food sources. Among reared shrimps, microbial infections are usually associated with high incidence of larval deformities and, mortality rates [1]. Vibriosis caused by *Vibrio harveyi* is one such prevalent bacterial disease which causes mass mortalities in larval cultures and shrimp production [2].

For sustainable-shrimp farming, systematic approaches to prevent and/or control diseases in particular at the early life-stages of shrimps are of great significance. An understanding therefore, of molecular responses and defense mechanism(s) in shrimps against bacterial pathogens is essential. Besides production of antimicrobial biomolecules, the innate immune/defense mechanisms in crustaceans involve phagocytosis, pathogen encapsulation, nodule formation, agglutination and prophenoloxidase (proPO) activating system [3]. However, the attempts to understand the molecular responses in the highly susceptible post larval stages of shrimps are rare.

Several genes have been shown to be involved in antibacterial response of shrimps. They have been isolated and characterized earlier. Nonetheless, responses at molecular level in the post larval stages of *Penaeus monodon* to *Vibrio harveyi* are not available. In this study, we aimed to identify the genes induced in early post-larval stages (PL10-14) of *P. monodon* challenged with a virulent strain of *V. harveyi*. We applied suppression subtractive hybridization (SSH) technique [4], a powerful tool that allows for isolating differentially expressed, tissue specific transcripts [5]. We used SSH to identify such transcripts in the 10-14 d old post-larvae following their exposure to a virulent strain of *V. harveyi*. We also quantified six major, defense related transcripts through quantitative PCR to ascertain the results of upregulated transcripts obtained following the SSH.

2. Materials and Methods

2.1. Immune challenge of postlarvae

Ten-day old post larvae (PL) of *P. monodon* (Crustacea, Decapoda) were obtained from a commercial hatchery and, held in the laboratory in a 500 L tank filled with seawater under continuous aeration. The

PL were fed twice daily with a commercial artificial feed and, maintained throughout the 3 day experimental period under aeration at 25°C and 29 psu salinity.

Replicates of two glass containers of 2-liter capacity were filled with one liter filtered autoclaved seawater. PL14 of *P. monodon* (60 nos/container) were acclimatized for a day under aeration. A strain of *V. harveyi* D3 (identity confirmed through partial sequencing of its 16 S rRNA gene) found to cause lethargy or mortality in all 3 trial runs was used for challenging the PL. Broth cultures of this strain grown for 24h in seawater nutrient broth were used for the experimental challenge. Cells were harvested by centrifugation and re-suspended in sterilized seawater and the PL were ‘bath’ challenged by adding the harvested cells to attain 2.5- 3.2 (X 10⁶) cells ml⁻¹ in the experimental containers for 48hrs. Two glass containers filled with one liter filtered autoclaved seawater holding ~60 PL with no added bacterial cells served as experimental controls.

2.2. RNA Isolation

Total RNA was isolated from both challenged and control populations. PL were first washed in sterile seawater and, 35 -40 whole PL (weighing ~500mg after blotting) were used for RNA extraction using Trizol Reagent (TRI, Sigma, USA). The concentration and purity of RNA samples were determined spectrophotometrically by measuring the absorbance at 260nm. An aliquot of RNA sample was run on 1% formaldehyde agarose gel for checking structural integrity. Later, poly A⁺ RNA was extracted from the control and tester sets of PL using Oligotex mRNA Kit (Qiagen, Valencia, CA).

2.3. Suppression subtractive hybridization (SSH)

SSH technique [4] was performed to generate subtracted cDNA library between *V. harveyi*-challenged (tester) and control (driver) PL using the PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol. Briefly, tester and driver cDNA products were purified by ethanol precipitation, and then digested with *Rsa* I at 37 °C overnight to obtain shorter, blunt-ended fragments. Following this, adaptor 1 and adaptor 2R were ligated to each strand of tester cDNA. Then, the ligated tester cDNA strands were hybridized at 68 °C for 8 h with an excess of driver cDNA after denaturation at 98 °C for 90s. After the first hybridization, two sets of adaptor ligated cDNA were mixed together without denaturation and hybridized again with freshly heat-denatured driver cDNA for 20h at 68 °C. The resulting mixture was diluted 1:50 and amplified by two rounds of PCR to enrich desired

cDNA (i.e., containing both adaptors) through exponential amplification. The primary PCR was performed with flanking primers against adaptor 1 and adaptor 2R and, the amplified products were used as template in the secondary PCR with nested primers. The efficiency of SSH was evaluated by another PCR protocol by comparing the abundance of β -actin gene in the subtracted and unsubtracted populations.

