Developmental and Comparative Immunology 42 (2014) 294–301

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Molecular signatures at imminent death: Hemocyte gene expression profiling of shrimp succumbing to viral and fungal infections

Priscila Goncalves [a,1,2], Cristhiane Guertler [a,2], Evelyne Bachère [b], Claudia R.B. de Souza [c], Rafael D. Rosa [a], Luciane M. Perazzolo [a,*]

[a] Laboratory of Immunology Applied to Aquaculture, Department of Cell Biology, Embryology and Genetics, Federal University of Santa Catarina, 88040-900 Florianópolis, SC, Brazil
[b] Ifremer, CNRS, Université Montpellier 2, IRD, Université Montpellier 1 (UMR5119 – Ecology of Coastal Marine Systems), Place Eugène Bataillon, CC80, 34095 Montpellier, France
[c] Laboratory of Molecular Biology, Institute of Biological Sciences, Federal University of Pará, 66075-110 Belém, PA, Brazil

ARTICLE INFO

Article history:
Received 14 August 2013
Revised 27 September 2013
Accepted 30 September 2013
Available online 9 October 2013

Keywords:
Litopenaeus vannamei
WSSV
Fusarium solani
Host–pathogen interactions
Molecular signature
High-throughput microfluidic RT-qPCR

ABSTRACT

Infectious diseases represent the most serious threat to shrimp farming worldwide. Understanding the molecular mechanisms driving shrimp-pathogen interactions is necessary for developing strategies to control disease outbreaks in shrimp production systems. In the current study, we experimentally reproduced mortality events using standardized infections to characterize the hemocyte transcriptome response of the shrimp *Litopenaeus vannamei* succumbing to infectious diseases. By using a high-throughput microfluidic RT-qPCR approach, we identified molecular signatures in shrimp during lethal infections caused by the White Spot Syndrome Virus (WSSV) or the filamentous fungus *Fusarium solani*. We successfully identified gene expression signatures shared by both infections but also pathogen-specific gene responses. Interestingly, whereas lethal WSSV infection induced the expression of antiviral-related genes, the transcript abundance of many antimicrobial effectors was reduced by lethal *F. solani* infection. To our knowledge, this is the first report of the immune-gene repertoire of infected shrimp at the brink of death.

1. Introduction

Interactions between host and pathogens have shaped the invertebrate immune system over the past million years. According to the evolutionary Red Queen hypothesis, pathogens are continuously evolving new strategies to challenge the immune response of hosts and, consequently, host immune systems have improved mechanisms of defense (Valen, 1974). Thus, an imbalance of host–pathogen interactions may result by either the successful elimination of the infectious agents or by causing a serious threat to the host. This is particularly true for farmed shrimp that have suffered frequent and severe mortality events worldwide (Flegel, 2012).

Penaeid shrimp harbor a wide range of natural and opportunistic pathogens with viruses being by far the most important risk to shrimp farming worldwide (Flegel, 2012; Flegel and Sritunyalucksana, 2011). Since its first appearance in the 1990's in Taiwan, the White Spot Syndrome Virus (WSSV) has been identified as the most serious pathogen infecting all cultivated penaeid shrimp as well as wild crustaceans (Flegel and Sritunyalucksana, 2011). Mass mortality events in shrimp farming due to infectious diseases have been linked to both environmental factors and aquaculture practices. Under stressful circumstances, some opportunistic microorganisms can become pathogenic, leading to the establishment of new diseases. The classical examples are filamentous fungi and bacteria of the genus *Vibrio* (Bachère et al., 2004; Lightner, 1988; Lightner et al., 2012). Opportunistic infections in shrimp have been associated with losses in both larval and juvenile stages (Saulnier et al., 2000).

