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Transcriptomic profiling revealed the signatures of intestinal barrier alteration and pathogen entry in turbot (*Scophthalmus maximus*) following *Vibrio anguillarum* challenge

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A B S T R A C T

The mucosal immune system serves as the frontline barriers of host defense against pathogen infection, especially for the fishes, which are living in the pathogen rich aquatic environment. The intestine constitutes the largest surface body area in constantly contact with the external pathogens, and plays a vital role in the immune defense against inflammation and pathogen infection. Previous studies have revealed that fish intestine might serves as the portal of entry for *Vibrio anguillarum*. To characterize the immune actors and their associated immune activities in turbot intestine barrier during bacterial infection, here we examined the gene expression profiles of turbot intestine at three time points following experimental infection with *V. anguillarum* utilizing RNA-seq technology. A total of 122 million reads were assembled into 183,101 contigs with an average length of 1151 bp and the N50 size of 2302 bp. Analysis of differential gene expression between control and infected samples at 1 h, 4 h, and 12 h revealed 2079 significantly expressed genes. Enrichment and pathway analysis of the differentially expressed genes showed the centrality of the pathogen attachment and recognition, antioxidant/apoptosis, mucus barrier modification and immune activation/inflammation in the pathogen entry and host immune responses. The present study reported the novel gene expression patterns in turbot mucosal immunity, which were overlooked in previous studies. Our results can help to understand the mechanisms of turbot host defense, and may also provide foundation to identify the biomarkers for future selection of disease-resistant broodstock and evaluation of disease prevention and treatment options.

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1. Introduction

The mucosal immune system serves as the first line of host defense against pathogen infection, especially for the fishes, which are living in the pathogen rich aquatic environment. The mucosal surfaces of fish (skin, gill and intestine) are exposed to a wide range of pathogens, and the immune events are happening all the time to prevent the pathogen attachment and invasion on these surfaces. It has been long hypothesized that the observed differences in disease susceptibility among fish species, strains and families are due to the different ability of the host to prevent attachment of pathogens and entry at mucosal epithelial sites on the mucus, skin, gill and intestine (Ellis, 2001; Palaksha et al., 2008; Rajan et al., 2011). In this regard, much efforts have been made to understand the fish mucosal immune system, aiming at elucidating mechanisms of fish defense against pathogenically hostile environments and furthermore developing mucosal vaccines to improve mucosal health over the last decades. However, our understanding of the structure and function, especially the mechanisms of antigen uptake and

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distribution, and the immune induction of the fish mucosal surfaces is still very limited.

The intestine constitutes the largest body area in constantly contact with the external pathogens, and plays a vital role in the immune defense against inflammation and pathogen infection (Rombout et al., 2011). Moreover, fish intestine is different from its mammalian counterparts in morphology and function. For example, there are no Peyer's patches, an organized lymphoid tissue, M cells, IgA and J-chain immunoglobulins (Rombout et al., 2011). The intestinal epithelium is a single cell layer targeted by many pathogens for disturbance. Many fish pathogens can gain entry through the intestinal epithelium very quickly. For example, the causative bacterium of enteric septicemia of catfish, *Edwardsiella ictaluri*, could be found in channel catfish kidney by 0.25 h after intragastric incubation, indicating its ability to fast pass through the intestinal epithelium (Baldwin and Newton, 1993). And many *Vibrio anguillarum* cells could be found in the spleen in turbot after oral infection, suggesting the intestine serves as the portal of entry for *V. anguillarum* (Oisson et al., 2006). Therefore, many studies have examined the molecular process of teleost intestinal immunology during infection in order to explore the mechanisms of fish intestinal immune defense. For instance, during *E. ictaluri* infection in catfish intestine, up to 1633 differentially expressed genes were captured with critical functional roles in immune activation and inflammation, and pathogen recognition (Li et al., 2012). The microarray studies about transcriptional responses observed in the $rag1^{-/-}$ zebrafish intestine, and responses in the gilthead sea bream intestine following myxosporean parasites infection have revealed the crucial role of intestine during infection (Davey et al., 2011; Jima et al., 2009). But our knowledge of the cellular actors and mechanisms governing intestinal mucosal barriers is still limited to a handful of teleost species.

