

Differential Gene Expression of Threatened Himalayan Snow Trout Challenged With *Aeromonas hydrophilla*

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ABSTRACT

The snow trout (*Schizothorax richardsonii*) is endemic to the high altitudinal Himalayan region and thrive well in a wide thermal regime (0-27°C). The species can be used as a model species to study the abiotic and biotic stress response of cold-water fish species. In the present study, we compared the liver transcriptomes of *Aeromonas hydrophilla* challenged (Ah+) and mock challenged (Ar-) *S. richardsonii*. The transcriptome database for the species has been developed with a cascade of immune-related differentially expressed genes using RNA sequencing (2×100 bp paired-end) carried out on the Illumina 2000 sequencing platform. Among 50,453 unigenes obtained from de novo assembly, 24,464 unigenes were annotated, among which 82 unigenes were associated with immune responses genes. There were 265 differentially expressed unigenes (189 upregulated, and 75 down-regulated) in Ah+ compared to Ah- group. The majority of genes involved in immune regulation during bacterial pathogenesis are linked to extracellular region, membrane integrity, ion binding, signal transduction, antigen processing and presentation, MHC class I protein complex, etc. Particularly, 15 unigenes associated with immune response and 9 unigenes related to signal transduction gene ontology terms were significantly dysregulated in the bacterial challenged group. Four immune genes Interleukin 1β1, prostaglandin-endoperoxide synthase 2a & 2b, and ankyrin repeat domain were found to be very active against *A. hydrophilla*. The present study provides preliminary insight into possible changes at the molecular level of this potential model fish during a pathogenic infestation.

INTRODUCTION

The snow trout (*Schizothorax richardsonii*) is a Himalayan fish that inhabits most of the rivers, rivulets in the states of Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh of India. This rheophilic and demersal cyprinid fish species thrive at a temperature range between 0-27°C and survive well in different ecological niches from mid to high altitudes of the Himalaya (Barat et al., 2012; Kamalam et al., 2019). The species is an integral part of regional livelihood and gaining attention as a potential candidate for aquaculture which necessitates the prior information on the overall adaptation process of the species in captive conditions using molecular tools. There are limited studies available on the adaptive mechanism of snow trout to different biotic and abiotic stresses. Barat et al. (Barat, Sahoo, et al., 2016) have studied the molecular response of the species at elevated temperatures to

reveal the putative thermal adaptive mechanism of the species. Also, Kamalam et al. (Kamalam et al., 2019) revealed that snow trout shows physiological plasticity to different environmental conditions. The bacterial diseases are also a great concern of captive aquaculture Therefore the present study was formulated to reveal the molecular response of snow trout to *Aeromonas hydrophilla* infections. We used deep RNA sequencing (RNA-Seq), a high-throughput transcriptomic approach to characterize the genes and molecular pathways involved in the snow trout's response to aeromoniasis.

RNA-seq uses deep-sequencing technologies to study the functional elements of the genome and reveal the major constituents of cells and tissues working during a biological process (Wang et al., 2009). The major advantage of RNA-seq for non-model organisms is its efficiency in detecting novel molecules and does not require any prior information about the

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genes (Qian et al., 2014; Wang et al., 2009). RNA-seq technology also helps to determine the ultimate pathways that are regulated by the putative functional regulators (Qian et al., 2014). The preventive measures formulated using specific information from native species is surely more applicable instead of adopting them from congeneric species or model organisms.

Aeromonad septicemia majorly affects the liver and kidney tissue and characterized by diverse pathological symptoms such as dermal ulceration, fin hemorrhages, fin rots, red sores, hemorrhages and necrosis of the visceral organs, etc. (Sun et al., 2014; Yardimci & Aydin, 2011). *A. hydrophila* is reported being primary bacterial pathogen affecting freshwater fishes like *Clarias gariepinus* (Angka et al., 1995), *Labeo rohita* (Sahu et al., 2007), *Sparus aurata* (Reyes-Becerril et al., 2011), *Magalobarma amblycephala* (Tran et al., 2015), *Tor pititora* (Kumar et al., 2017) Also, high mortality has been reported among thermally stressed fishes due to *Aeromonas* infection (Noga, 2010; Ortega et al., 1995). The study of the defence mechanism of fishes against *A. hydrophila* can be very beneficial to control and prevent disease outbreaks (Peatman & Liu, 2007). Therefore, the present study has been formulated to compare the liver transcriptomes of *A. hydrophilla* challenged (Ah+) and mock challenged (Ar-) *S. richardsonii*.

