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Pre-exposure to infectious hypodermal and haematopoietic necrosis virus or to inactivated white spot syndrome virus (WSSV) confers protection against WSSV in *Penaeus vannamei* (Boone) post-larvae

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Abstract

Larvae and post-larvae of *Penaeus vannamei* (Boone) were submitted to primary challenge with infectious hypodermal and haematopoietic necrosis virus (IHHNV) or formalin-inactivated white spot syndrome virus (WSSV). Survival rate and viral load were evaluated after secondary *per os* challenge with WSSV at post-larval stage 45 (PL45). Only shrimp treated with inactivated WSSV at PL35 or with IHHNV infection at nauplius 5, zoea 1 and PL22 were alive (4.7% and 4%, respectively) at 10 days post-infection (p.i.). Moreover, at 9 days p.i. there was 100% mortality in all remaining treatments, while there was 94% mortality in shrimp treated with inactivated WSSV at PL35 and 95% mortality in shrimp previously treated with IHHNV at N5, Z1 and PL22. Based on viral genome copy quantification by real-time PCR, surviving shrimp previously challenged with IHHNV at PL22 contained the lowest load of WSSV (0–1 $\times 10^3$ copies μg^{-1} of DNA). In addition, surviving shrimp previously exposed to inactivated WSSV at PL35 also contained few WSSV (0–2 $\times 10^3$ copies μg^{-1} of DNA). Consequently, pre-exposure to either IHHNV or inactivated WSSV resulted in slower WSSV replication and delayed mortality. This evidence suggests a protective role of IHHNV as an interfering virus, while protection obtained by inactivated WSSV might result from non-specific antiviral immune response.

Keywords: infectious hypodermal and haematopoietic necrosis virus, *Penaeus vannamei*, viral co-infection, viral inactivation, viral interference, white spot syndrome virus.

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Introduction

Viral diseases have led to severe mortalities of cultured penaeid shrimp all over the world (Flegel 1997; Lightner 1999). In Ecuador, the development and sustainability of the cultured white shrimp, *Penaeus vannamei* (Boone), has been threatened by the occurrence of several viral pathogens, mainly infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Jiménez, Barniol, de Barniol & Machuca 1999), Taura syndrome virus (Hasson, Lightner, Poulos, Redman, White, Brock & Bonami 1995) and white spot syndrome virus (WSSV) (Rodríguez, Bayot, Amano, Panchana, de Blas, Alday & Calderón 2003).

Taura syndrome virus was earlier known as Taura syndrome (Jiménez 1992). Its viral aetiology was demonstrated (Hasson *et al.* 1995) and the agent characterized (Bonami, Hasson, Mari, Poulos & Lightner 1997). A later genomic characterization revealed its similarity with members of the genus

Cricket paralysis-like viruses (Mari, Poulos, Lightner & Bonami 2002).

White spot syndrome virus is a tailed, enveloped, rod-shaped double-stranded circular DNA virus that contains a very large genome of 300 kb (van Hulten, Witteveldt, Peters, Kloosterboer, Tarchini, Fiers, Sandbrink, Lankhorst & Vlak 2001; Yang, He, Lin, Li, Pan, Zhang & Xu 2001). It is extremely virulent (Wang, Lo, Chang & Kou 1998) and has a broad host range, infecting all cultured shrimp (penaeid and non-penaeid), and some species of crab, lobster and crayfish (Chang, Chen & Wang 1998; Corbel, Zuprizal, Shi, Huang, Sumartono, Arcier & Bonami 2001; Dupuy, Bonami & Roch 2004). The virus can cause 100% mortality in 3–10 days in farmed shrimp. Clinical signs of WSSV infection include the appearance of white spots inside the carapace and a reddish discolouration of the body. Histological analysis shows hypertrophied nuclei in the cuticular epithelial cells, connective tissue cells and haemocytes (Lightner 1996). Although WSSV may cause losses at any time during grow-out, high mortality from WSSV disease often occurs within the first 40 days after the shrimp post-larvae have been stocked into ponds (Brock & Bullis 2001; Rodríguez *et al.* 2003).

