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Gene expression and protein levels of thioredoxin in the gills from the whiteleg shrimp (*Litopenaeus vannamei*) infected with two different viruses: The WSSV or IHHNV

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ABSTRACT

The thioredoxin (TRX) system in crustaceans has demonstrated to act as a cell antioxidant being part of the immune response by dealing with the increased production of reactive oxygen species during bacterial or viral infection. Since the number of marine viruses has increased in the last years significantly affecting aquaculture practices of penaeids, and due to the adverse impact on wild and cultured shrimp populations, it is important to elucidate the dynamics of the shrimp response to viral infections. The role of Litopenaeus vannamei thioredoxin (LvTRX) was compared at both, mRNA and protein levels, in response to two viruses, the white spot syndrome virus (WSSV) and the infectious hypodermal and hematopoietic necrosis virus (IHHNV). The results confirmed changes in the TRX gene expression levels of WSSV-infected shrimp, but also demonstrated a more conspicuous response of TRX to WSSV than to IHHNV. While both the dimeric and monomeric forms of LvTRX were detected by Western blot analysis during the WSSV infection, the dimer on its reduced form was only detected through the IHHNV infectious process. These findings indicate that WSSV or IHHNV infected shrimp may induce a differential response of the LvTRX protein.

1. Introduction

Invertebrates lack an adaptive immune system, but during their long evolutionary story they selectively fixed effective innate immune responses capable of protecting the organism against pathogens [1]; however, this complex system is still poorly understood [2]. Thus, as invertebrates lack the cells for the highly specific recognition, and a long-term memory found in vertebrates [3], the study of the innate factors dealing with cell protection against foreign and self-produced toxic substances, such as the redox systems, is relevant. The role of sulphydryl groups (−SH) in response to oxidative stress, and the thioredoxin systems in the maintenance of the cell redox homeostasis was highlighted by Grant [4].

Aerobic metabolism continuously produces reactive oxygen species (ROS) [5,6], that can be helpful, and one of the most important components of the host response of crustaceans against noxious microorganisms [7–9]. It is well known that the redox balance may be perturbed by the generation of ROS caused by exogenous sources (such as pathogens), and cells are shifted into a state of oxidative stress. To protect them from oxidative stress, cells possess a set of antioxidant enzymes and systems, which maintain the intracellular ROS at proper levels [10]. The thioredoxin (TRX) system is one of the major intracellular redox regulating systems, and it has been reported that in shrimp, it is a potent component of the antioxidant response [11]. Besides, an indirect anti-viral function of the Chinese white shrimp (*Fenneropenaeus chinensis*) TRX has been suggested, as its transcripts and protein levels were significantly up-regulated after challenging the shrimp by injection with a WSSV inoculum [12].

Viruses are an integral and important component of the marine ecosystem and all the living organisms inhabiting the marine environment may be prone to diseases and death caused by viral infections [13]. Interest in marine viruses has increased due to the severe economic consequences caused on commercially exploited marine animals such as shrimp. Outbreaks of shrimp viruses are

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now recognized as one of the most serious problems delaying the development and expansion of the shrimp farming industry worldwide [14]. Among the known viruses capable to infect penaeid shrimp, only four, the White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV) cause a serious threat to cultured and wild shrimp populations [15].

WSSV, an enveloped rodshaped virus that contains a circular double-stranded DNA genome of about 305 kb [16–18], is one of the most lethal pathogens in shrimp aquaculture [19]. WSSV is the causative agent of a disease leading to cumulative mortalities that can reach up to 100% within 3–10 days of the onset of clinical signs in cultured shrimp [20]. Furthermore, it is worth mentioning that despite considerable advances on the knowledge about factors, mechanisms and strategies used by this virus to infect and replicate in susceptible host cells, at present there is no treatment available to interfere with the unrestrained occurrence and spread of the disease.

IHHNV is a single-stranded DNA-containing virus belonging to the family Parvoviridae [21]. This virus was recognized as the etiological agent of high mortalities (over 90%) in juveniles and subadult individuals of the blue shrimp *Litopenaeus stylirostris* farmed in Hawaii in 1981 [22,23]. Even when the information related to IHHNV is still scarce, it has been reported that this pathogenic virus caused a serious decline of the wild shrimp populations in the Gulf of California in northern Mexico [24]. IHHNV is also considered a low-impact virus in *L. vannamei* culture; however, Kalagayan et al. [25], provided epidemiological and histopathological evidence for the hypothesis that infection by IHHNV induces a chronic disorder known as the Runt-Deformity Syndrome (RDS) in *L. vannamei*. Thus, not only the growth rate of infected organisms is affected, but a variety of cuticular deformities of the rostrum, antenna, and other thoracic and abdominal areas, may be identified in IHHNV infected specimens [26,27]. Finally, IHHNV shows a slow replication rate, showing a doubling time of about 22 h, while in WSSV, it was estimated to be of 1.9 h, which may be due to the limited genetic information contained within parvoviral particles and to the high dependence of this virus on their host cells for replication [28].