MATERIALS AND METHODS

A total of 40 live specimens of the snow trout (*S. richardsonii*) were collected using cast net in the tributary of Kosi River near Ratighat (29° 27.48' N; 79° 28.81' E) located in the Kumaon hills of Uttarakhand, India. The specimens were identified using the taxonomic key of Talwar and Jhingran (Talwar & Jhingran, 1991). The fishes were transported to wet lab and acclimatized in the aerated water at ambient temperature (10 ± 2 °C) for one month before the experiment. Fishes were fed twice daily with a formulated diet (Wagle et al., 2016) based on 5% of the body weight. Constant aeration and water flow were maintained in experimental tanks. Water temperature and physicochemical parameters were recorded at regular intervals using multi-parameter auto-analyser (Hach®, Colorado, US). All the protocols were performed in accordance with animal welfare act approved by the ethical body (corresponding author's affiliation).

Experimental Design and Sampling:

A. hydrophilla (MTCC 646) was cultured in LB medium and suspended in PBS to 1×10^6 CFU/ml. Median lethal dose (LD50) was initially calculated using 6 fishes in each group and bacterial concentration of 1×10^4 , 10^5 , 10^6 , 10^7 CFU/mL for 24 h in a separate experiment. After the acclimation period (1 week), fishes were transferred randomly to two 1000 L FRP tanks. In each tank, 5 fishes (100–150 gm) were kept, and the water temperature was constantly monitored using a mercury thermometer. The first group was injected with 1×10^5 CFU of *A. hydrophilla* (bacterial challenged, Ah+) and the second group with PBS, which served as control (mock challenged, Ah-). Three fishes from each tank were collected for sampling at 6 h post-challenge. Fishes were anesthetized using an overdose of tricaine methanesulfonate (400 mg/L) (Sigma, St. Louis, USA) before

tissue sampling. Liver tissue was collected from each fish sampled at 6 h and used for transcriptome profiling.

RNA isolation, quality analysis, library preparation, and de-novo assembly

Total RNA was extracted from 50–100 mg of each liver sample using the PureLink® RNA Mini Kit (Life Technologies, Carlsbad, USA) as per the manufacturer's instructions. RNA was treated with DNase at 30°C for 30 min to remove the genomic DNA contamination. The concentration and purity were measured using NanoDrop® 2000 (Thermo Fisher Scientific, Wilmington, USA), and its integrity was visualized using agarose gel electrophoresis (1.2 % MOPS/ formaldehyde agarose gels). The quality of total RNA was further analyzed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA), with an acceptable RNA integrity (RIN) value ≥ 8 . The RNA samples in each group (Ah+ and Ah-) collected at 6 h were pooled (3 biological replicates) in equal concentration for library preparation. Two paired-end RNA-Seq libraries (one each for Ah+ and Ah-) were prepared using the TruSeq® RNA sample preparation kit (Illumina, San Diego, USA), according to the manufacturer's instructions. RNA-Seq libraries were sequenced on Hi-Seq 2000 (Illumina, San Diego, USA) for generating 2×100 bp sequencing reads at SciGenom Laboratories (SciGenom, Kochi, IND). The fastq files were trimmed separately to avoid specific sequence bias and low-quality bases using CLC Genomics Workbench v.7.5.4 (Qiagen, Hilden, DEU). The pooled trimmed reads were then assembled using SOAPdenovo2 (Luo et al., 2012) 31 mer algorithm with default options. The trimmed reads of all samples were aligned to the assembled transcriptome using Bowtie Program (Langmead et al., 2009). It was allowed up to 3-mismatches in the seed region (length = 36 bp). We retrieved raw sequence data of SRR1503433 (bio-project PRJNA 253239) of the same species and pooled to generate reference de novo transcriptome. Of all filtered reads, 61-70% of reads from each sample were aligned back to the assembled transcriptome. The assembled unigenes were annotated using an in-house pipeline (CANoPI-Contig Annotator Pipeline of SciGenom Labs, IND), and unigenes were compared with the NCBI nonredundant protein database using BLASTx tools. Matches with e-value $\leq 10^{-5}$ and similarity score $\geq 60\%$ were retained for further annotation. Gene ontology (GO) annotations were performed using Blast2Go program (Conesa et al., 2005). The Kyoto encyclopedia of genes and genomes (KEGG) assignment was done by running BLASTx against the KEGG database (Ogata et al., 1999) after availing KO information of each unigenes. The differential gene expression analysis was performed using the DESeq program (Anders & Huber, 2010) following the guidelines available in the web link. Minimum cut-off value for differentially expressed genes was ≥ 5 fold at $p=0.01$. The expression levels of genes were compared between Ah- and Ah+ after the reads were remapped to the contigs. The number of reads was calculated as reads per kilobase per million mapped reads (RPKM) for DeSeq analysis.

