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Transcriptome profiling of grass carp (*Ctenopharyngodon idellus*) infected with *Aeromonas hydrophila*

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ABSTRACT

Aeromonas hydrophila is the causative pathogen of intestinal hemorrhage which has caused great economic loss in grass carp aquaculture. In order to understand the immunological response of grass carp to infection by *A. hydrophila*, the transcriptomic profiles of the spleens from infected and non-infected grass carp groups were obtained using HiSeq™ 2500 (Illumina). An average of 63 million clean reads per library was obtained, and approximately 80% of these genes were successfully mapped to the reference genome. A total of 1591 up-regulated and 530 down-regulated genes were identified. Eight immune-related categories involving 105 differently expressed genes were scrutinized. 16 of the differently expressed genes involving immune response were further validated by qRT-PCR. Our results provide valuable information for further analysis of the mechanisms of grass carp defense against *A. hydrophila* invasion.

1. Introduction

Grass carp (*Ctenopharygodon idella*) is an economically important species and its global production is more than 4.5 million tones per year, which making it the most highly consumed freshwater fish worldwide [1]. However, infectious intestinal hemorrhage caused by *Aeromonas hydrophila* has been severe for years [2], resulting in great economic loss and threatening the development of grass carp aquaculture. *A. hydrophila* is facultative anaerobic, motile, and Gram-negative rods in the family Aeromonadaceae [3]. It is often found in association with hemorrhagic septicemia in cold-blooded animals including fish, reptiles and amphibians. However, this organism has also attracted attention as an emerging human pathogen, and has been considered to have a significant impact on public health [4]. Therefore, identification of host factors in response to *A. hydrophila* infection has a great significance for disease prevention and control in grass carp culture.

Transcriptome analysis is a powerful tool for leading to a better understanding of the underlying pathways and mechanisms controlling cell fate, development, and disease progression [5]. Over the years, several technologies have been developed to survey transcriptomes in a high-throughput manner. High-throughput sequencing (HTS) technologies permit genome-wide transcriptomic analysis at a higher resolution, and these technologies have been widely used to study pathogenic processes during bacterial infections [6]. Hegedus et al. firstly used Solexa/Illumina's DGE system to study zebrafish transcriptome after *Mycobacterium marinum* infection, it showed the feasibility and superiority of high-throughput sequencing technology in the field of fish immune research [7]. Then RNA-Seq have been applied to immune-related gene and signaling pathway analysis of several fish species such as Mandarin fish, Large yellow croaker and Lates calcarijer [8–10].

The results of sequencing of the transcriptome of juvenile grass carp after *A. hydrophila* infection and non-infection were presented in this study. High-quality cDNA sequences were obtained by using Illumina RNA-Seq method and the sequencing reads were mapped to the reference genome database of grass carp [11]. Additionally, a great number of immune related genes that were differently expressed upon *A. hydrophila* infection were obtained and functionally annotated, and the gene expression patterns of some of these genes were verified by qRT-PCR. These results provide a valuable resource for further research into the mechanism of anti-infection immunity of grass carp.

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2. Materials and methods

2.1. Fish and bacteria

Healthy juvenile grass carps of 20 g ± 2 g body weight were kindly provided by the Hold-one aquatic breeding center in China and reared under pathogen-free conditions. Fish were maintained in aerated tap water at 28 °C in aquaria with Eheim biofilters until use. The bacterial strain *A. hydrophila* used for the experiments was isolated from diseased grass carp, and has been deposited in the China Center for Type Culture Collection under preservation number CCAM05068. The bacteria was cultured in Luria-Bertain (LB) at 28 °C for 24 h with constant shaking (150 rpm), then harvested by centrifugation at 6000 rpm for 5 min, washed once by PBS and centrifuged again. Bacterial pellets were then re-suspended in saline adjusted the concentration to 5×10^7 colony forming units (CFU) ml^{-1}.

2.2. Challenge experiments and RNA preparation

Healthy fish were randomly divided into two groups (30 fish per group). For bacterial infection group, fish were injected intraperitoneally (i.p.) with 0.1 ml *A. hydrophila* suspension abovementioned. The fish treated with 0.1 ml 0.65% physiological saline, were used as the control. After 6 h of injection, the spleen were collected and immediately stored in liquid nitrogen until RNA extraction.

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen, USA) according to the manufacture's protocol. The quality and quantity of the RNA samples were examined by use of the Agilent 2100 Bioanalyzer (Agilent Technologies) and the integrity was assessed by electrophoresis on 1% agarose gel. For each group, equal amount of RNA from the nine fish individuals per line were pooled to provide templates for RNA-Seq library construction.