2.4. Cloning, sequencing and sequence similarity analyses

The secondary PCR products were purified and cloned into pGEM-T easy vector (Promega, Madison, USA) and transformed into *E.coli* Top10 electrocompetent cells through electroporation. The transformed mixture was plated on agar plates containing ampicillin, X-gal, and IPTG. Randomly selected white colonies were single pass sequenced. All raw sequences were manually screened for vector sequences using Vecscreen available at the <http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>. After removal of vector and exclusion of poor quality regions from the sequences, the ESTs were analyzed using BLASTX and BLASTN algorithms. Database searches were limited to ESTs > 100 bases in length. Blast matchings with E-value < 10^{-3} were considered as significant and only these were considered for categorization of ESTs into different groups.

2.5. Expression analyses of selected genes by Real time PCR (qPCR)

To further confirm the changes in expression induced by bacterial challenge, real time gene expression analyses of six different immune related transcripts were carried out. Total RNA was treated with DNaseI at 37 °C for 30 min to ensure genomic DNA-free RNA preparation. The first strand cDNA was synthesized from 1 μ g of DNA-free total RNA with oligo-dT primers and Superscript III reverse transcriptase (Invitrogen). Relative expression levels of hemocyanin, ferritin, TCTP, lysozyme, penaeidin and MnSOD were measured using the primer pairs listed in Table 1. Shrimp β -actin gene was used as an internal control during all the qPCR runs. The relative fold of induction was calculated using comparative C_T method for relative quantification. The amplifications were carried out in a 96 well plate in a total volume of 25 μ l using 4 μ l of cDNA, 200nM of each gene-specific primers pair with 2X SYBR Green Jump Start Taq Ready Mix (Sigma) in an ABI 7500 Real-Time PCR system. Cycling parameters were 95°C for 15 min, followed by 40 cycles of 95 °C for 15s and 60°C for 1 min. The specificity of

PCR products was confirmed by dissociation curve analysis performed at the end of qPCR by continuously heating from 55 °C to 95 °C with an increment of 0.1 °C. Data were analyzed using the ABI 7500 software version 2.0.1. The relative fold of induction was determined by $\Delta\Delta C_T$ method [6]. The fold change was calculated using the formula $2^{-\Delta\Delta C_T}$.

3. Results

3.1. SSH and functional categories

The efficiency of SSH was first evaluated by PCR with a house-keeping gene, β -actin. In this PCR, the depletion of β -actin transcript in the subtracted *vis a vis* the unsubtracted cDNA was considered as the basis for determining the efficiency of SSH. While β -actin PCR product was detected in the unsubtracted cDNA, they were not detected in the subtracted cDNA under the same PCR conditions. This suggests that a drastic reduction of highly abundant cDNAs was achieved.

From the subtracted library, 960 clones were randomly picked and sequenced. Only sequences with E-value $<10^{-3}$ were considered for categorization of genes into different functional categories. After removal of low quality sequences and contaminants, as many as 790 ESTs were available for further analyses. Out of these 240 (37%) did not match to any of the sequences in GenBank. As many as 410 ESTs (51.89 %) were quite similar to sequences in the Database. These ESTs represented 45 different genes (Table 2). Apart from these, 20 hypothetical proteins (Table 3) were also identified. The remaining 140 ESTs had database matching of E-value $>10^{-3}$.

The identified ESTs could be grouped into 12 categories based on their physiological and functional roles (Fig 1). Among these, three genes are likely to be immunorelevant and, eight others, related to antioxidant and ATP metabolism. The largest group comprised of the muscle and cytoskeletal related genes which were represented by 12 different transcripts followed by the enzymes which comprised of eight different genes.

The most abundant immune related genes in the subtractive library were ferritin, hemocyanin and translationally controlled tumor protein (TCTP). Various genes encoding antioxidant molecules, such as cytochrome C-oxidase subunit III, cytochrome C-oxidase subunit I and NADH dehydrogenase subunit-I were also identified. This category of genes contributed to 6% of the total identified transcripts. ATP

metabolism genes such as F1F0-type ATP synthase subunit G and H⁺ transporting ATP synthase involved in ATP metabolism were also found in large numbers.