RESULTS AND DISCUSSION

A total of 65,428,283 and 62,387,440 bp of Ah⁻ and Ah⁺ samples, respectively, were generated from Illumina HiSeq 2000 by 2×100 bp paired-end sequencing. The sequence data accounts for approximately 13.22 and 9.48 Gb bases with GC% of 46.64 and 46.37, respectively, for Ah⁻ and Ah⁺ samples. The raw sequence data were submitted to NCBI GenBank under the Bioproject PRJNA253239. After trimming, a total of 62,378,245 and 57,820,543 reads were retained for analysis (Ah⁻ and Ah⁺, respectively). The de novo assembly of PRJNA253239 resulted in a total of 50,453 unigenes. Among 50,453 unigenes, 24,464 (48.49%) were annotated with BLASTx similarity and 24,237 (99%) contigs were found to have ≤1e-5 cut-off value. Uniprot annotation was also obtained for 14,291 unigenes. Approximately, 80.28% unigenes of BLASTx result showed similarity with *Danio rerio*, 4.19% *Oreochromis niloticus*, 2.82%, *Oryzias latipes*, 9.57% with other species, etc. (Figure 1). The details (Uniprot ID and name, protein and gene names, species name, Gene ontology IDs, Inter-Pro IDs GenBank accession, etc.) of BLASTx hit result has been listed in Supplementary file 1. The highest number of gene ontology (GO) terms identified for molecular function (16,211) followed by biological processes (11,491) and cellular component (8,014). The top 25 terms in each category were shown in Figure 2a-2c. There were 82 unigenes assigned as “immune responses” (GO ID: 0006955) in the biological process. The unigenes like chemokine, MHC class I & II antigens, cascades of complement components, interleukins, toll-like receptors, etc. were identified under GO “immune response” in this transcriptome profile. The highest number of unigenes (377) were assigned to “proteolysis” GO term in the biological process. Interestingly, 49 unigenes were assigned to the apoptotic process (GO ID: 0006915), which also includes a cascade of caspases (initiator caspase 2, -8, and -9 and executioner caspase 3, -6, -7). The caspase 1 (first discovered caspase in human and called interleukin 1b converting enzyme) was also identified among differentially expressed unigenes. Caspases are the members of conserved intracellular cysteine-dependent aspartate-specific cysteine proteases that play a significant role in regulating inflammation and cell death (Li et al., 2019). The caspases are active in cysteine-type endopeptidase activity, proteolysis, regulation of the apoptotic process, and response to a chemical stimulus (Chang & Yang, 2000; Li et al., 2019). Long et al. (Long & Sun, 2016) proposed that caspase genes 1, 2, 3, and 9 are essential in optimal defense against bacterial infection in fish. Executioner Caspase 3 protein mediates macrophage apoptosis during *Aeromonas hydrophila* infection in the head kidney of *Clarias batrachus* (Banerjee et al., 2012). Additionally, caspase 3 and 6 are supposed to play an essential function in apoptotic cell death during the red sea bream iridovirus infection (Imajoh et al., 2004).

The annotated sequences were mapped to the functional pathways of the KEGG database, and 8,247 unigenes were successfully assigned to 402 KEGG pathways in 6 categories (Figure 3). Most unigenes were categorized under human diseases (4,917), followed by the organismal system (3,274), metabolism (3,198), environmental information processing

(2,197), cellular processes (1,579) and genetic information processing (1,080). However, the highest number of unigenes were characterized under subcategory signal transduction (1912) of environmental information processing, followed by infectious disease: viral (1149) in human diseases, immune system (946), and endocrine system (900) subsections of the organismal system.

