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Delayed mortality of juvenile shrimp *Penaeus vannamei* challenged to White spot syndrome virus (WSSV) previously exposed to Infectious hypodermal and haematopoietic necrosis virus (IHHNV) or inactivated WSSV

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Abstract

In Ecuador, the development and sustainability of the cultured white shrimp, *Penaeus vannamei*, has been threatened by the occurrence of several viral pathogens, Infectious hypodermal and haematopoietic necrosis virus (IHHNV) and White spot syndrome virus (WSSV) mainly. The aim of the present study was to evaluate the exposition of *P. vannamei* juveniles to IHHNV and formalin-inactivated viruses (inactWSSV or inactIHHNV) to induce a protective response in *P. vannamei* juveniles against WSSV infection. *P. vannamei* were challenged to WSSV by intramuscular injection. Shrimp mortalities appeared at day 1 post-injection (p.i.) in positive control and inactIHHNV treatment, while IHHNV and inactWSSV treatments presented onset of mortalities at day 2 p.i. Positive control and inactIHHNV treatment presented 100% mortality at day 4 p.i., while IHHNV and inactWSSV treatments reached similar mortality at day 6 p.i. Statistical analysis revealed that WSSV-induced mortalities in juvenile *P. vannamei* of IHHNV and inactWSSV treatments had a significant delay ($P < 0.05$) compared to both the inactIHHNV-treatment and positive control. Our results showed that preliminary exposure to IHHNV or to formalin-inactivated WSSV can induce delayed mortality in *Penaeus vannamei* following challenge with WSSV via intramuscular injection. In case of IHHNV infection, viral interference could be the biological phenomenon involved, mediated by competition between IHHNV and WSSV. Regarding to WSSV inactivated by formalin, a "vaccination" response would be responsible for the delay, evidencing a possible specific antiviral immune response from the host.

Key words: *Penaeus vannamei*, WSSV, IHHNV, viral interference, "vaccination", delayed mortality.

Introduction

Shrimp aquaculture is one of the most important forms of animal production in the world (31). However, the intensive nature of this type of aquaculture has allowed the development and transmission of infectious diseases. Diseases caused by infection with Infectious hypodermal and haematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV), White spot syndrome virus (WSSV) and Yellow head virus (YHV) represent the most serious threats to cultured shrimp all over the world (18).

In Ecuador, the development and sustainability of the cultured white shrimp, *Penaeus vannamei*, has been threatened by the occurrence of TSV, IHHNV and WSSV.

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In recent years, IHHNV and WSSV have been predominate pathogens in the local shrimp cultures (23).

WSSV is the causative agent of widespread disease associated with high mortality rate in cultured shrimp (17). It causes up to 100% mortality within 10 days in commercial shrimp farms, resulting in huge losses to the shrimp farming industry (10). Histological analysis show hypertrophied nuclei in the cuticular epithelial cells, connective tissue cells and haemocytes (17). Approximately 4-6 billion US$ of economic losses have been estimated in Asia and more than 1 billion US$ in America, between 1992 and 2001 (19) and presently this virus has spread world wide.

Meanwhile, IHHNV is a cosmopolitan virus, reported for most penaeid shrimp (17). It was discovered due to high mortalities in cultured *P. stylirostris* (20). Nevertheless, IHHNV infection is not lethal to *P. vannamei*, but causes the disease named Runt & deformity Syndrome (RDS) (13). Typical signs include deformed rostrums, wrinkled antennal flagella, cuticular roughness and cuticular abnormalities of the abdominal segments and tail fan. Growth retardation is greater than 30% for many populations and may approach 90%, causing heavy economic losses (38). Cowdry type A nuclear inclusion bodies can be observed on histologic examination of multiple organs of ectodermal and mesodermal origin (20).

The occurrence of simultaneous viral infections, i.e. the presence of two or more viruses in a same host, infecting the same tissue or cell, is frequent in shrimp (11, 35). As a consequence, one biological phenomenon called viral interference has been described in several species of marine shrimp, such as *P. stylirostris* (33), *P. monodon* (15) and *P. vannamei* (4). This phenomenon occurs when a host population previously virus-infected resists to a subsequent viral challenge with another virus (33).