Expression of genes related to cytoskeleton motility was at a higher level in the challenged PL. Contributing to 10% of the total, this group of genes was the largest in this study. Furthermore, cDNA fragments encoding for allergens such as arginine kinase, myosin light chain and sarcoplasmic calcium binding protein were also present. Genes encoding for different enzymes such as pyruvate kinase, trypsin, catalase, xylose isomerase and phosphoglycerate kinase were detected, albeit in small numbers. Two genes related to crustacyanin pigment were also identified from the subtracted library.

3.2. Relative quantification of differentially expressed immune related genes

A significant increase in the relative expression of all the genes examined was observed. The values for mean C_T and $\Delta\Delta C_T$ for challenged and control samples are presented in (Table 4). The hemocyanin transcript showed an 11.63 fold upregulation in the challenged PL. Whereas TCTP gene had 6.47 fold and ferritin a 2.04 fold rise in their expression (Fig. 2a). Lysozyme gene had 5.49 fold, penaidin 2.09 fold and MnSOD 3.28 fold rise respectively (Fig 2b). The expression of all six genes was normalized to β -actin, the internal control.

4. Discussion

In the present study, we applied SSH method to investigate the *V. harveyi* responsive genes in postlarvae of *P. monodon*. Also, we chose the postlarval stages as the mortalities due to *V. harveyi* has been proven to be predominant in these stages [7]. Primarily, the frequency of immune-related genes is reported to be very high in the hemolymph and other tissue homogenates of adult *P. monodon* challenged with *V. harveyi* [8, 9]. It is inferable from this first study on gene expression in postlarval stages of *P. monodon* challenged with *V. harveyi* that many components of defense mechanisms in the postlarvae are upregulated. These observations may prove useful for elucidating primary avenues for controlling luminous vibriosis in marine shrimps in their early stages. As such, the ESTs from the SSH cDNA library could be grouped into 12 different functional categories with the representing molecules having diverse functional roles.

4.1. Genes related to defense and immunity

In this category, hemocyanin and ferritin were the most frequently encountered clones. Earlier studies [10, 11] had also found that the level of ferritin gene transcripts were up-regulated after challenge with white spot syndrome virus (WSSV). This iron storage protein is crucial in the metabolism of iron as well as in the detoxification of this metal. Its expression is known to play a key role in maintaining iron homeostasis inside the cell [12]. Binding of free iron is considered bacteriostatic, as bacterial cell growth is inhibited if no iron is available for synthesis of essential iron-containing compounds [12]. It may also have a role in clotting, as it has been shown in *Drosophila* [13]. The high representation of ferritin-ESTs in our library indicates its upregulation due to the *V. harveyi* challenge, as was also confirmed by the qPCR.

The EST fragments bearing 98 % similarity to the hemocyanin subunit L gene of *Marsupenaeus japonicus* were also numerous. As the principal component of haemolymph, haemocyanin contributes to 95% of the total amount of serum proteins [14]. Earlier studies have shown the evidence of generation of antibacterial and antifungal peptides from the C terminus of haemocyanin of shrimp [15]. Haemocyanin gene is multifunctional respiratory pigment performing important immune functions in crustaceans. Haemocyanin helps in activation of prophenoloxidase activity by partial proteolysis and also sometimes, can even function by itself as a phenoloxidase under certain conditions [16]. It is proved to be an important immune factor against WSSV [17] and its diverse peptides are reported from *P. monodon* (18). From the occurrence of this gene in our subtracted library of *P. monodon*, its role as a major defense molecule against challenged *V. harveyi* is ascertainable.

ESTs relating to zinc proteinase Mpc1 and Mpc2 subunits were also identified in the library. Zinc proteinase belongs to family of metalloendopeptidases that have many different roles in biological systems like connective tissue remodeling and removal of signal sequences from nascent proteins [19]. In general, metal binding proteins such as ferritin and zinc proteinase are involved in animal's immune responses, and some are also involved in activating or degrading signal molecules [20]. This suggests that metal binding proteins such as ferritin and zinc proteinases may participate in shrimp's humoral response to the bacterial infection.

A single transcript coding for manganese superoxide dismutase (MnSOD), with proven role in antioxidant defenses [21], was recorded. MnSOD plays an important role in crustacean immune defense by eliminating oxidative stress [22]. MnSOD tissue-specific expression in crustaceans may respond to particular types of invaders, as also seen in previous studies on *Macrobrachium rosenbergii* challenged with *Lactococcus garvieae* [23]. Thus over 3 fold upregulation of MnSOD suggests its role in the clearance of pathogens [22], and the defense processes of *P. monodon*. Though we did not get any clone bearing peaeidin and lysozymes inserts in the SSH library, they appear important in immune functions as was evidenced through qPCR analyses.