Differential Gene Expression

The read count, RPKM, and differential gene expression analysis results were provided in Supplementary file 1. The normalized RPKM value distribution is Ah⁺ and Ah⁻ sample are given in Figure 4. A total of 265 unigenes were differentially expressed (p-value <0.01) between Ah⁻ and Ah⁺. Of the total 265, 189 unigenes were upregulated (Figure 5) and 75 were downregulated (Figure 6) in Ah⁺ compared to Ah⁻. Among 189 upregulated genes, 96 were assigned to GO term, and in the case of down-regulated genes, 21 out of 75 genes were assigned to GO term. Among 189 up-regulated GO assigned unigenes, five unigenes [Interleukin 17c (2), tissue inhibitor of metalloproteinase 2b, chemokine CXC-C1c, granulocyte colony-stimulating factor 3, Thrombospondin-1b] were predicted to localize in extracellular regions. GO classification of 8 unigenes [4 signal transduction genes, i.e., Fibrinogen beta polypeptide (2), ADP-ribosylation factor 4, Rho family GTPase 1 like (3), A kinase (PRKA) anchor protein (gravin) 12b (3), and 4 immune specific unigenes, i.e., MHC class I α antigen, Interleukin 6, Chemokine CXC-C1c, Granulocyte colony-stimulating factor (2), were assigned under immune response (Table 1 and Figure 2). In these immune functions, a cascade of interleukins were either specifically expressed in Ah⁺, e.g., interleukin 17c (∞ fold), interleukin 6 (∞ fold), or significantly overexpressed, e.g., interleukin IL-1 β (>10.2 fold). The interleukins, a group of cytokines, play a major role in the activation and differentiation of immune cells and also in pro- and the anti-inflammatory response of the organism. The expression of interleukin 17c (IL 17c) was significantly up-regulated under bacterial infection in the trout RTS-11 cell line by LPS, poly I: C (Wang et al., 2010). IL-6 was reported to be first discovered in the Japanese pufferfish (*Fugu rubripes*) among the teleost fishes (Bird et al., 2005). LPS, poly I:C, and IL-1 β induced the expression of IL-6 in the trout macrophage cell line RTS-11 (Costa et al., 2011). Faikoh et al. (Faikoh et al., 2014) studied the increased expression of endogenous IL 6, IL-1 β , and other interleukins when zebrafishes were immersed in liposome-encapsulated cinnamaldehyde (LEC) as a measure to enhance immunity against bacterial infection. Some notable genes, prostaglandin-endoperoxide synthase 2a, and 2b were involved in response to oxidative stress/response to a chemical stimulus with >6.09 to 8.5-folds overexpression in Ah⁺ samples to Ah⁻. Prostaglandin endoperoxide synthase (PTGS) expression is known to be responsive to inflammatory stimuli such as interferon γ and lipopolysaccharides (LPS). The expression and activity of PTGS have been demonstrated in a few teleost fishes, *Danio rerio*, *Oncorhynchus mykiss*, *Dicentrarchus labrax*, *Gadus morhua*, and *Salmo salar* (Montero et al., 2016). The expression level of PTGS2 was significantly up-regulated (>4.7 fold) in orange-spotted grouper (*Epinephelus coioides*) against *Vibrio* *harbeyi*

(Maekawa et al., 2017). Other up-regulated genes viz. chemokine C-X-C motif, immunity-related GTPase family, complement component, interferon-induced transmembrane protein1, cytochrome p450, toll-like receptor 5, ATP binding cassette, etc. were significantly differentially expressed (fold change within 4.0- 33.8, $p < 0.01$) in Ah⁺ samples. Similarly, few unigenes, i.e., novel immune type receptor, immunoglobulin heavy chain, complement c3-Q1, MHC class 1 antigen, were down-regulated with a fold change between -23.8 to -1.4 folds ($p < 0.01$) in Ah⁺ samples in-comparison to Ah⁻. There were also two solute transporter genes (GO characterized as integral to the membrane), slc16a3 (monocarboxylic acid transporters), and slc5a2 (sodium/glucose cotransporter) with >5.2 and 6.8 fold expression, respectively in Ah⁺ compared to Ah⁻. Monocarboxylic transporter was reported to be involved in the transport of metabolite and functioning of brain development in zebrafish embryos (Tseng et al., 2013). The expression analysis of Sodium/glucose cotransporter (slc5a2) was also observed in golden mahseer (*Tor putitora*) by Barat et al. (Barat, Kumar, et al., 2016). There are many types of glucose transporters identified on the cell surface, playing an important role in the transportation of Na⁺ mediated glucose to maintain the metabolic process. But there were also possibilities to develop efficient anti-viral strategies or therapeutic methods using Glut1 as reported by (Huang et al., 2015) in their studies on white spot syndrome virus (WSSV) infection to shrimp.

There were around 22 unigenes related to the immune response such as MHC class I antigens, antigen processing, etc. were down-regulated (< 2.0-23.8) in Ah⁺ samples than the Ah⁻. These changes could be the results of the inactivation of major histocompatibility complex class I, which failed to display the antigen peptides on the cell surface and facilitate easy entry for the pathogens. It was also observed the downregulation of few unigenes like immunoglobulin heavy chain, integrin, etc. which could be negatively regulated by pathogen might cause due to pathogenic factors overcoming the host immune response. Luder et al. (Luder et al., 1998) also studied the changes due to the downregulation of MHC Class I and II molecules in murine macrophages when treated with *Toxoplasma gondii*. They argued that the downregulation of these molecules might enable the pathogens to establish within the host avoiding the host's immune response (Luder et al., 1998).