The aim of the present study was to evaluate and compare the changes in the thioredoxin gene expression profile and protein levels in gills of the whiteleg shrimp *L. vannamei* caused by two different viruses, WSSV and IHHNV.

2. Materials and methods

2.1. The WSSV challenge

2.1.1. Experimental shrimp for WSSV infection

L. vannamei breeding shrimp (44.0 ± 5.8 g) were obtained from a larval production laboratory located in San Agustin Bay, Sonora, Mexico and transported to the lab. Shrimp were randomly distributed in eight 450 L fiberglass tanks in a recirculating seawater system at 34 ppt, 28 ± 1 °C, and air stones to provide constant aeration. Animals were acclimatized for 10 days to these laboratory conditions. Water quality parameters were measured daily, and titanium heaters with temperature controllers were used. Animals were fed with a commercial pelletized feed containing 35% protein (Camaronina, Mexico) with a daily ration of 3% of total biomass per tank. At the end of acclimatization, two shrimp were randomly sampled from each tank and their pleopods were dissected, then DNA was isolated from pleopods and tested by PCR amplification for WSSV to ensure they were pathogen free (data not shown).

2.1.2. WSSV inoculum preparation and shrimp challenge

WSSV inoculum was prepared from naturally infected (PCR-positive) whiteleg shrimp *L. vannamei* kindly provided by an aquaculture facility. Approximately, 6 g of abdominal muscle were dissected, minced and then homogenized at 1:4 (w/v) ratio with sterile TN buffer (20 mM Tris; 400 mM NaCl, at pH 7.4) at 4 °C. The homogenate was first centrifuged at 500× *g* for 10 min at 4 °C to separate gross debris, and the supernatant was centrifuged at 12,000× *g* for 20 min at 4 °C, then it was filtered through a 0.45 μm membrane and kept frozen in aliquots at −20 °C for further use.

During the WSSV challenge, acclimatized shrimp ($n = 52$) were individually injected intramuscularly into the third abdominal somite using a 1 mL tuberculin syringe containing 20 μL of the WSSV inoculum (0.05×10^6 copies/ng of DNA), and then isolated in fiberglass tanks connected to a recirculating seawater system. Mock-infected group shrimp ($n = 31$) were injected with 20 μL of saline solution (400 mM NaCl, pH 7.4) in the same manner as infected animals and isolated in fiberglass tanks connected to a separate recirculating seawater system. After injection, all animals were maintained as described above and 4 shrimp from the control group and 8 from the WSSV-challenged group were sampled at 0, 6, 12, 24, 36 and 48 h post-injection (hpi). Individual samples of haemolymph (250 μL) and gills were taken each time. Haemolymph was extracted from the base of the fifth pereiopod with a 1 mL syringe containing two volumes of pre-cooled shrimp anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM Na_2-EDTA, 10 mM HEPES, pH 7.3) [29]; immediately it was centrifuged at 600× *g* for 5 min (4 °C) to separate the haemocytes from plasma, then haemocytes were preserved at −80 °C for further analysis. Gills and pleopods were individually dissected (~100 mg) and immediately frozen at −80 °C.

2.1.3. Quantitative assessment of the WSSV- DNA by qRT-PCR

Viral load was quantified by measuring WSSV-DNA copies in the pleopods of all mock-infected and WSSV-challenged shrimp as follows. The DNA from all samples was isolated by using the DNA extraction kit IQ2000™ WSSV Detection and Prevention System, following the manufacturer instructions. Real-time PCR was performed in triplicate reactions according to Durand and Lightner [30] in a BioRad ICycler® real-time PCR system using 20 ng of total DNA, a FAM/TAMRA-labeled taqman probe and the 2× Taqman master mix (Applied Biosystems). To construct a standard curve, a plasmid containing a WSSV DNA insert was kindly donated by Dr. Donald Lightner from the University of Arizona. Dilution series of WSSV DNA (10–10 million copies) were amplified with WSSV-specific primers (see [30]). The standard curve was calculated by linear regression analysis using threshold cycle (C_T) values and the log copies number (log Co) obtained from the serial dilution analysis. The copy number of unknown samples was calculated as follows: $10^{[(CT\ value-b)/m]}$ where b is the *y* intercept, and m is the slope.

2.2. The IHHNV challenge

2.2.1. Experimental shrimp for IHHNV infection

L. vannamei adult shrimp weighing 12 ± 1 g were received from an aquaculture facility and confirmed as specific pathogen free. During the IHHNV bioassay shrimp were randomly distributed and acclimatized during 10 days as described above in eight plastic tanks in aerated seawater at 34 ppt, 28 °C, and water quality was kept constant. Shrimp were fed twice a day with a 35% protein commercial pelletized feed (Api Camaron 2, Malta Cleyton). After acclimatization, shrimp were tested for IHHNV infection by PCR analysis to ensure that they were not infected as follows. Haemolymph was extracted as previously mentioned from each shrimp