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Transcriptome analysis of hybrid tilapia (*Oreochromis* spp.) with *Streptococcus agalactiae* infection identifies Toll-like receptor pathway-mediated induction of NADPH oxidase complex and piscidins as primary immune-related responses

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ABSTRACT

Streptococcus agalactiae infection is one of the most significant bacterial diseases in tilapia aquaculture. Identification of immune-related genes associated with *Streptococcus agalactiae* infection may provide a basis for breeding selection or therapeutics to augment disease resistance. Therefore, we utilized transcriptome profiling to study the host response in tilapia following *Streptococcus agalactiae* infection. Based on GO and KEGG enrichment analyses, we found that differentially expressed genes are widely involved in immune-related pathways, including the induction of antimicrobial peptides. Moreover, the main components of two immune-related pathways (Toll-like receptor signaling and leukocyte transendothelial migration) and four environmental information processing pathways (TNF, PI3K-Akt, Jak-STAT and MAPK) were identified. Finally, a time-course expression profile for several of the identified transcripts including tilapia piscidin 3 (TP3), tilapia piscidin 4 (TP4), TLR2, TLR5, MyD88, TRAF6, p38, and interleukin components was performed by qRT-PCR. Collectively, these results provide a starting point to study molecular mechanisms of tilapia immune response to *Streptococcus agalactiae* infection and may be applied as a basis for developing disease resistant strains by breeding selection.

1. Introduction

Tilapia is one of the most important commercial and aquaculture fishes in the world, and the common name encompasses three distinct genera: *Oreochromis*, *Sarotherodon* and *Tilapia*. *Oreochromis niloticus* (Nile tilapia) is among the largest tilapia in the world and plays an important economic role worldwide. In this fish, disease outbreak from *Streptococcus agalactiae* infection has brought about severe economic losses in various countries and threatened the further development of tilapia aquaculture [1]. Because of these economic concerns, most farmers use antibiotics or probiotics to control *Streptococcus agalactiae* infection [2]. Many articles have reported that *Streptococcus agalactiae* will cause meningoencephalitis, mastitis, pneumonia, and bacterial sepsis in a wide range of hosts [3]. Furthermore, *Streptococcus agalactiae* has been isolated from several animal species, including humans and many types of fish [4]. In aquacultured fish, *Streptococcus agalactiae* infection has been known to be a cause of septicemic disease since 1966 [5], and thus, this bacterial species represents a serious problem for the tilapia aquaculture industry. However, misuse of antibiotics may lead to accumulation of the drugs in the aquatic environment, which can be harmful to the aquatic organisms. As such, development of safer alternative therapeutic or prophylactic methods to combat *Streptococcus agalactiae* infection in tilapia are urgently needed. In order to achieve this goal, a detailed understanding of the tilapia immune response to *Streptococcus agalactiae* infection will be of great benefit. With this knowledge, effective and precise methods may be rationally designed to target selected functional

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genes or pathways that contribute to disease resistance [6]. For example, after identifying gene or signal transduction pathways that are involved in disease resistance, the genetic basis of immune-related traits may then be examined and exploited by selective breeding. While the identified genetic factors may contribute to a host of different immune responses, they should be explicitly useful for preventing *Streptococcus agalactiae* infection in tilapia to maximally benefit the tilapia aquaculture industry [7].

The complete genome of *Streptococcus agalactiae* (Lancefield group B; GBS) strain S25, isolated from peritoneal liquid of Nile Tilapia, was previously published. This study revealed several essential and unique components of the bacterial cells, such as bacterial DNA and peptidoglycan (PGN), which may serve as efficient signal molecules for tilapia to detect bacterial invasion [8]. In recent years, next-generation sequencing (NGS) technologies have provided a new approach for identifying genes that are associated with bacterial infections in fish [9]. Understanding the underlying molecular mechanisms that control different effects of *Streptococcus agalactiae* infection in tilapia is critical to developing strategies for decreasing disease outbreaks and minimizing economic risks in aquaculture [10]. In order to gain such understanding, we have previously used NGS technology to analyze the response of *Epinephelus lanceolatus* to infection by *Vibro alginolyticus*. In that study, we found that the bacterial flagellum probably stimulates TLR5 activity and causes effects via a MyD88-dependent pathway. The resulting production of IL-1β and IL-8 by NF-κB activation, induces pro-inflammatory responses, including chemotactic effects [9]. In another study, we utilized NGS to demonstrate that the complement system and hepcidin may be induced after *Epinephelus coioides* infection by *Vibro alginolyticus* [11]. Our research results on *Epinephelus coioides* or *Epinephelus lanceolatus*, along with results from other groups, have clearly shown that NGS technologies represent a powerful tool for the large-scale identification of immune-related genes and the analysis of their expression in tilapia with limited genomic information [12–14].