On the other hand, immunization as prophylactic measure and a protective management tool is designed to aid in the prevention of disease. Vaccination as immunization strategy implies to confer long lasting protection through immunological memory requiring primary challenge with antigen (specific recognition) and clonally derived lymphocyte sub-sets in vertebrates only (2). However, certain evidences concerning specific pathogen recognition in invertebrates (16, 21, 28) support results already known in shellfish aquaculture obtained from experimental bioassays. Indeed, the development of resistance to infection by probable immune response like mechanism following “vaccination” has been documented against bacterial pathogens in crustaceans (1, 30, 34). As a matter of fact, this “vaccination” could be also one of the potential strategies to overcome viral infections in crustaceans. The notion of shrimp “vaccination” is strongly supported by recent studies in which protection against viral infections has been demonstrated, by using inactivated WSSV and WSSV recombinant proteins to mitigate infections caused by this virus in *Marsupenaeus japonicus* (24), *P. monodon* (37), *Fenneropenaeus indicus* (5) and *P. vannamei* (38).

Regarding this matter, a previous study in *P. vannamei* postlarvae has showed that preliminary exposure to IHHNV or formalin-inactivated WSSV before to WSSV challenge test resulted in slower WSSV replication and delayed mortality, suggesting a protective role of IHHNV as interfering virus and inactivated WSSV simulating a vaccination strategy (22).

In this investigation, we evaluated similar treatments in older stages (e.g. juvenile shrimp) for inducing a protective response in the host against WSSV infection. Evaluated treatments were based on: IHHNV, formalin-inactivated IHHNV and formalin-inactivated WSSV. Cumulative mortalities and viral loads were assessed after WSSV challenge by intramuscular injection.

Material and Methods

Specific pathogen free *P. vannamei* postlarvae (PL12) acquired from Shrimp Improvement Systems (FL, USA) were randomly asigned to each of 4 treatments: a) IHHNV, b) formalin-inactivated IHHNV, c) formalin-inactivated WSSV, and d) positive control. These cultures were carried out at the CENAIM-ESPOL Laboratory, in separate rearing units with one, 5,000 L fibreglass tank, for control shrimp and 1,000 L, plastic tanks for formalin-inactivated IHHNV, formalin-inactivated WSSV and IHHNV exposed shrimp. Standardized rearing parameters included 30 postlarvae/m^2 as stocking density in filtered and UV sterilized (300000 µWs) sea water, with constant aeration. Daily water exchange rate was 10 to 15%. Salinity and temperature were kept at 35 g/L and 29±1 °C, respectively. Shrimp were fed daily twice with artificial feed (Molino 50™). Inactivated IHHNV and WSSV were applied at PL60 and PL75 stages; while IHHNV was applied at PL25 stage only. IHHNV and WSSV inocula preparations and formalin-inactivation procedures for both shrimp viruses were performed according to Melena et al. (22). Treatment inoculations were waterborne at a dose of 1 mL/L during 12 h. Water in treatment units were completely exchanged after 12 hours. Experimental shrimp were randomly sampled for viral PCR detection during treatments application and prior to WSSV challenge. The gill DNA was isolated with Cetyltrimethylammonium bromide (CTAB) based extracting protocol (22). For WSSV, a nested PCR test was used to amplify the following WSSV-DNA sequences: one external 982 bp fragment (982F: 5'-ATCATGGCTGCTTCACAGAC-3'; 982R: 5'-GGCTGGAGAGGACAAGACAT-3') and one internal 570 bp fragment (570F: 5'-TCTTCATCAGATGCTACTGC-3'; 570R: 5'-TAACGCTATCCAGTATCACG-3') (14). For IHHNV, an one-step PCR test was used to amplify a 600 bp sequence of IHHNV-DNA: (600F: 5’-GGACTCTTCCAAGAATACG-3’; 600R: 5’-CGGCTTCCTTAGTTGATAG-3’) (25). PCR products