Infectious hypodermal and haematopoietic necrosis virus is a small, icosahedral, non-enveloped virus that contains a single-stranded, 4.1 kb DNA genome (Bonami, Trumper, Mari, Brehelin & Lightner 1990). IHHNV was found to cause high mortalities in cultured *P. stylirostris* (Stimpson) (Lightner, Redman & Bell 1983). Nevertheless, IHHNV infection is not lethal to *P. vannamei*, but it causes a disease named runt and deformity syndrome, which results in cuticular deformities and a lower growth rate (Kalagayan, Godin, Kanna, Hagino, Sweeney, Wyban & Brock 1991), observed as soon as post-larvae 35 (PL35) (Lightner & Redman 1998).

Recent investigations focusing on viral interactions in *P. stylirostris* (Tang, Durand, White, Redman, Mohney & Lightner 2003) and on the evaluation of antiviral 'vaccination' in *P. japonicus* (Bate) (Namikoshi, Wu, Yamashita, Nishizawa, Nishioka, Arimoto & Muroga 2004), *P. monodon* (Fabricius) (Witteveldt, Cifuentes, Vlak & van Hulten 2004a; Witteveldt, Vlak & van Hulten 2004b) and *P. indicus* (Milne-Edwards) (Bright Singh, Manjusha, Somnath Pai & Philip 2005) suggested possible induced resistance to WSSV infection in shrimp. To date, there is no relevant information concerning both topics in *P. vannamei*.

In this study, three parallel larval cultures of *P. vannamei* were challenged with IHHNV or formalin-inactivated WSSV at different stages of development. Later, they were infected *per os* with WSSV at PL45. Survival and viral load were evaluated.

Materials and methods

Experimental shrimp

A batch of nauplii ($n = 600\,000$) at nauplius 4 stage (N4) of specific pathogen-free (SPF) *P. vannamei* provided by Shrimp Improvement Systems (Islamorada, FL, USA), were distributed in three parallel cultures: (a) untreated SPF, (b) SPF to be exposed to formalin-inactivated WSSV and (c) SPF to be challenged with IHHNV.

These larval cultures were performed at the CENAIM-ESPOL Laboratory, in separate facilities with one, 5000-L fibreglass tank for SPF larvae and 1000-L plastic tanks for both inactivated WSSV and IHHNV challenged larvae. Standardized rearing parameters included 100 nauplii L^{-1} as stocking density in filtered sea water, sterilized by ultraviolet light (300 000 μWs) with constant aeration and in a closed system until the zoea 3 stage. After zoea 3, water exchange rate was 10–15% daily. Salinity was 35‰ and water temperature 30–31 °C. Larvae and early PL were fed every 2–3 h with a living mixture of phytoplankton and *Artemia* sp., and artificial feed (Molino 50^{TM}, ALIMENTSA, Guayaquil, Ecuador). IHHNV and inactivated WSSV suspensions were applied by immersion at early larval stages. Early stages were chosen in order to stimulate shrimp by inactivated WSSV during the initial recognition of non-self material and to mimic a vertical transmission of IHHNV. Inactivated WSSV was applied to: zoea 1 (Z1), zoea 2 (Z2), zoea 3 (Z3) and mysis 1 (M1); while IHHNV was applied at nauplius 5 (N5) and Z1 stages. Both viral suspensions were added to culture tanks at 1 mL L^{-1}. Shrimp larvae were exposed to viral suspensions for 6 h at each stage before a total water exchange.

At PL15, shrimp from these three cultures were sorted into 10 treatments (Fig. 1) prior to challenge with WSSV. Each treatment ($n = 800$) was maintained in one, 1000-L tank. PL were fed every 5–6 h with artificial feed (Ziegler^{TM}, Zeigler Bros,