2.3. Library construction and illumina sequencing

After DNase I treatment, mRNA was purified using oligo (dT)25 magnetic beads (Dynabeads® oligo (dT)25, Invitrogen) and subsequently interrupted to short fragments of 200–250 nucleotides using RNA fragmentation reagent (Ambion, USA). Then sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The cDNAs were checked by Agilent 2100 Bioanalyzer (Agilent, USA) and ABI StepOnePlus Real-Time PCR System (ABI, USA). The mixed DNA libraries were diluted to 4–5 pM for sequencing by Illumina Hiseq 2500™ platform.

2.4. Sequence annotation

Raw reads were first cleaned by removing adaptor sequences, low quality sequences (Sanger base quality < 20) and reads with unknown nucleotides larger than 10%. Clean reads were mapped to the *Ctenopharyngodon idellus* reference genome (http://www.ncgr.ac.cn/grasscarp/) using HISAT (version 0.1.6) [12]. The HTSeq (version 0.6.1) [13] was utilized to calculate the number of aligned reads per exon through annotation of the grass carp genome. Subsequently, the transcripts were subjected to BLASTX similarity searching against NCBI non-redundant protein database (NR) with an E-value threshold of 10^{-5}, and the unigenes were identified.

2.5. Differential expression analysis

Expression levels were measured in reads per kilo base of exon per million mapped reads (RPKM) method. The distribution of gene expression was analyzed. Statistical comparison between two groups was conducted using DEGseq (version 1.18.0) [14]. To assess the significance of differential gene expression, the threshold of FDR was set at ≤ 0.01 and the absolute value of log2 ratios (fold change between non-infected/infected samples) at ≥1. Differential expression genes (DEGs) were further annotated by Gene ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. GO annotation was acquired using the GOseq (version 1.16.2) and WEGO [15,16]. P-values generated from the enrichment analysis were subjected to multiple hypothesis testing, with *p*-values ≤ 0.05 considered significant. Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg) [17]. After multiple test correction, pathways with Q-values ≤ 0.05 were considered to be significantly enriched in DEGs.

2.6. Confirmation using quantitative real-time RT-PCR (QPCR)

To examine the reliability of the RNA-Seq results, a selected subset of differently expressed genes involved in immune responses were selected for validation using quantitative real-time RT-PCR (qRT-PCR). 16 genes differently expressed between the infected and non-infected groups, including Chemokine C motif receptor 1 (XCR 1), interleukin-1 beta (IL-1β), tumor necrosis factorα (TNF-α), C-type lysozyme (LysC), interferon regulatory factor 4 (IRF4), B cell receptor CD22, coagulation factor 7 (F7), complement component 1 (C1), complement component 3 (C3), complement component 7 (C7), toll-like receptor 22 (TLR 22), MHC class I antigen (MHC-I Ag), MHC class II antigen (MHC-II Ag), macrophage expressed gene 1 (MPEG 1), heat shock 70 kDa protein (HSP 70), and α-2-macroglobulin (α2MG) were selected for qRT-PCR assay. The housekeeping gene β-actin was used as the reference gene. Suitable primers were designed using Primer Express 3.0(Table 1) and synthesized by Sangon Bothech (Shanghai) Co., Ltd. QRT-PCR with SYBR Green dye (TaKaRa, Dalian, China) was performed on an ABI PRISM 7500 Fast Real-Time PCR System according to the manufacturer's protocol. Each treatment group was created by combining equal amounts of RNA from three replicate pools (three individual fish per pool). All reactions were performed in triplicates. The qRT-PCR conditions were as follows: 30 s at 95 °C, followed 40 cycles of 5 s at 95 °C, 30 s at 58 °C, 34 s at 72 °C. Dissociation curve analysis was performed to determine the target specificity. The relative expression ratio of the target genes versus β-actin gene was calculated using 2-ΔΔCT method and all data were given in terms of relative mRNA expression.

3. Results

3.1. Transcriptome sequence and reads mapping

Infected and non-infected groups were analyzed by RNA-Seq respectively. RNA sequencing resulted in about 65.5 million (infected) and 63.4 million (non-infected) raw reads of 125 bp (Table 2). After filtration, 64.6 million of infected groups and 62.6 million of non-infected groups clean reads were obtained, and these sequences were carried forward for additional analysis. Average 80% of the clean reads were mapped to the reference genome, 24507 genes of infected group and 24383 genes of non-infected group were detected. These sequences were compared with the NCBI non-redundant (NR) protein for functional annotation. Raw sequencing reads data has been submitted to Sequence Read Archive in NCBI, the SRA accession numbers are SRR3045340 and SRR3045341.