were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide solution at 0.5 mg/mL. Later, all treatments and positive control were challenged with WSSV by intramuscular injection. Briefly, 36 juvenile shrimp (6 g, average weight) from each treatment and control were transferred and equally distributed in 3 glass aquaria, with 40 L of sterilized, aerated sea water (35 g/L and 29 °C). Shrimp were acclimated during two days prior the WSSV challenge. Later, shrimp were intramuscularly injected with 0.1 mL of WSSV inoculum previously diluted 40,000-fold into the second abdominal segment using a sterile 1 mL tuberculin syringe. In case of negative control, shrimp were injected with sterile sea water. The challenged shrimp were maintained in seawater at room temperature and were fed with pelleted artificial feed twice daily at 8% of their mean body weight. After injection, mortality was registered daily and moribund shrimp were preserved in Davidson′s fixative (whole body) and 95% ethanol (gills) for routine histology following standard methods (3) and Real-time PCR, respectively. The viral loads were quantified in randomly selected moribund shrimp (n = 4) from all treatments and positive control, in the Aquaculture Pathology Laboratory (UAZ, USA) (9, 32).

In addition, data were analysed employing a Kruskal-Wallis 1-way analysis of variance on ranks to test the effect of treatments ($P < 0.05$).

Results

Experimental shrimp came IHHNV-treatment were PCR-positive for IHHNV prior the WSSV challenge (Fig. 1). In addition, tested shrimp from all treatments and positive control were PCR-negative for WSSV (data not shown).

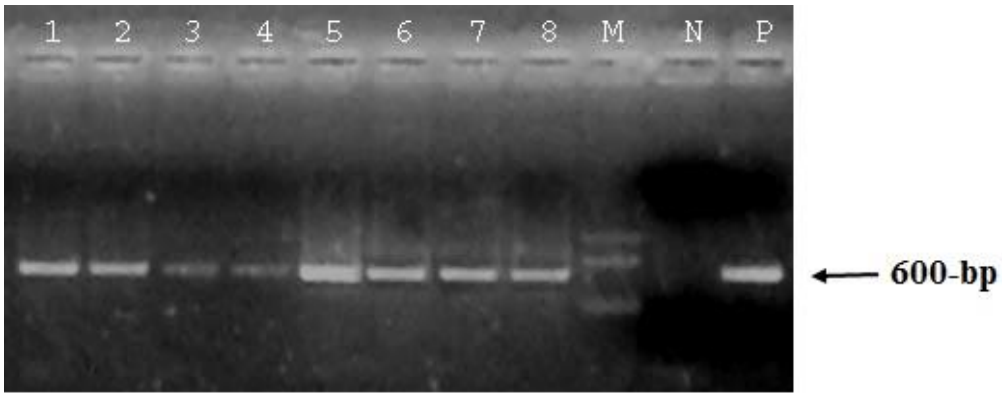

Figure 1. Juvenile shrimp *P. vannamei* from IHHNV-treatment tested by PCR for IHHNV prior the WSSV challenge. Lane 1 – 8: Individual juvenile shrimp showing a 600-bp amplicon corresponding to IHHNV DNA. Lane M: Molecular weight marker (848 bp, 630 bp, 333 bp). Lane N: Negative control (IHHNV non-infected shrimp). Lane P: Positive control (IHHNV infected shrimp).

During the WSSV challenge, shrimp mortalities appeared at day 1 post-injection (p.i.) in positive control and inactIHHNV treatment, while IHHNV and inactWSSV treatments presented dead shrimp at day 2 p.i. According to this fact, positive control and inactIHHNV treatment displayed 100% mortality to day 4 p.i., while IHHNV and inactWSSV treatments reached similar mortality to day 6 p.i. No mortalities were observed in the negative control (Table 1).

Table 1. Time-course mortality (expressed as percentage) of treated juvenile *P. vannamei* during WSSV challenge via intramuscular injection. Data shown are the mean of three replicates.

	Days post-injection					
Treatment	1	2	3	4	5	6
InactWSSV	0	0	42	67	92	100
InactIHHNV	8	8	50	100	100	100
IHHNV	0	0	33	58	92	100
C +	8	17	58	100	100	100
C -	0	0	0	0	0	0

InactWSSV = Formalin-inactivated WSSV; InactIHHNV = Formalin-inactivated IHHNV; IHHNV = active IHHNV; C + = Positive control; C - = Negative control.

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Based on these data, shrimp mortalities observed in InactWSSV and IHHNV treatments were slower than in positive control and inactIHHNV treatment, with an elapsed time of 2 days.