In the present study, our goal is to extend our previous results with *Vibro alginolyticus* and examine the immune responses that are induced by *Streptococcus agalactiae* infection in tilapia. To elucidate the components of tilapia immune response after *Streptococcus agalactiae* infection, we examined the transcriptional profiles of a mixture of liver, brain and intestine after infection. Utilizing transcriptome technology, we identified 1748 differentially expressed genes, many of which play critical functional roles in immune-related pathways. One especially relevant pathway was leukocyte transendothelial migration, which proceeds by several recognized steps, involving both endothelial cells and leukocytes. This pathway was represented by differential expression of rolling, firm adhesion, and diapedesis-specific molecular regulators [15]. Hepoxilin A3 and leukotriene B4 are known to coordinate bacterial-induced neutrophil transepithelial migration, and distinct cellular sources of these molecules have been suggested to be important in tilapia [16]. These research results contribute to better understanding of the mechanism of *Streptococcus agalactiae* infection in tilapia, and inducers of these immune-related genes may be suitable for inclusion as fish fodder in the aquaculture industry.

2. Materials and methods

2.1. Tilapia and Streptococcus agalactiae culture

Oreochromis spp. and *Oreochromis niloticus* were purchased from a private tilapia culture company in the Changhua region of Taiwan. The *Oreochromis niloticus* (average length 4 ± 1 cm; average weight of 2.5 ± 1.0 g) and *Oreochromis* spp. (average length 3.5 ± 0.3 cm; average weight of 1.22 ± 0.13 g) were acclimated in an FRP tank under 28 °C for 7 days. The *Streptococcus agalactiae* culture was previously described and used for infection experiments [17]. To evaluate the bacterial distribution in the organs (brain, liver, or intestine) after *Streptococcus agalactiae* infection in tilapia, 1.1×10^8 *Streptococcus agalactiae* cfu/fish were injected into tilapia. Pilot dosing studies were performed by injecting tilapia with a range of serial dilutions of *Streptococcus agalactiae* and monitoring the survival rate. The dose of 1.1×10^8 *Streptococcus agalactiae* cfu/fish was based on preliminary results showing that this level of pathogen could reliably produce infections that were sublethal for at least 48 h. After 1, 3, 6, 12, 24 or 48 h, tilapia were killed and the organs were removed (brain, liver and intestine). Organs were soaked in PBS buffer, then *Streptococcus agalactiae* were counted in the plate after 16 h incubation (Supplementary Fig. 1). All animal experimental procedures were in accordance with Academia Sinica guidelines and approved by the Animal Care and Use Committee of Academia Sinica. In this study, a standard procedure for euthanasia of fish was used. Buffered tricaine methane sulfonate (TMS, MS-222, Sigma, Cat: A5040) was administered to induce loss of consciousness and death. Briefly, a 10 g/L stock solution was first prepared and buffered by sodium bicarbonate (pH 7.0–7.5). One-part stock solution was diluted with 39 parts of water, making a 250 mg/mL working solution. Fish to be euthanized were transferred into a transfer container with diluted MS-222. It usually required 10–15 min for death to occur. Fish were considered to be dead 10 min after the loss of gill movement. Verified dead fish were stored at 4 °C.

2.2. RNA extraction and next generation sequencing

The *Oreochromis niloticus* were injected with *Streptococcus agalactiae* (40,000 cfu/fish) and the tilapia were sacrificed 720 min after infection. Tilapia that were only injected with Bacto™ Brain Heart Infusion served as a control. Total RNA from liver, intestine and brain were extracted according to the manufacturer's protocol for Trizol reagent (Invitrogen, Carlsbad, CA, USA) for next generation sequencing [11]. The RNA samples for gene expression analysis by quantitative real-time PCR (qRT-PCR) were extracted from *Oreochromis* spp. sacrificed at 1, 3, 6, 12, 24 and 48 h after infection ($n = 5$). The assembly and functional annotation for NGS analysis was described previously [11].

2.3. Bioinformatic analyses and identification of differentially expressed contigs

Gene ontology (GO) enrichment analysis and pathway enrichment analysis of the Kyoto encyclopedia of genes and genomes (KEGG) followed our previous publications [9,11]. The sequences were considered as orthologous if the reciprocal BLAST e-value was $<10^{-40}$ [18]. The *P* value corresponds to the gene expression test between the *Streptococcus agalactiae* infection group and the control group, and the false discovery rate (FDR) adjusted *P* value was used [7]. The GO terms from the database (http://www.geneontology.org) were applied and mapped with the transcriptome data. A gene ontology functional enrichment analysis and pathway enrichment analysis were used for GO and KEGG databases to study biological pathways.

2.4. Quantitative real-time PCR for analysis of gene expression

The tissue (liver, intestine and brain) samples were homogenized in Trizol Reagent and extracted to obtain the mRNA for qRT-PCR to analyze gene expression. Primers for qRT-PCR were designed as shown in Table 1 qRT-PCR was performed as previously described [11]. These three tissues were selected because they are known to