The economic impact of mass mortalities in shrimp farming has intensified the efforts of researchers and aquaculture producers to develop strategies to overcome disease outbreaks. In this context, many transcriptome- and proteome-wide studies have been performed in the last decades in order to characterize shrimp immune effectors and immune responses to infections (reviewed in (Bachère et al., 2004; Hauton, 2012; Robalino et al., 2009)). These studies have provided valuable information for understanding the molecular mechanisms driving shrimp–pathogen interactions. Although the published high-throughput molecular studies have explored the shrimp immune response upon different viral and microbial infections, no previous studies have investigated the gene expression profile of shrimp during mortality events. Here, we improve upon knowledge of shrimp immune response with the identification of hemocyte gene expression signatures associated with imminent death resulting from pathogenic infections.

* Corresponding author. Tel.: +55 48 3721 5528; fax: +55 48 3721 9672.
E-mail address: l.m.perazzolo@ufsc.br (L.M. Perazzolo).
[1] Present address: Department of Biological Sciences, Faculty of Science, Macquarie University, 2109 Sydney, NSW, Australia.
[2] These authors contributed equally to this work.

0145-305X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2013.09.017

Using standardized viral and fungal infections, we assessed the hemocyte transcriptome response of the penaeid shrimp *Litopenaeus vannamei* succumbing to two unrelated lethal infections. High-throughput microfluidic RT-qPCR analyses provided a general overview of the molecular functions regulated by lethal infections and also evidenced pathogen-specific gene expression signatures. While lethal WSSV infection induced the expression of many antiviral-related genes, in shrimp succumbing to infection by the opportunistic filamentous fungus *Fusarium solani* the mRNA levels of most of the analyzed genes were reduced. This study represents the first transcriptome analysis of the immune functions affected in penaeid shrimp at imminent death by pathogenic infections.

2. Materials and methods

2.1. Shrimp

Specific pathogen-free (SPF) *Litopenaeus vannamei* (10 ± 2 g) were obtained from the Laboratory of Marine Shrimp (Federal University of Santa Catarina, Brazil) and transferred to aerated aquaria (23 ± 4 °C; salinity: 32–34‰) for at least 72 h prior to their use. Only male and female animals at intermolt stage were used in the experiments.

2.2. Experimental viral and fungal infections

To assess transcriptomic responses of shrimp succumbing to pathogenic infections, experimental infections were carried out with the shrimp viral pathogen White Spot Syndrome Virus (WSSV) and the opportunistic filamentous fungus *Fusarium solani*. For both pathogens, we standardized doses sufficient to cause 100% mortality within 6 days. WSSV inoculum was prepared from WSSV-infected shrimp tissue following the protocol previously described by (Prior et al., 2003) with slight modifications. Briefly, abdomen tissues from infected shrimp were homogenized in a sterile saline solution (330 mM NaCl, 10 mM Tris, pH 7.4) (1:10 w/v) using a hand blender. The mixture was centrifuged (2000*g*, 20 min, 4 °C) and the supernatant filtered through 0.45 μm membrane filter. Tissue homogenate from WSSV-free shrimp was used as control. Fungal inoculum was prepared from cultures of the shrimp pathogen *F. solani*, following the method described by (Goncalves et al., 2012). Briefly, pure cultures were grown in potato dextrose agar at 30 °C in the dark for 10 days. Spores were then harvested from the agar surface in sterile saline solution, filtered through cheese cloth, and centrifuged (500*g*, 10 min). Spore pellets were washed twice with sterile saline solution and the concentration was adjusted in a Neubauer chamber.

Lethal experimental infections were carried out by the intramuscular injection of 50 μl of viral (1.3×10^7 viral particles) or fungal (5×10^6 spores) inoculum into the shrimp dorsal region. Naïve shrimp (*i.e.* non-injected animals at time 0 h) and unchallenged shrimps (*i.e.* injected with WSSV-free inoculum or sterile saline solution) were used as controls for both viral and fungal infections. Mortalities were monitored twice a day. Kaplan–Meier cumulative survival curves were generated for both infections and compared with log–rank tests. WSSV and *F. solani* infections were confirmed in infected shrimp by using the PCR-based assay previously described by (Lo et al., 1996) and (Lee et al., 2000), respectively.

