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Expression profile of bio-defense genes in *Penaeus monodon* gills in response to formalin inactivated white spot syndrome virus vaccine

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ABSTRACT

White spot syndrome virus (WSSV) is the most devastating pathogen of penaeid shrimp. While developing technology to vaccinate shrimp against WSSV, it is imperative to look into the immune response of the animal at molecular level. However, very little information has been generated in this direction. The present study is an attempt to understand the expression of bio-defense genes in gill tissues of *Penaeus monodon* in response to formalin inactivated WSSV. A WSSV vaccine with a viral titer of 1×10^9 DNA copies was prepared and orally administered to *P. monodon* at a rate of 1.75×10^6 DNA copies of inactivated virus preparation (IVP) day^{-1} for 7 days. The animals were challenged with WSSV on 1st and 5th day post vaccination, and temporal expression of bio-defense genes in gill tissues was studied. Survival of 100% and 50% were observed respectively on 1st and 5th day post vaccination challenge. The humoral immune genes prophenoloxidase (proPO), alpha 2-macroglobulin (α2M), crustin and PmRACK, and the cell mediated immune genes caspase and Rab7 were up regulated in gill tissue upon vaccination and challenge. The expression of humoral gene crustin and cellular gene Rab7 was related to survival in IVP administered shrimp. Results of the study suggest that these genes have roles in protecting shrimp from WSSV on vaccination.

1. Introduction

White spot syndrome virus (WSSV) is an enveloped virus with circular double stranded DNA genome of about 300 kb belonging to family Nimaviridae under the genus *Whispovirus* (van Hulten et al., 2001; Escobedo-Bonilla et al., 2008). Once introduced into culture systems it spreads rapidly and uncontrollably (Yi et al., 2003) and causes total mortality (Lightner, 1996). The crustacean immune system is characterized by humoral and cellular factors (Flegel and Sritunyalucksana, 2011), which include phagocytosis, melanization, clotting, encapsulation, antimicrobial peptides, reactive oxygen intermediates (ROI), agglutination and non-self recognition factors such as lectins, lipoproteins and cell to cell communication molecules (Soderhall and Cerenius, 1992). There was a belief that invertebrates did not posses specific immunoglobulin like molecules which delayed attempts to vaccinate shrimp against WSSV (Faye, 1990; Singh et al., 2005). In this light a specific and active viral accommodation theory had also been proposed which suggested that exposure to inactivated viral particles or subunit viral proteins might result in innocuous, persistent viral infections rather than mortality upon subsequent viral challenge (Flegel and Pasharawipas, 1998). Meanwhile, in invertebrates, several immunoglobulin super family (IgSF) molecules have also been documented (Mendoza and Faye, 1996) such as cadherins, Ig – like proteins, extra cellular matrix proteins, tigrin (Johansson, 1999), peroxinectin (Johansson et al., 1995) haemolin (Bettencourt et al., 1997), limulus agglutination – aggregation factor (LAF), haemocytin, A74 protein, croquemort, plasmocyte spreading peptide (PSPI), Drosophila Toll/Cactus/Dorsal proteins (TCID) (Arala-Chaves and Sequeira, 2000) and down syndrome cell adhesion molecules (DSCAM) (Watthanasurorot et al., 2011).

Bio-defense genes regulate the production of biological defense molecules in an organism in response to invading pathogen and foreign materials. These include immunity related molecules, homeostasis, host pathogen interaction and other cellular processes. The role of bio-defense genes in shrimp immunity against WSSV infection has been studied extensively using high

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throughput methods (Leu et al., 2007; Zhao et al., 2007). Aoki and Hirono (2005) characterized 27 Expressed Sequence Tags (ESTs) for bio-defense genes in *Marsupenaeus japonicus* and found that immunostimulation with peptidoglycan resulted in transcription of the genes for prophenoloxidase, prophenoloxidase-activating factors, masquerade protein, trans glutaminase, clottable protein, lysozyme, α-2-macroglobulin and penaeidin. Temporal analysis of 17 genes related to cellular defense mechanisms in *Penaeus monodon* in response to WSSV resistance was investigated and found induction of prophenol oxidase (proPO) pathway genes and JAK-STAT pathway and down regulation of clotting related and antimicrobial peptide genes (Chen et al., 2008).

Namikoshi et al. (2004) claimed the first vaccination in shrimp against WSSV by intramuscular injection of formalin inactivated WSSV. Singh et al. (2005) demonstrated the same by oral administration. Since then there ware many reports on possible vaccination in crustaceans against WSSV (Rout et al., 2007; Ha et al., 2008; Mustaq and Kwang, 2011; Caipang and Fagutao, 2013; Kulkarni et al., 2013). Besides the impracticality of injection as the mode of vaccination (Witteveldt et al., 2004) the recent study by Kulkarni et al. (2013) showed that the shrimp mid gut could take up and process the WSSV vaccines effectively. Formalin inactivation is a process during which virus proteins are cross linked maintaining the antigenic epitops sufficient to induce protective immune response (Barteleng and Woortmeyer, 1984; Singh et al., 2005), and is superior to heat inactivation (Namikoshi et al., 2004). Investigations on the expression of bio-defense genes in response to formalin inactivated oral vaccine in shrimp are scanty. Meanwhile, expression of phagocytosis activating protein (PAP) gene in response to intramuscular injection of formalin inactivated vaccine was studied by Deachamag et al. (2006) and Chotigeat et al. (2007), and recent studies reported up regulation of LGBP and STAT after oral vaccination with baculo viral expressed VP28 (Mustaq and Kwang, 2011) and fortilin with recombinant VP28 (Caipang and Fagutao, 2013).

