Fish & Shellfish Immunology 33 (2012) 788–794

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Cryptocaryon irritans infection induces the acute phase response in *Lates calcarifer*: A transcriptomic perspective

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ARTICLE INFO

Article history:
Received 18 May 2012
Received in revised form
9 July 2012
Accepted 17 July 2012
Available online 27 July 2012

Keywords:
cDNA microarray
Host–pathogen
Lates calcarifer
Cryptocaryon irritans
Acute phase response (APR)

ABSTRACT

Cryptocaryoniasis (also known as marine white spot disease) is mediated by *Cryptocaryon irritans*. This obligate ectoparasitic protozoan infects virtually all marine teleosts, which includes *Lates calcarifer,* a highly valuable aquaculture species. Little is known about *L. calcarifer–C. irritans* interactions. This study was undertaken to gain an informative snapshot of the *L. calcarifer* transcriptomic response over the course of *C. irritans* infection. An in-house fabricated cDNA microarray slides containing 3872 probes from *L. calcarifer* liver and spleen cDNA libraries were used as a tool to investigate the response of *L. calcarifer* to *C. irritans* infection. Juvenile fish were infected with parasites for four days, and total RNA was extracted from liver tissue, which was harvested daily. We compared the transcriptomes of *C. irritans*-infected liver to uninfected liver over an infection period of four days; the comparison was used to identify the genes with altered expression levels in response to *C. irritans* infection. The greatest number of infection-modulated genes was recorded at 2 and 3 days post-infection. These genes were mainly associated with the immune response and were associated in particular with the acute phase response. Acute phase proteins such as hepcidin, C-type lectin and serum amyloid A are among the highly modulated genes. Our results indicate that an induced acute phase response in *L. calcarifer* toward *C. irritans* infection is similar to the responses observed in bacterial infections of teleosts. This response demonstrates the importance of first line defenses in teleost innate immune responses against ectoparasite infection.

1. Introduction

Mariculture of finfish is one of the fastest growing sectors in Asian aquaculture. The Asian sea bass (*Lates calcarifer*), also known as Barramundi, is a valuable edible fish species cultured in Southeast Asia [1]. In Malaysia, aquaculture of *L. calcarifer* is a lucrative industry and ranks as the third largest contributor of aquacultured marine species after the tiger prawn and Hawaiian White Shrimp [2]. However, diseases are one of the major risk factors in commercial aquaculture, and millions of dollars are lost annually due to disease, greatly impeding both the economic and social development of many countries [3]. Notably, parasite infections were reported to be major pathogenic agents that contributed to fish mortality [4].

Cryptocaryoniasis, a disease caused by the obligate holotrichous ciliate protozoa [5], *Cryptocaryon irritans,* is a major problem in the *L. calcarifer* industry [6]. This ectoparasitic protozoan rapidly proliferates and invades the integument of its host, which severely impairs the physiological functions of the skin and gills [7]. The parasite undergoes a homogeneous host quadriphasic life cycle, which consists of a theront (free swimming infective stage), trophont (embedded and feeding on the host stage), protomont (upon leaving the host and encyst stage) and tomont (host-independent reproductive stage). Being an opportunistic histophagous parasite, the theront invades and penetrates to the basal layer of the epidermis, where it further undergoes differentiation and growth in the same host [8]. In the gills and skin, cryptocaryoniasis causes localized lymphocytic infiltration, focal necrosis and varying degrees of epithelial proliferation; similar pathological effects have been reported for its freshwater counterpart, *Ichthyophthirius multifiliis* [8]. Cryptocaryoniasis has been reported to be pathogenic to *Epinephelus coioides*, *L. calcarifer* and *Lutianus johni* larvae [9,10], and it also caused mortality in *Sparus aurata*, *Dentex dentex* and *Seriola dumerili* broodstock [11]. Cryptocaryoniasis caused

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http://dx.doi.org/10.1016/j.fsi.2012.07.005

substantial losses in mariculture [12,13], particularly at the hatchery and nursery stages in Malaysia [14]. These losses have prevented the expansion of the industry.

Although the biology of *L. calcarifer* has been increasingly studied using molecular approaches [15–17], the knowledge of host–pathogen interactions for this species is still limited. Large scale transcriptome studies utilizing the microarray approach enables the profiling and identification of enormous numbers of genes that have differentially altered expression levels [18]. This approach has been widely adapted in teleost research for profiling the host response toward bacterial [19], parasitic [20], fungal [21] and viral infections [22]. With the availability of the genomic data on this species [23,24], the enigma of *L. calcarifer*-pathogen interactions can be further explored and elucidated.

Therefore, to better understand the host–pathogen interactions in *L. calcarifer*, we investigated the time-resolved liver transcriptome response of *L. calcarifer* toward *C. irritans* infection. Although *C. irritans* infection occurs in skin and gill, this study focused primarily on the liver tissue as it is a central metabolism organ that controls many physiological functions, and is a major organ for production of acute phase reactants during infection [25–27]. Thus, the objective of this study was to gain a representative and reliable snapshot of the transcriptional response toward infections caused by *C. irritans* using a targeted microarray. The results of our study indicated that an induced acute phase response (APR) in *L. calcarifer* occurred as a result of *C. irritans* infection.