KEGG Pathways of DEGs

Out of 189 differentially expressed genes, 84 (74 upregulated and 10 downregulated) were assigned to KEGG IDs. Among 74 upregulated genes, 58 genes were successfully assigned with KEGG automatic annotation server (KAAS) ID, and they were classified into five major groups which were 1) metabolism, 2) environmental information processing, 3) cellular processes, 4) organismal system, and 5) Human diseases. These 58 genes were related to 132 KEGG pathways. The unigenes were most abundant in human diseases (72) with bacterial (12) and viral (16) category, followed by the immune system (17) under the organismal system and signal transduction (23) under environmental information processing. The most important pathways under signal transduction pathway were Ras signaling (Ras and Rab interactor 1, fibroblast growth factor 21), MAPK

signaling (Interleukin 1 β 1, fibroblast growth factor 21), NF-Kappa signaling, TNF signaling pathway (Interleukin 1 β 1, B-cell lymphoma 3 protein homolog, Prostaglandin endoperoxide synthase 2a), PI3-Akt signaling pathway, etc. The KEGG pathway analysis also revealed that a single gene was active in several different pathways, e.g., Interleukin 1 β 1, Prostaglandin endoperoxide synthase 2a, Ankyrin repeat domain-containing protein 34B, etc. were co-expressing in MAPK signaling, TNF signaling pathway. The primary role of the TNF signaling pathway is the regulation of immune cells to inhibit tumorigenesis and viral replication (Aggarwal, 2003; Zou & Secombes, 2016). In TNF signaling pathway, there were 3 genes, Interleukin 1 β 1(IL1b) (>10.2 fold, $p=0.002$) as inflammatory cytokines, B-cell lymphoma 3 protein (Bcl3) (>5.97 fold, $p=0.004$) as intercellular signaling and prostaglandin-endoperoxide synthase 2a (>7.56 fold, $p < 0.00$) as the synthesis of inflammatory mediators) were up-regulated (Supplementary Figure 1). IL7 signaling pathways comprised 3 genes, FOS like antigen 1 (>4.64-fold, $p < 0.00$) as DNA binding factor, Prostaglandin endoperoxide synthase 2 as cytokines and Interleukin 1 β 1 as inflammatory gene (Supplementary Figure 1). There were 11 pathways related to the immune system under the organismal system. Nine immune responsive genes were found to be up-regulated in the immune system pathway. Among them, Interleukin 1 β 1 shared 7 different categories (hematopoietic cell lineage, toll-like receptor signaling, NOD-like receptor signaling, cytosolic DNA sensing, C-type lectin receptor signaling, Th17 cell differentiation, and IL-17 signaling) of immune system pathways (Supplementary Figure 2). It was observed to be >10.2 fold over-expressed in Ah⁺ samples compared to Ah⁻.

Total 69 down-regulated unigenes were analysed for KEGG pathway and only 9 unigenes were assigned to KAAS ID. There were 9 pathways detected under metabolism, organismal system, and human diseases category. One unigene, cytochrome oxidase I (contig ID: 1049604; KASS ID: K02256) was co-expressed in multiple pathways like metabolism, cardiac muscle contraction, thermogenesis and neurodegenerative diseases (https://www.kegg.jp/kegg-bin/find_pathway_object) and the expression of this gene was observed to be downregulated due to pathogenesis (Sukumaran et al., 2004). The functional annotation and KEGG pathway analysis of all the differentially expressed genes revealed the enrichment of immune responsive genes due to pathogenic infection to the fish.

CONCLUSION

The snow trout belongs to Schizothoracinae, a subfamily of the Cyprinidae that comprises 10-13 genera with about 100 species. The group represent an important taxon where fishes can survive at an extreme cold and pathogen prone environment. Although the present study did not conclude any concrete mechanism exhibited by snow trout in *Aeromonas hydrophila* infection, the study did highlight few explorable mechanisms in fish immune response. Various cell surface receptors including toll like receptors and c-type lectin receptors might play important role in immune response of snow trout. Cytokines (IL1 β 1, B-cell lymphoma 3 protein homolog, Prostaglandin-

endoperoxide synthase 2b) and caspases could be responsible for modulating the balance between humoral and cell-based immunity and regulation of cascade of immune function in bacterial infection. At present, a number of outstanding questions remain to be addressed to understand the snow trout's immune response in bacterial infection that require functional studies to reveal the role of specific effector during pathogenesis.

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