4.2. Cell death/apoptosis related proteins

Interestingly, the TCTP with 25 clones was one of the most frequently encountered transcripts present in our library. Also known as fortilin, it is believed to protect cells from death under toxic conditions. Fortilin/TCTPs are highly conserved throughout the animal and plant kingdoms, and shrimp fortilin has a 64% identity in amino acid composition with human fortilin [24]. Earlier, fortillin gene was detected in WSSV-infected *P. monodon* using SSH. Its expression was reported to decrease during the terminal stage of the infection [24]. TCTP has a proven role in ‘histamine release factor’ (HRF), triggering histamine release from leukocytes [25]. TCTP also displays more general ‘cytokine-like’ activities, as it also induces the production of interleukins from basophiles and eosinophiles [26] and also reported to be induced by certain cytokines [27]. Our study reveals for the first time the upregulation and/or over expression of TCTP due to bacterial challenge. Thus, TCTP may play a critical role in their response to bacterial infection, through regulation of a cell death pathway [24].

4.3. Muscle and cytoskeleton related proteins

Contributing to 18% of the total ESTs identified, the muscle and cytoskeletal proteins constituted a major category of genes identified in the library. As the cDNA library was prepared out of the whole animal tissue, transcripts of muscle and cytoskeletal specific genes such as actin, myosin, troponin and sarcoplasmic calcium binding protein were observed more frequently. Actins are conserved proteins that participate in muscle contraction, cell motility, cell division, and cytoskeletal structure [28]. The upregulation of these genes result in effective measures to clear away the invading pathogens.

Interestingly, nine transcripts of sarcoplasmic calcium binding protein (SCP) were also found in our library. The SCPs are an important Ca^{2+} -binding system in the muscles of both vertebrate and invertebrate species [29]. Although expressed ubiquitously, it is usually abundant in muscle. Its increased expression might suggest modulation of calcium ion concentration in *P. monodon* due to bacterial challenge.

4.4. ATP -metabolism related genes

Contributing ~6% of the total ESTs identified, genes related to ATP-metabolism such as ATP synthase, F1F0 type ATP synthase subunit g and H^+ transporting ATP synthase were quite frequent. ATP synthase (or H^+ -ATP synthase) is the terminal complex of the respiratory chain and catalyzes the formation of ATP from ADP and inorganic phosphate [30]. The activation of ATP synthase genes could indicate a high increase in ATP demand which may be due to the increased demand for energy in the bacteria challenged postlarvae.

4.5. Antioxidant metabolism

Proteins, particularly those encoded by mitochondrial genes and those involved in oxidative phosphorylation and antioxidant metabolism, contributed to 5% of the identified ESTs, and represented cytochrome C-oxidase (16 clones) and NADH dehydrogenase (15 clones). Similar to the observations from WSSV infection studies [31], it is likely that bacterial afflictions also modulate expression of energy metabolism related genes in *P. monodon* PL. Expression of the above mentioned mitochondrial genes related to oxidative phosphorylation, might suggest a functional response to the challenge. Thus the present study is useful to suggest that bacterial infection might also modulate the amount of mitochondrial mRNAs.

4.6. Enzymes

The enzymes encountered during this study were either proteolytic such as trypsin, and retinoid inducible serine carboxypeptidase or, metabolic such as xylose isomerase, pyruvate kinase, phosphoglycerate kinase. As many as eight percent of the total ESTs were related to metabolic and proteolytic enzymes. The reason for high incidence of this category of genes may be due to the fact that

the mRNA copy numbers for all these genes would have been very high as we had macerated the whole postlarva for RNA isolation.

4.7. Pigments and other genes

The cDNA clones encoding for crustacyanin and rhodopsin formed a minor group in the subtracted library. Rhodopsin, an eye pigment protein, derived from the contaminated compound eye in the whole animal macerate was more frequent. Fragments encoding both crustacyanin C1 and crustacyanin A2 subunit were identified in this study. Crustacyanin a member of lipocalin family of proteins. It is involved in various physiological functions, such as binding with small hydrophobic molecules [32,33] forming complexes with other macromolecules for various physiological processes such as invertebrate cryptic coloration [34] and the regulation of cell homeostasis [35,36]. Crustacyanin was first identified in European lobster, *Homarus gammarus* [32] and has been shown to bind astaxanthin which gives blue coloration.