2.3. RNA extraction and specific target amplification

Shrimp hemolymph was withdrawn into a modified Alsever solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) (Rodriguez et al., 1995) from challenged and unchallenged animals at 24 and 48 h post-injection. Hemocytes were separated from the plasma by centrifugation (800*g*, 10 min, 4 °C) and the cell pellet was used for RNA extraction.

Total RNA was extracted from pooled hemocyte samples (3 pools of 3 animals per condition) using TRIzol® reagent (Invitrogen) and treated with DNase I (Fermentas) to eliminate contaminating genomic DNA. Total RNA concentration and purity were checked by using a NanoVue (GE General Eletric Healthcare) spectrophotometer. Following heat denaturation (70 °C for 5 min), reverse transcription was performed using 1 μg of purified total RNA with 50 ng/μl oligo$(dT)_{12-18}$ in a 20 μl reaction volume containing the ImProm-II™ reverse transcriptase (Promega), according to the manufacturer's instructions.

In order to increase the number of cDNA templates, a preamplification PCR was performed using 1.25 μl of cDNA, 1.25 μl of a mixture of forward and reverse primers (200 nM each) and 2.5 μl of 2 × TaqMan®PreAmpMaster Mix (Applied Biosystems) (Jang et al., 2011). PCR cycling conditions were as follows: polymerase activation at 95 °C for 10 min, followed by 14 cycles of 95 °C for 15 s and 60 °C for 4 min. Preamplification products were diluted to 1:5 in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at −20 °C.

2.4. High-throughput RT-qPCR using 96.96 microfluidic dynamic arrays

A total of 68 hemocyte-expressed genes of broad relevance to immune system were chosen, including antimicrobial proteins/peptides (*e.g.* penaeidins, anti-lipopolysaccharide factors, crustin), proteases and protease inhibitors (*e.g.* cathepsins, alpha2-macroglobulins, serpins), prophenoloxidase system-related proteins (*e.g.* prophenoloxidase, prophenoloxidase-activating enzymes), antioxidants (*e.g.* superoxide dismutase, catalase, glutathione S-transferase), cytokines and signaling molecules (*e.g.* astakine, toll receptor, STAT), homeostase and coagulation (*e.g.* hemolymph clottable protein, transglutaminase), antiviral-related molecules (*e.g.* Dicer proteins, argonautes, Sid-1), apoptosis and autophagy (*e.g.* caspase 3, inhibitor of apoptosis, Rab7, Ras-related protein Rab-11) and other immune-related genes (*e.g.* HSP70, HSP90, histones H1 and H3) (Appendix A). The expression levels of the selected genes were evaluated by using the high-throughput microfluidic RT-qPCR platform BioMark™ (Fluidigm 96.96 dynamic array systems) (Jang et al., 2011). The sample reaction mixtures were performed in a final volume of 5 μl containing 1.25 μl of preamplified cDNA (diluted 1:5), 2.5 μl of 2 × TaqMan®Gene Expression Master Mix (Applied Biosystems), 0.25 μl of 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.25 μl of 20× EvaGreen (Biotium) and 0.75 μl of TE buffer. Primer reaction mixtures were made in the same volume of 5 μl containing 2.5 μl of 2× Assay Loading Reagent (Fluidigm), 1.25 μl of 20 μM of forward and reverse primer mix and 1.25 μl of TE buffer. Both sample and primer reaction mixtures were loaded into the dynamic array chip that was subsequently placed on the NanoFlex™ 4-IFC Controller for loading and mixing. After approximately 50 min, the chip was transferred to the BioMark™ Real-Time PCR System.

The cycling program used consisted of 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 1 min at 60 °C. Melting curves analysis was performed after completed RT-qPCR collecting fluorescence between 60–95 °C at 0.5 °C increments. Data were analyzed using the BioMark™ Real-time PCR analysis software to obtain Cq values. The 40S ribosomal protein S6 (*Lv*RpS6; GenBank: FE080516/FE095908) and S3A ribosomal protein (*Lv*RpS3A; GenBank: BF023924) were selected as endogenous reference genes. Results were presented as changes in relative expression normalized with the arithmetic mean of the Cq values of the two reference genes (Livak and Schmittgen, 2001). Statistical significance was