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Expression profile of immune-related genes in *Lates calcarifer* infected by *Cryptocaryon irritans*

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ABSTRACT

Cryptocaryon irritans causes Cyptocaryonosis or white spot disease in a wide range of marine fish including *Lates calcarifer* (Asian seabass). However, the immune response of this fish to the parasite is still poorly understood. In this study, quantitative polymerase chain reaction (qPCR) was performed to assess the expression profile of immune-related genes in *L. calcarifer* infected by *C. irritans*. A total of 21 immune-related genes encoding various functions in the fish immune system were utilized for the qPCR analysis. The experiment was initiated with the infection of juvenile fish by exposure to theronts from 200 *C. irritans* cysts, and non-infected juvenile fish were used as controls. Spleen, liver, gills and kidney tissues were harvested at three days post-infection from control and infected fish. In addition, organs were also harvested on day-10 post-infection from fish that had been allowed to recover from day-4 up to day-10 post-infection. *L. calcarifer* exhibited pathological changes on day-3 post-infection with the characteristic presence of white spots on the entire fish body, excessive mucus production and formation of a flap over the fish eye. High quality total RNA was extracted from all tissues and qPCR was performed. The qPCR analysis on the cohort of 21 immune-related genes of the various organs harvested on day-3 post-infection demonstrated that most genes were induced significantly ($p < 0.05$) in all tissues, particularly liver (11/21 genes) and kidney (11/21). The expression profile demonstrated that induction of the MHC Class IIα gene was the highest compared to the other genes followed by serum amyloid A, CC chemokine and hepcidin-2 precursor genes. In fish that were allowed to recover from the *C. irritans* infection (10 days post-infection), expression of the immune-related genes was down-regulated to levels similar to the control fish. These results provide insights into the interaction between *C. irritans* and *L. calcarifer* and suggest that the innate immune system plays an important role in early defence against parasite infection allowing the fish to eventually recover from the infection.

1. Introduction

Cryptocaryon irritans, one of the most common and destructive protozoan pathogens of marine fish, is the causative agent of cryptocaryonosis, also known as 'white spot disease' [1]. Cryptocaryonosis in marine fish is not limited to the natural environment, and is becoming an issue of concern in aquaculture due to the high mortality of infected stocks [2]. In Malaysia, occurrence of cryptocaryonosis is a significant problem for marine fish aquaculture especially *Lates calcarifer* (Asian seabass) both at hatchery and nursery stages [3,4]. As a consequence, the disease has caused severe economic losses to the aquaculture industry due to high mortality as well as high cost of control measures and treatment during outbreaks. Although chemical treatment has been widely used to control the outbreaks [5], major drawbacks include toxicity [6] as well as development of drug resistance in tomonts or the encysting stage of *C. irritans* [4]. Thus, vaccination against the parasite may provide an alternative to chemical treatment and previous immunization of *L. calcarifer* [7] and *Epinephelus coioides* [8] with *C. irritans* elicited a strong antibody response against the parasite. The protective immunity, however, takes a few weeks to develop and could be defined as sluggish.

It is evident that immune defence mechanisms play a crucial role in protecting fish from various pathogenic organisms. The innate immune system is generally the first line of host defence in

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overcoming the invading pathogenic organism(s) and to deal with any foreign material until the acquired immune system is sufficiently activated [9,10]. Many earlier studies have documented the expression levels of various immune-related genes in different fish models especially in response to various parasite infections such as *Ichthyophthirius multifiliis* [11], *Trypanosoma carassii* [12] and *Enteromyxum leei* [13]. The identification of *L. calcarifer* immune-related genes upon challenge with bacterial lipopolysaccharide (LPS) was reported previously using the suppression subtractive hybridization (SSH) technique [14]. We recently utilised an in-house developed microarray to study the transcriptional response of genes in the liver of *L. calcarifer* following *C. irritans* infection. The study identified several group of genes that are likely to contribute to the anti-parasitic immune responses [15]. Fish hosts, however, do possess an intricate immune system and it is likely that various components interact extensively to give the host optimal protection against ciliate-infections. To validate our earlier observations, we extended the study to analyse mRNA transcriptional changes in a cohort of pivotal immune-related genes of the systemic (spleen, liver and kidney) and local immune (gills) organs by real-time quantitative PCR (qPCR). We demonstrated that mRNA transcript levels of this panel of genes including those encoding for α-2-macroglobulin, serum amyloid A, C-type lectin, MHC-IIβ and MHC-IIα, were significantly increased in the different tissues of infected seabass during the acute phase of infection [3 days post-infection (pi)] when compared to expression in non-infected fish. In addition, *L. calcarifer*, generally recover from cryptocaryonosis suggesting a diminished immune response that allows the fish to improve from the infection. We measured RNA transcript levels during the recovery phase [10 days pi] and demonstrate that expression levels declined as the fish recovered.