Apart from these, transcripts coding for tetraspanin and smooth endoplasmic reticulum calcium, ATPase were identified in this study. These two proteins are integral components of membranes. Besides these, as many as 20 hypothetical proteins were also identified. Among them, many have been reported for the first time from this shrimp. Many of these might be the products of novel genes with interesting roles in shrimp immune-modulation or in stress management.

4.8 Quantitative analysis of relative gene expression

The qPCR quantification of expression of three immune related genes (TCTP, ferritin and hemocyanin) with reference to the internal control β -Actin supports the data generated by SSH with respect to the upregulation of defense related genes in particular. The hemocyanin gene, with a higher upregulation of 11.6 fold, seems to be active in the defense against bacterial pathogens. Clearly, its importance is significant in processes such as activation of prophenoloxidase pathway for elimination of pathogens. Furthermore, increased expression of TCTP and ferritin is generally considered as first line of defense in the invertebrate immune system. These observations are in accordance with previous studies of differential expression of such genes in various species of shrimps upon microbial stress [9, 37, 38]. Also the upregulation in levels of MnSOD, penaeidin and lysozyme proves their previously established roles in innate immune response of crustaceans. The data obtained from our work clearly indicates the

differential expression of the three immune related transcripts on *V. harveyi* challenge. To our knowledge this the first study depicting the upregulation of such proteins on bacterial challenge in the postlarval stages of *P. monodon*.

5. Conclusion

Results from this study provide information on individual genes that constitute the shrimp postlarval response towards bacterial pathogens. Application of SSH and Real Time PCR is very useful for identification of *V. harveyi* responsive genes in *P. monodon* postlarvae. All together, we could identify 45 known genes from this study. About 37% of sequences from this analysis had no matches in the databases. Real time analyses of cDNA from challenged PL for defense response-related genes like ferritin, hemocyanin, TCTP, lysozyme, MnSOD and penaeidin brought out noteworthy upregulation of these genes. Although the numbers of genes related to immune functions were less, many genes related to various other processes such as ATP/energy metabolism, antioxidant metabolism and apoptosis/cell death were preponderant. Implicitly, the frequency of occurrence of such genes in the library and their gene expression suggests that they are up-regulated due to the bacterial challenge. We believe that many of the transcripts identified from this study will be of great value in understanding the shrimp immune system and also in studying the response of early developmental stages of shrimp to bacterial stress.

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Figure legends:

Fig 1. Per cent of different categories of transcripts identified from the subtracted cDNA library prepared through suppression subtractive hybridization from *Penaeus monodon* postlarvae bath challenged with the strain *Vibrio harveyi*D3

Fig 2a. Relative expression of immune related genes, hemocyanin, ferritin and TCTP. Expressions normalized with respect to the internal control gene β -Actin. The error bars in the histogram represent the maximum and minimum changes of the relative expression fold changes. β -Actin is compared to itself as a reference and represented here as one fold change in its expression

Fig 2b. Relative expression of immune related genes lysozyme, penaeidin and superoxide dismutase MnSOD. Expressions normalized with respect to the internal control gene β -Actin. The error bars in the histogram represent the maximum and minimum changes of the relative expression fold changes. β -Actin is compared to itself as a reference and represented here as one fold change in its expression