2. Materials and methods

2.1. Experimental fish and C. irritans infection

Juvenile *L. calcarifer* (27.5–36.0 cm in total length, 219–479 g) were obtained from floating cages in Bukit Tambun, Penang, Malaysia. Fish were screened for ectoparasites via body and gill smears, acclimatized for 2 weeks (28–30 ppm salinity, natural photoperiod, 27–30 °C) and later randomly assigned to infected and control groups. Fish were hosted individually in a 13 L aquarium with filtered and UV sterilized water; sterilization was conducted utilizing the Sanitron UV water purifier S50C (Sanitron, USA). *C. irritans* isolation was conducted utilizing methods reported previously [28]. The encysted tomont was cleaned and incubated in sterile seawater that was filtered and maintained at 27 °C. Each individual fish in the infected group was challenged with theronts from 20 cysts. Three controls and three infected fish tissue samples were collected 1, 2, 3 and 4 days post-infection (dpi) for RNA extraction. Fish were euthanized and dissected, and liver tissues were immediately snap frozen in liquid nitrogen prior to storage in a −80 °C freezer until use. Each fish was also sampled for the presence of *C. irritans* via body and gill smears for confirmation of infection.

2.2. RNA preparation and hybridization

Individual frozen liver tissues were homogenized with a mortar and pestle prior to total RNA extraction with TRI REAGENT® (Molecular Research Center, USA), which was conducted according to the manufacturer's instructions. The quality and quantity of the total RNA were determined using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, USA) and an Agilent 2100 Bioanalyzer, which utilized the RNA 6000 LabChip® kit (Agilent, USA). A dual-labeled experimental design for the microarray hybridization was employed. Briefly, 10 μg of total RNA (RIN 7 and above for both samples) were used for first strand synthesis of aminoallyl-labeled cDNA, and this was followed by covalent coupling to the NHS-ester of the Cy3 or Cy5 cyanine fluor using dual color Post labeling CyScribe™ (Amersham Biosciences, USA), which was conducted according to manufacturer's protocol. Uncoupled dye-ester to the aminoallyl-labeled cDNA was removed by CyScribe™ GFX™ column purification (Amersham Biosciences, USA) prior to hybridization. Each hybridization experiment included dye-swaps to compensate for cyanine fluor effects. Thus, the entire microarray experiment contained 24 hybridizations as follows: 4 time-points (1, 2, 3 & 4 dpi) × 3 biological replicates × 2 types of labeling (normal & dye swap). An in-house fabricated *L. calcarifer* cDNA microarray slide [29], which contained 3872 probes from spleen and liver cDNA libraries [30] was use for the hybridization. Prehybridization of the slides was conducted according to methods described previously [31]. Purified and labeled cDNAs were hybridized onto the *L. calcarifer* cDNA slides, and hybridizations were completed in the dark and inside an incubation chamber (Genetix, UK) that was submerged in a 45 °C water bath for 18 h. Application of the probe to the microarray slide and hybridization were performed according to the Post labeling CyScribe™ instructions (Amersham Biosciences, USA).

2.3. Microarray analysis

Slides were washed according to the manufacturer's instructions (Corning Microarray Technology) and scanned immediately with the GenePix 4100A scanner (Axon Instruments, USA). Slides were scanned at a resolution of 10 μm. The Cy3 and Cy5 cyanine fluors were excited at 532 nm and 635 nm, respectively; the photomultiplier tube settings were adjusted within slides to balance the Cy5 and Cy3 channels. Images obtained were processed and analyzed with GenePix™ Pro 6.0 (Axon Instruments, USA) to align the spot and to quantify the spot intensity. Extracted raw spot intensity from GenePix™ Pro 6.0 was subsequently used for array data analyses using GeneSpring GX 7.3.1 (Agilent Technologies, USA). The data were subjected to transformation, normalization and quality filtering as follows: 1) dye swap to specific samples and 2) a whole array Lowess normalization was performed (using 20% smoothing/cut-off 0.001). The normalized data were grouped on the basis of the experimental conditions (infection time points) and filtered using a Volcano plot. Differentially expressed genes were defined as those having a p-value of ≤ 0.01 and an absolute change of $\geq$ 2-fold or $\leq$ 0.5-fold for *C. irritans*-infected tissue at 1 dpi, 2 dpi, 3 dpi and 4 dpi relative to the uninfected respective control tissue. One-way ANOVA tests were employed to identify genes that had significantly altered expression levels at the four time points. The ANOVA parameters included the following: parametric test, variances not assumed equal (Welch ANOVA), a p-value cutoff 0.01 and a multiple testing correction (Benjamini and Hochberg False Discovery Rate). The array data for this publication was deposited into NCBI's Gene Expression Omnibus database and are accessible via GEO Series accession number GSE34745. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34745).

2.4. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed on a Mastercycler® ep realplex (Eppendorf, Germany) to quantify the expression of hepcidin (HEP), acute phase serum amyloid A (A-SAA) and ferritin heavy subunit (FerH) genes. To control for variation in the RNA concentration, cycle threshold (Ct) values were normalized to β-actin and 18S ribosomal RNA. Primers were either designed using Oligo Explorer version 1.1.2 (http://oligoexplorer.software.informer.com/) or adopted from previous reports [32]; primer sequences are shown in Table 1. Briefly, 25 μl reactions were performed using the iScript™One-Step RT-PCR kit with SYBR green according to the manufacturer's instructions (BioRad Laboratories, USA); the reaction contained