Here we propose that oral administration of formalin inactivated WSSV elicit innate humoral and cellular responses in shrimp, and the resulting immunity is a collective action of more than one bio-defense mechanisms, however, these responses are short lived. Hence the present study was undertaken to elucidate the response of known cellular and humoral bio-defense genes of *P. monodon* to the oral administration of formalin inactivated WSSV. To check the persistence of the efficacy of vaccination in terms of expression of immune genes, two challenge experiments were carried out, one immediately after 7 days vaccination and the other 5 days later.

2. Material and methods

2.1. Experimental animals

Specific pathogen free (SPF) shrimp (from WSSV and MBV) (250 Nos.) maintained in a Recirculating Aquaculture System (RAS) (Oriental Aqua Marine Biotech, India Pvt. Ltd., Coimbatore, India) at National Centre for Aquatic Animal Health (NCAAH), India, were used for the experiment. Absence of WSSV and MBV was confirmed through Nested PCR (Lo et al., 1996; Belcher and Young, 1998) during various stages of culture and before start of the experiment.

2.2. Extraction of WSSV

Virus was extracted from infected shrimp tissue as described earlier (Sudheer et al., 2012). All extractions and macerations were carried out in a laminar air flow chamber. In brief, 15 g gill and soft tissues from cephalothorax were macerated in 100 ml sterile sea water with sterile glass wool fiber and centrifuged at 8200×*g* for 20 min at 4 °C. The supernatant was collected in a sterile glass container. The remaining pellet was subjected to three freeze–thaw cycles and re-extracted. After each freeze–thaw cycle the extract was filtered through fine meshed sterile muslin cloth (100 μm mesh size). The filtrate was centrifuged at 8200×*g* for 20 min at 4 °C to get rid off shrimp cellular debris. The supernatant was collected, pooled and stored in sterile bottles. A batch of SPF juvenile *P. monodon* (10 Nos.) was injected at 6th abdominal segment with 10 μl WSSV suspension to assess its infectivity. The presence of WSSV in moribund animals was proved through nested PCR (Lo et al., 1996).

2.3. Real time PCR to determine WSSV titer

WSSV titer of the tissue extract used for preparing vaccine was determined by real time PCR through standard curve method using plasmid containing cloned VP28 gene as control. The PCR mix (25 μL) contained 1 μL template DNA, 1 μL each of forward and reverse primer targeting VP28 gene (586F-GGGAACATTCAAGGTGTGGA-3′ and 586R-GGTGAAGGAGGAGGTG TTGG), 12.5 μL Power SYBR Green® master mix (Applied Biosystems, USA, California), and 9.5 μL MiliQ water. The reaction conditions were initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 68 °C for 45 s. All reactions were done in triplicates and data analyzed using StepOne™ Software v2.2.2 (Applied Biosystems).

2.4. Inactivated virus preparation (IVP)

WSSV (see Section 2.1) was inactivated by adding formalin to the suspension to obtain final concentration of 0.2% V/V, maintained at room temperature (RT) for 48 h (Singh et al., 2005) and designated as inactivated virus preparation (IVP). Its sterility was checked by streaking on ZoBell's Marine agar and Saboraud Dextrose agar plates. The virus inactivation was assessed by injecting 10 μL IVP intramuscularly on 6th abdominal segment of SPF shrimp (10 Nos.) and observing for clinical signs of the disease for 10 days, and confirming by nested PCR (Lo et al., 1996).

Vaccine-feed was prepared by soaking 280 g commercial shrimp feed (Higashimaru Feeds India Limited, Kochi, India), in 140 ml diluted IVP solution containing 1×10^9 viral DNA copies per ml. This was done to obtain 3.5×10^6 DNA copies g^{-1} shrimp feed and to deliver 1.75×10^6 DNA copies day^{-1} $shrimp^{-1}$ (as each shrimp consumed approximately 0.5 g feed per day). The above calculations were based on a previous study conducted to determine the lowest DNA copies of the virus required per gram shrimp feed to get highest survival in shrimp juveniles against challenge (unpublished). The vaccine coated feed was dried overnight at 4 °C and coated with a binder (Trubind, Wockhardt, Mumbai, India) and dried for 2 h at room temperature (RT) (28 °C ± 1) and stored at 4 °C in a sterile container.

2.5. Vaccination and WSSV challenge

Vaccination and challenge were carried out in the bioassay facility of NCAAH. Before start of the experiment all utensils used such as tanks, tubes, air stones and nets were washed with chlorinated water (100 ppm available chlorine) and sun dried. Aged sea water (25 gL^{-1} salinity) used for the experiment was filtered through 100 μm bag filter. The experiment was conducted in 100 L fiber reinforced plastic (FRP) tanks under constant aeration, and water quality maintained through 10% water exchange daily.