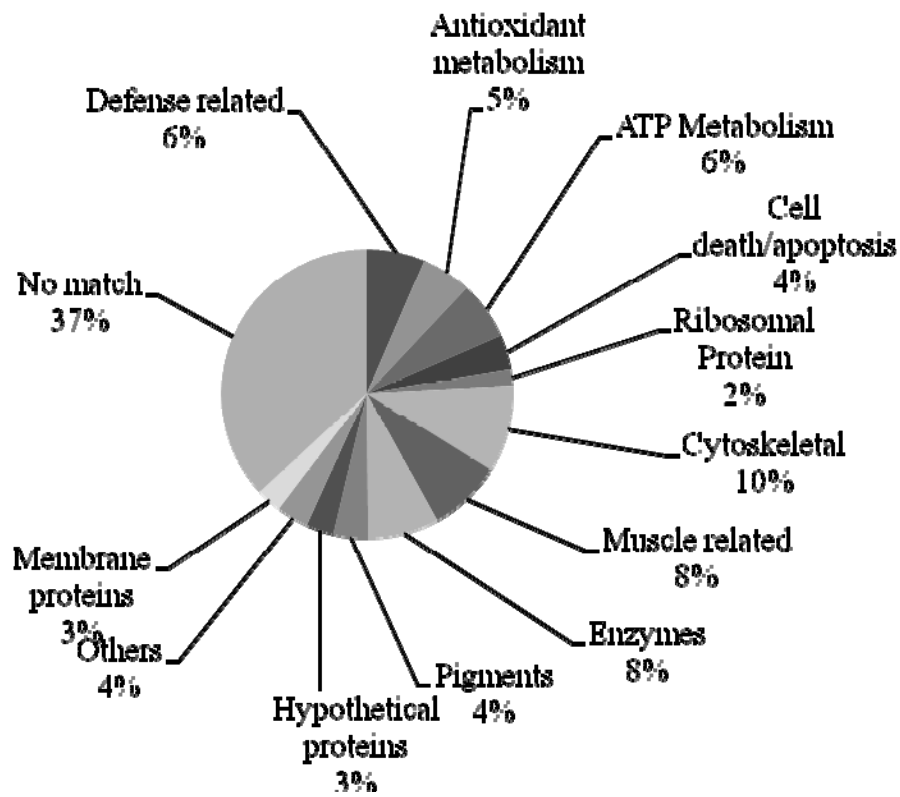


FIGURE 1

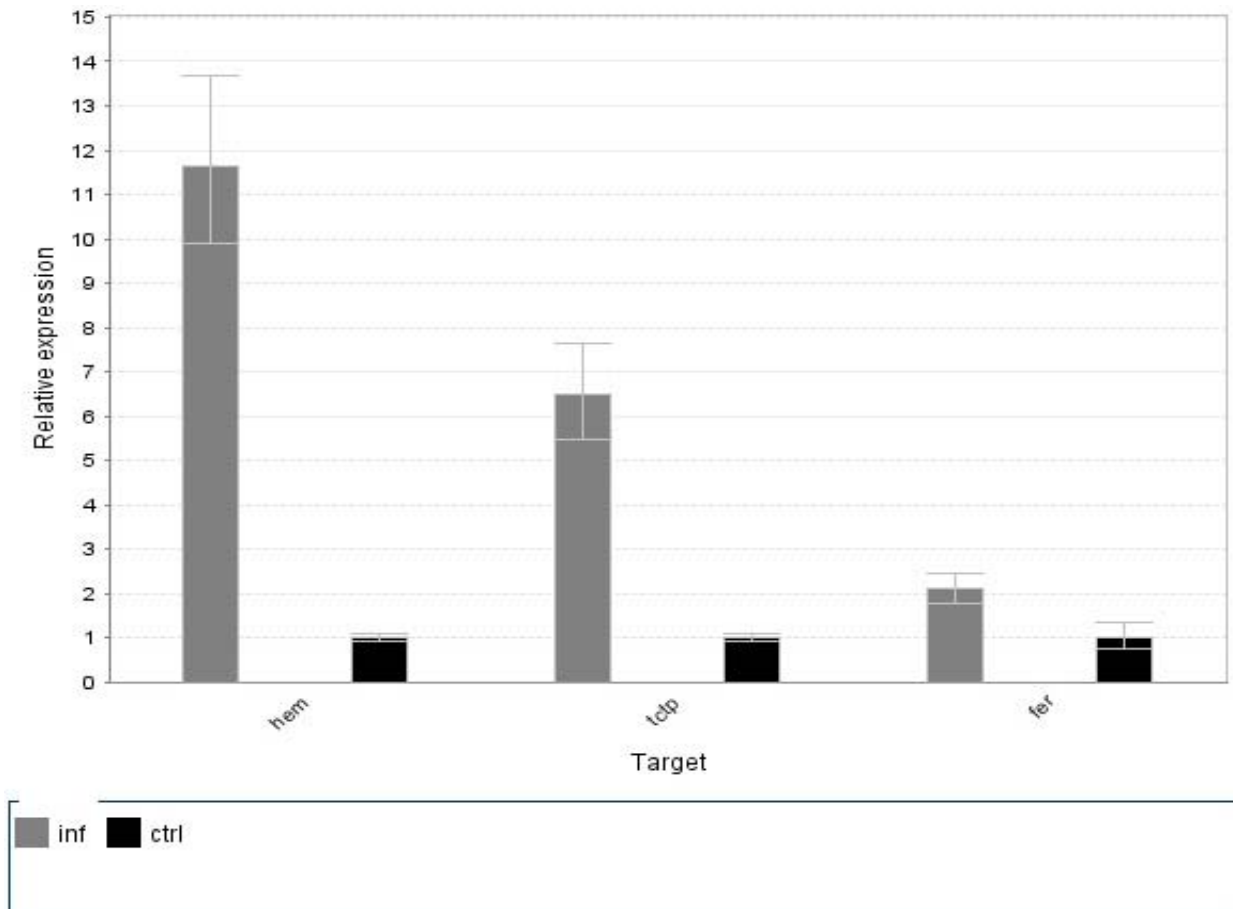