2. Materials and methods

2.1. Fish and rearing conditions

For the experimental infection, 30 healthy juvenile Asian seabass (*L. calcarifer*) (115 g mean body weight) were transported from the Marine Aquaculture Breeding Centre at Tanjong Demong, Terengganu to the Fisheries Research Institute, Penang, Malaysia. The juvenile fish were kept in 1000 L capacity tanks with running sterilized seawater at 28 ppt salinity and on a simulated natural photoperiod. The fish were acclimatized for three weeks prior to the experiments and were fed twice daily with a commercial pellet diet. The seawater temperature fluctuated between 25 °C and 27 °C during the experiment. For parasite collection, 10 adult *L. calcarifer* (500–700 g) were obtained from open sea cages at Bukit Tambun, Penang, Malaysia. The fish were reared in 150 L aquariums filled with 100 L of seawater at 28 ppt salinity [16].

2.2. Parasites

C. irritans was isolated from infected adult *L. calcarifer* using the method described by Lokanathan et al. [16]. Ice bags were placed into the aquariums twice daily to lower the water temperature from 27 °C to 19 °C and induce disease. Once white spots were visible to the naked eye, glass petri dishes were placed at the bottom of the aquariums. The following day, the petri dishes were collected and replaced with new ones. The adhering cysts were gently scrapped from the petri dishes and were cleaned with autoclaved seawater. The cysts were incubated at 27 °C until further use.

2.3. Infection experiment

All infections were performed in individual tanks at 27 °C. Juvenile fish were each exposed to approximately 20 000 to 22 000 theronts hatched from 200 *C. irritans* cysts whilst non-infected juvenile fish were used as controls. Ten fish were used for each condition; controls, 3-day post-infection and 10-day post-infection. The control and 3-day post-infection fish were sacrificed on day-3 post-infection. The group of fish referred to as 10-day post-infection was transferred to a fresh tank on day-4 to allow them to recover from the infection. For these fish, seawater was changed every day until day-10. All fish were anaesthetized with clove oil and spleen, liver, gills and kidney tissues were harvested on day-3 and day-10 post-infection. Tissues were wrapped in RNase free aluminium foil and immediately snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction [14].

2.4. Isolation of total RNA and cDNA synthesis

The harvested frozen tissues were homogenised in 5 mL TRI Reagent® (Molecular Research Center, Inc., USA) on ice using a handheld motorized homogenizer (IKA® T10 Basic Ultra-Turrax®, Germany). Three biological replicates were randomly picked from controls, day-3 post-infection and day-10 post-infection samples. The RNA was extracted using TRI Reagent® (Molecular Research Center, Inc., USA), DNase treated to remove contaminating genomic DNA and purified using the Qiagen kit (Qiagen, Germany) according to the manufacturer's protocol. The RNA integrity and concentration was determined on the Agilent 2100 Bioanalyzer (Agilent Technologies) and Nanodrop ND-1000 spectrophotometer (Agilent Technologies). First strand cDNA was synthesized using 1 μg of total RNA for each sample (spleen, liver, gills and kidney) and Superscript III transcriptase following the manufacturer's instructions (Invitrogen, USA). The synthesized cDNA samples were stored at −20 °C until further use.

2.5. Primer design

The immune-related genes chosen for analysis by qPCR were selected from the *L. calcarifer* EST database (http://www.genomemalaysia.gov.my/cbass/). A total of 21 genes were selected (Table 1) based on immune-related genes that were reported in other fish species from previous studies. The primers used in the study were designed using Oligo Explorer version 1.1.2 (http://oligoexplorer.software.informer.com/). The primer sets for housekeeping genes were based on the 18S (GenBank Accession No: GQ507431) [16] and Ef1-α (GenBank Accession No: GQ507427) gene sequences [17] (Table 1). A dilution series was used to generate a standard curve by plotting the quantification cycle (C_q) for each dilution point against the starting quantity of cDNA. Standard curves were generated and the efficiency (E) of the primer sets calculated according to the equation $E = 10^{(-1/\text{slope})}$ [18] ranged from 1.9 to 2.1.

2.6. qPCR analysis

The resulting single strand cDNA generated from 1 μg RNA was diluted 10 times and assayed using primers for 21 different genes (Table 1). The qPCR on the immune-related genes selected was performed with the Mastercycler® ep realplex 4 (Eppendorf, Germany) using Ssofast™ EvaGreen® according to the manufacturer's protocol (Biorad Laboratories, USA). Briefly, the 20 μl of reaction including 10 μL SsoFast EvaGreen Supermix, 1 μL each primer (10 μM) and 2 μL diluted cDNA was initially denatured at 95 °C for 2 min and then amplified for 40 cycles (95 °C, 5 s, 59 or 60 °C, 15 s and 68 °C, 20 s). Data from each of the assays was normalized to the housekeeping or reference genes, *18S rRNA* [16] and *Ef1-α* [17], to standardize the results by eliminating variation in both mRNA and cDNA synthesis efficiencies. At the end of each cycle, melting curve