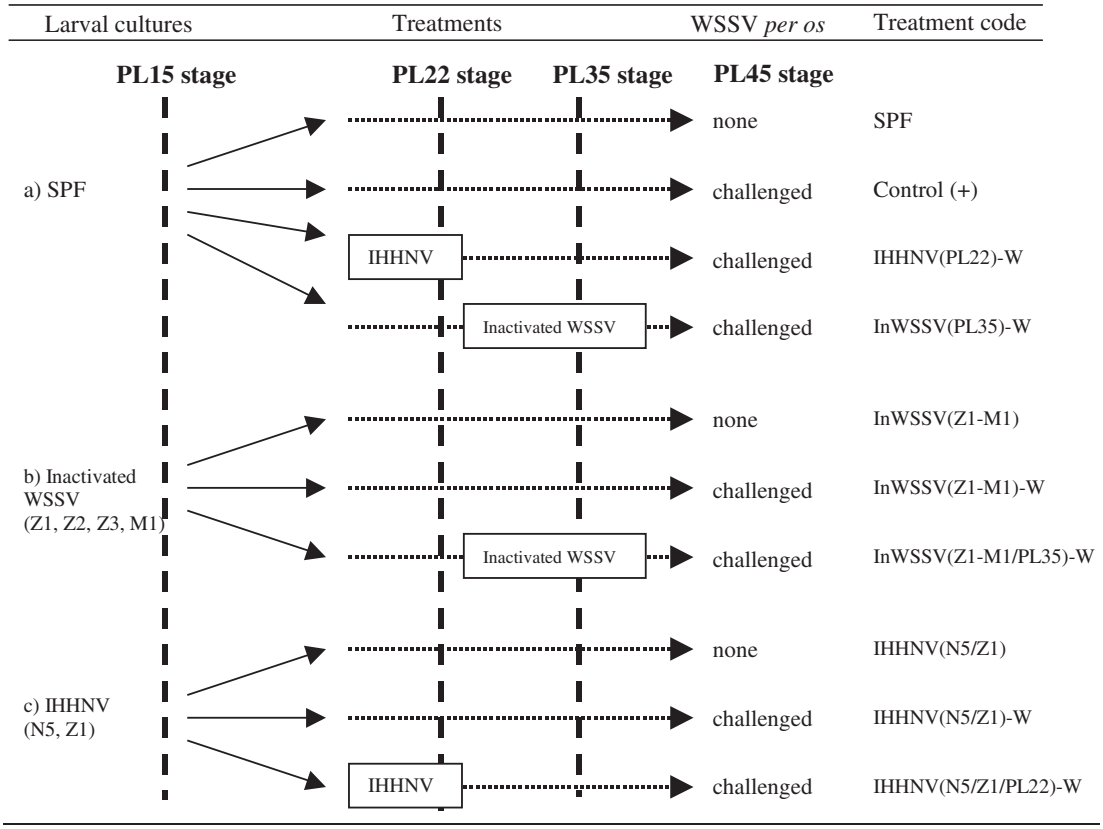

Figure 1 Experimental design showing parallel larval cultures, subsequent treatments after PL15 and *per os* challenge with white spot syndrome virus (-W) at PL45.

Inc., Gardners, PA, USA). Water quality parameters were similar to those described previously, except for a lower water temperature (26–27 °C). Of the 10 treatments, seven were challenged with WSSV *per os* at PL45. Prior to WSSV challenge, two treatments were immersed in IHHNV at PL22, while two others were immersed in inactivated WSSV at PL35. Different PL stages were selected for both exposures because of the slow replication rate of IHHNV and the lack of replication of inactivated WSSV. Thus, 3 weeks for IHHNV infection and 10 days for inactivated WSSV were considered as suitable periods of pre-exposure in order to test the performance of these agents against WSSV infection. Viral suspensions were added to culture tanks as described above.

A strategy for supplying viral suspensions by immersion was adopted as a practical way to deliver prophylactic agents in larval and PL stages, as it does not involve animal manipulations and is less time consuming (Alabi, Jones & Latchford 1999). On the other hand, exposure to IHHNV was made early in the sequence of infection because its replication rate is slower than that of WSSV (Tang & Lightner 2001).

Preparation of virus suspensions

White spot syndrome virus was prepared using a protocol modified from Huang, Zhang, Zhang, Xiao, Wu, Chen & Li (2001). Briefly, WSSV-infected *P. vannamei* with severe clinical signs of disease were collected in October 2004 from a previous experimental WSSV infection at CENAIM. Infected tissues (gills, appendages and exoskeleton) were resuspended in four parts of TN buffer (0.02 M Tris-HCl; 0.4 M NaCl, pH 7.4) and then homogenized for two 45-s intervals in a blender at 4 °C. After centrifugation at 4500 ***g*** for 5 min at 4 °C, the supernatant was cleared once again at 20 000 ***g*** for 15 min at 4 °C in a SORVALL A-1256 rotor (SORVALL, Discovery^{TM} 90SE, Kendro Laboratory Products, Newtown, CT, USA). Finally, supernatant was centrifuged at

20 000 ***g*** for 1 h at 4 °C. The pellet containing WSSV was resuspended in 10 mL TN and stored at −80 °C until use. To generate WSSV-infected tissues, SPF *P. vannamei* broodstock was injected intramuscularly into the 2nd abdominal segment with a volume of viral stock solution previously diluted 1000-fold, equal to 0.5% of the mean body weight.