Turbot (*Scophthalmus maximus*), one of the extensively maricultured species in China, suffers from a wide range of disease outbreaks due to a number of pathogens, including *Edwardsiella tarda*, *Streptococcus iniae*, and different species of *Vibrio*. Among these, *V. anguillarum*, a Gram-negative bacterium, is usually considered as a secondary pathogen in turbot fish diseases, and appears to have emerged as a primary pathogen. This pathogen is commonly associated with widespread mortalities through both acute and chronic infections, which has caused significant economic losses. Understanding the mechanisms of the host immune responses to prevent pathogen attachment and entry is critical for developing effective disease treatments and prevent economic losses. In turbot, although several studies have elucidated the gene activities during the infection process, most of the immune related studies were limited to examine specific gene targets, such as IgM (Gao et al., 2014), CC chemokine (Chen et al., 2010), HSP70 (Wang et al., 2013), cathepsin B (Zhou et al., 2014) and cytochrome P450 (Sun et al., 2013). Next-generation RNA-seq sequencing allows us not only to identify the gene and characterize the gene expression levels at the same time, but also to elucidate the interactions of host-bacterial (Chen et al., 2010). However, up to date, only very few immune studies in turbot have been performed using RNA-seq following infection (Robledo et al., 2014). No studies have systematically characterized the immune elements in turbot intestine following *V. anguillarum* infection at transcriptomic level.

To characterize the immune actors and their associated immune activities in turbot intestine barrier, here we examined the gene expression profiles of turbot intestine at three time points following experimental infection with *V. anguillarum* utilizing RNA-seq technology. We have detected a total of 2079 significantly expressed genes which involved in antioxidant, cell cycle, cytoskeletal rearrangement, immune activation and inflammation, pathogen recognition, lysosome regulation, and apoptosis. Our results can set the foundation for further developing of biomarkers, characterizing the mechanisms of intestinal mucosal barriers for vaccine development, and expanding our knowledge of teleost immunology.

2. Methods

2.1. Experimental animals and tissue collection

Turbot (average size 33 ± 0.45 g) were purchased from a turbot hatchery (Haiyang, Shandong, China). All fish were maintained in the laboratory for at least one week prior to experimental use. Fish were challenged in 30 L (20 L water) aquaria with one control and three treatment groups. The bacterial isolate of *V. anguillarum* was provided by the disease lab in Qingdao Agricultural University (Qingdao, Shandong, China). Bacteria were re-isolated from a single symptomatic fish and biochemically confirmed to be *V. anguillarum* using standard procedure. Briefly, the bacteria were inoculated in LB broth and incubated in a shaker (180 rpm) at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 ml of 10-fold serial dilutions onto LA agar plates. During challenge, the treatment groups were immersed for 2 h at a final concentration of 5×10^7 CFU/mL. Meanwhile, control fish were immersed in sterilized media alone. Aquaria were randomly assigned for 1 h, 4 h, and 12 h post-treatment sample collection, respectively, and 0 h control with twenty fish in each aquarium. The water temperature was kept at 28 ± 0.5 °C. At each time point following challenge, 15 fish were collected from the appropriate aquaria and euthanized with MS-222 (300 mg/L). The intestine from 15 fish was dissected and pooled (5 fish per pool). Samples were flash-frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

2.2. RNA extraction, library construction and sequencing

Samples were homogenized with mortar and pestle in the presence of liquid nitrogen. Total RNA was extracted from tissues using the RNeasy® Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit™ RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA 6000 Nano Assay Kit with the Agilent 2100 Bioanalyzer system (Agilent Technologies, CA, USA). For each time point, equal amounts of RNA from the three replicates were pooled for RNA-seq library construction.

Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext® First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext® Adaptors with hairpin loop structure were ligated to prepare for hybridization. The library fragments were purified with AMPure XP system (Beckman Coulter, Inc., Beverly, USA) to select cDNA fragments of preferentially 150–200 bp in length. Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by

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