After WSSV challenge, histopathological analysis confirmed that moribund shrimp came from IHHNV-treatment were co-infected with IHHNV and WSSV (Fig. 2 and 3). Accordingly, pathognomonic lesions of WSSV infection were found in moribund shrimp from remaining treatments and positive control (data not shown).

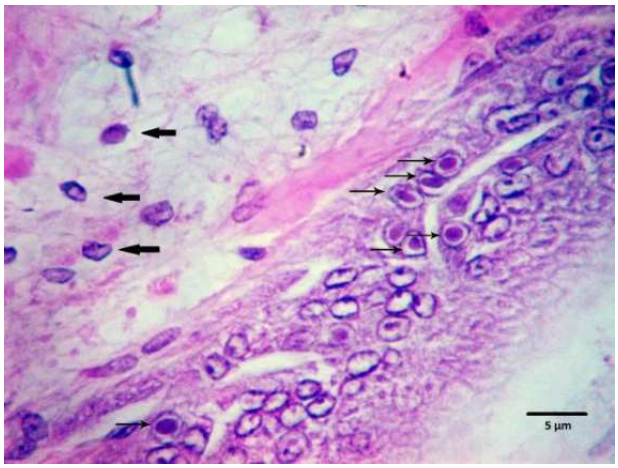

Figure 2. Histopathology of Mayer-Bennett hematoxylin/eosin-phloxin (H&E) from co-infected juvenile *P. vannamei* with IHHNV and WSSV from IHHNV-treatment and subsequently challenged with WSSV. Epithelium cells of the stomach showing the presence of nuclear hypertrophy and chromatin margination by WSSV (large arrows) and Cowdry type A nuclear inclusion bodies of IHHNV (small arrows).

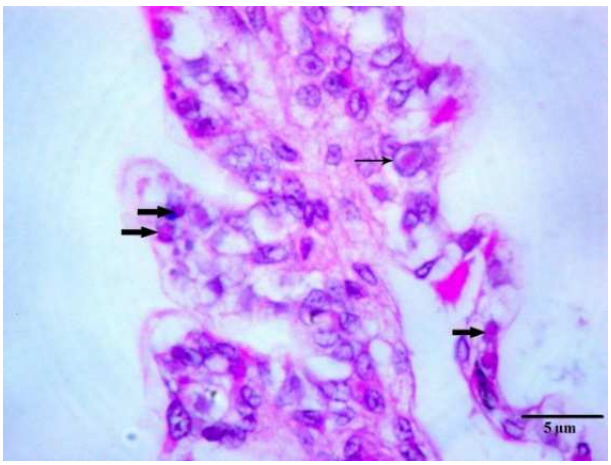

Figure 3. Mayer-Bennett hematoxylin/eosin-phloxin (H&E) histopathology from co-infected juvenile *P. vannamei* with IHHNV and WSSV from IHHNV-treatment and subsequently challenged with WSSV. Gill tissue cells showing the presence of nuclear hypertrophy and chromatin margination by WSSV (large arrows) and Cowdry type A nuclear inclusion bodies of IHHNV (small arrow).

Concerning to Real-time PCR analysis, moribund shrimp (n = 4) from IHHNV-treatment had IHHNV loads ranging from 80 to 1.7×10^5 copies per µg DNA. In turn, WSSV loads were higher with quantities of 8.5×10^7 to 1.9×10^8 copies per µg DNA in moribunds (n = 4) from IHHNV treatment, 3.4×10^8 to 3.9×10^9 copies per µg DNA in moribunds (n = 4) from InactIHHNV treatment, 2.1×10^8 to 7.4×10^8 copies per µg DNA in moribunds (n = 4) from InactWSSV treatment and 2.9×10^8 to 7.0×10^8 copies per µg DNA in moribund shrimp (n = 4) from positive control.

Finally, Kruskal-Wallis analysis revealed that WSSV-induced mortalities in juvenile *P. vannamei* from IHHNV and InactWSSV treatments showed a significant delay ($P < 0.05$) compared to both the InactIHHNV-treatment and positive control.

Discussion

As our experimental data suggest, preliminary exposure to IHHNV or to formalin-inactivated WSSV can induce delayed mortality in *Penaeus vannamei* following challenge with WSSV via intramuscular injection. In case of IHHNV infection, viral interference could be the biological phenomenon involved, mediated by competition between IHHNV and WSSV, which is likely to be result of shared cellular replication machinery. Regarding to WSSV inactivated by formalin, a vaccination response would be responsible for the delay, evidencing a possible specific antiviral immune response from host.