FIGURE 2A

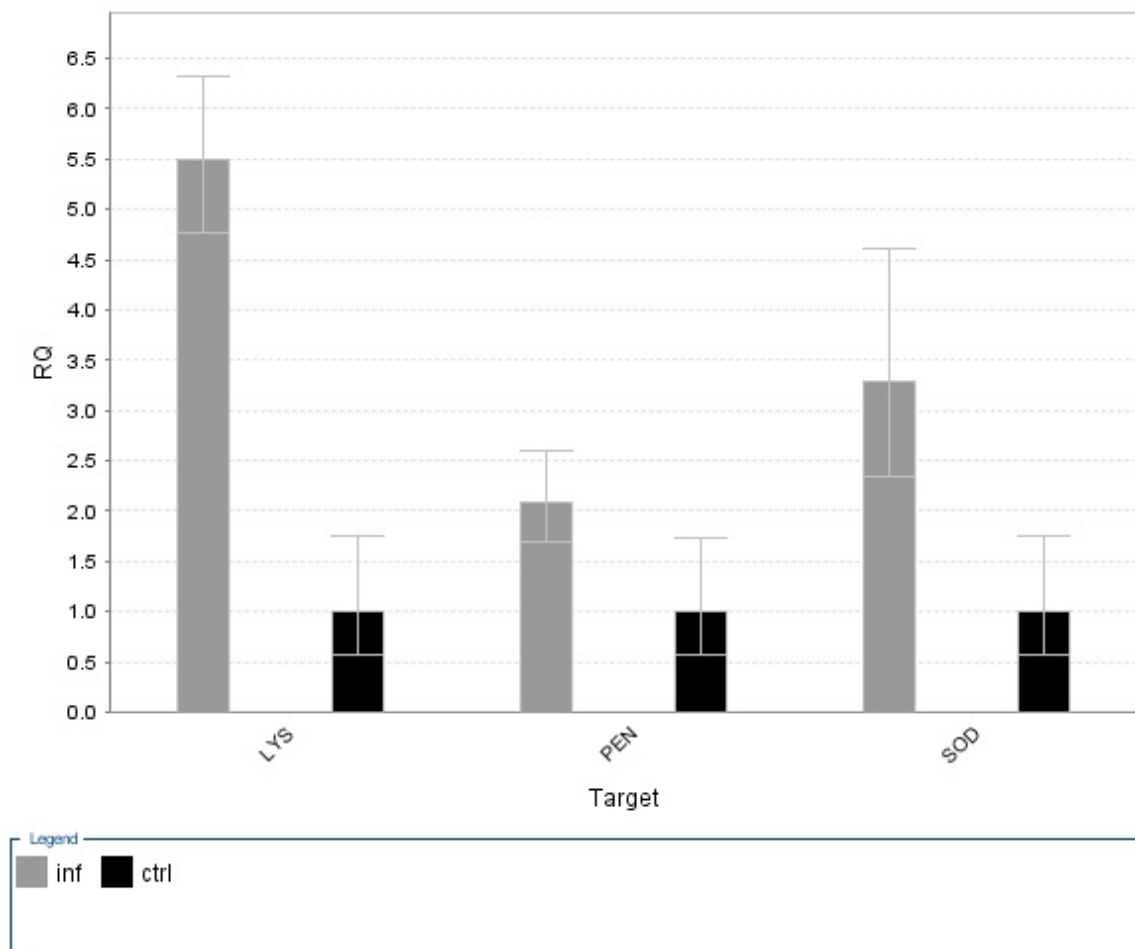


FIGURE 2B