Viral interference has been documented in several vertebrates and invertebrates aquatic species, such as fish (6, 7, 8) and marine shrimp (4, 22, 33). In some cases, protein factors such as cytokines (e.g. Interferon), might be involved in interfering with the viral replication, which could explain the protection observed in co-infected host (7, 8). However, the lack of such evidences in other cases (4, 6, 22, 33) suggests that the viral interference is a competition for host biological resources (12). Indeed, infection with one virus may block entry of another virus by down-regulating production of cellular receptors or through competition for a common receptor (29). Alternatively, an existing viral infection can shutdown host cell functions required for replication of a second virus (40). In shrimp, the presence of innate antiviral factors released into haemolymph could interfere with subsequent infection by another virus, but the activity of compounds such type I Interferon homologues have not yet been demonstrated in any crustacean (27).

On the other hand, much of the research on the "vaccination" response of shrimp to virus infection has been led to produce candidate vaccines. To date, "vaccination" of shrimp against only a single virus (WSSV) has been reported. An early indication that "vaccination" of shrimp may be possible came when it was discovered that previous exposure to WSSV could protect shrimp from future challenge with the virus (36). The

observation of protection by prior pathogen exposure was followed by investigations of the efficacy of the protective response following vaccination with inactivated virus via intramuscular injection (24) or oral feeding (5), which shows that protective immune responses in shrimp are not reliant on the presence of viable virus. In parallel, several groups have investigated the efficacy of subunit vaccines, that is vaccines made from recombinantly expressed virus particle proteins (37, 38).

The delay in WSSV-induced mortality was associated to IHHNV and InactWSSV treatments, which showed a significantly delayed ($P < 0.05$) cumulative mortality compared to both the InactIHHNV-treatment and positive control. Similar behavior was observed in shrimp exposed to IHHNV and formalin-inactivated WSSV by immersion in *P. vannamei* postlarvae subsequently challenged with WSSV *per os* (22) and in juvenile *P. vannamei* infected with IHHNV *per os* for 30, 40 or 50 days and challenged with WSSV *per os* later (4). Interestingly, the delay on the mortality rates for the IHHNV and InactWSSV treatments challenged with WSSV seems not be depending on age and size of experimental animals, occurring in postlarvae and juvenile stage in a similar way.

Meanwhile, Real-time PCR analyses showed that moribund shrimp from IHHNV-treatment challenged with WSSV had IHHNV loads lower (10^5 copies per µg DNA in average) than those found (10^8 copies per µg DNA in average) in *P. vannamei* experimentally infected with IHHNV (31). The reduction of IHHNV load during this bioassay could be attributed to WSSV challenge. Similar fact was reported when shrimp pre-infected with IHHNV for 40 days had 2.6×10^9 copies per µg DNA before WSSV challenge and only 9.7×10^7 copies per µg DNA in moribund or dead shrimp after WSSV challenge (4). Although the IHHNV quantitation was not performed in shrimp from IHHNV-treatment before the challenge with WSSV, the PCR results showed an unambiguous IHHNV-content in tested samples (Fig. 1). On the other hand, similar WSSV loads quantified in samples from InactWSSV treatment and positive control would suggest that survival to WSSV infection it is not related with WSSV load, which can be explained by the inability of PCR methods to differentiate between infective and noninfective viruses (26), so that, the viral load detected in samples from InactWSSV treatment after WSSV challenge probably would be comprised of infective and noninfective WSSV (inactive viral particles).

By turn, the mortality levels of InactIHHNV treatment, which was proposed to elucidate a possible innate antiviral immune response in *P. vannamei*, were similar to positive control, suggesting that inactive IHHNV caused no interference and that the responsible factor for viral interference was not present in the capside proteins.

In conclusion, the delay observed in WSSV-induced mortality suggest that is related to a competition between IHHNV and WSSV for host biological resources and a WSSV-specific response elicited from a prior exposure to this viral pathogen or components.

Further studies on viral interference can contribute to the understanding of the interactions of viruses and possibly provide a means of improving "vaccination" strategies. In the same way, "vaccination" as preventive health strategy can aid to mitigate viral infections in shrimp.

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