From a presumptive IHHNV-infected *P. vannamei* broodstock population, approximately 200 shrimp showing clinical signs (cuticular deformities) were collected in November 2004 from a maturation facility at CENAIM. Approximately 30 shrimp were confirmed to be IHHNV positive by a one-step polymerase chain reaction (PCR) test. Infected tissues (gills, appendages and exoskeleton) were resuspended in six parts of TN buffer and then homogenized as described above. After centrifugation at 4500 ***g*** for 5 min at 4 °C, the supernatant was cleared again at 20 000 ***g*** for 15 min at 4 °C (SORVALL A-1256 rotor). Finally, the supernatant was centrifuged at 40 000 ***g*** for 45 min at 4 °C. The pellet containing IHHNV was resuspended in 10 mL TN and stored at −80 °C until use. Quantification of both viruses was carried out from respective infected tissue samples by real-time PCR. In both cases, viral suspensions were diluted (1:9) in TN buffer just before addition to culture tanks.

WSSV inactivation

Inactivated WSSV was prepared according to Namikoshi *et al.* (2004). Briefly, the virus was incubated for 10 min at 25 °C with formalin (0.5% v/v). Formalin was removed by dilution with TN followed by two centrifugations at 30 000 ***g*** for 1 h at 4 °C (SORVALL A-1256 rotor). Finally, the pellet was resuspended in TN buffer, adjusted to the same volume as the original viral suspension and used immediately.

Detection of virus by PCR in larvae

During the rearing period, shrimp larvae were randomly sampled every 7 days by taking 100 nauplii/zoeae, 40 mysis/PL1/PL10 or 20 late PL per larval culture, fixed in 95% ethanol solution, until PL42 stage. Template DNA was prepared and extracted from PL whole bodies and cephalothoraxes, without eyes. Tissues (*c.* 200 mg) were crushed with a plastic pestle and homogenized in a plastic microfuge tube containing a lysis buffer (500 μL of CTAB solution, 0.2 mg mL^{-1} of proteinase K) and incubated at 55 °C for 2 h, then deproteinized by successive phenol–chloroform, chloroform and isoamyl alcohol extractions, with alternate centrifugations (10 000 ***g*** for 8 min). Finally, DNA was recovered by ethanol precipitation, dried and resuspended in TN buffer. Concentration of total DNA in each sample was estimated using a spectrophotometer (Eppendorf, Hamburg, Germany).

For IHHNV, a one-step PCR test using primers designed by Quéré, Commes, Marti, Bonami & Piquemal (2002) was used to amplify a 600-bp sequence of IHHNV-DNA: (600F, 5′-GGACTC TTCCAAGAATACG-3′; 600R, 5′-CGGCTTCC TTAGTTGATAG-3′). The PCR amplification (Thermocycler; MJ Research, Watertown, MA, USA) was carried out in a 25-μL reaction mixture containing 1.5 μL of template DNA (*c.* 100 ng), 1X PCR buffer, 1.5 μM $MgCl_2$,0.5 μM of each primer, 200 μM of each deoxynucleotide triphosphate (Promega, Madison, WI, USA) and 1 U of *Taq* DNA polymerase (Promega). The PCR protocol comprised one start cycle at 95 °C for 2 min, three cycles at 95 °C for 45 s, 56 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. DNA from IHHNV-negative PL was used as negative control.

For WSSV a nested PCR method, using primers designed by Kimura, Yamano, Nakano, Momoyama, Hiraoka & Inouye (1996), was used to amplify the following WSSV-DNA fragments: one external 982 bp fragment (982F, 5′-ATCATG GCTGCTTCACAGAC-3′; 982R, 5′-GGCTGG AGAGGACAAGACAT-3′) and one internal 570-bp fragment (570F, 5′-TCTTCATCAGATG CTACTGC-3′; 570R, 5′-TAACGCTATCCAG TATCACG-3′). Briefly, nested PCR amplifications were carried out with the second pair of primers (570F/570R) using the amplified product of the first pair (982F/982R) as a template. In both cases, PCR amplification was carried out in a 25 μL reaction mixture containing 1.5 μL of template DNA (*c.* 100 ng), 1X PCR buffer, 1.5 μM $MgCl_2$, 0.2 μM of each primer, 200 μM of each deoxynucleotide triphosphate (Promega) and 1 U of *Taq* DNA polymerase (Promega). The PCR protocol comprised one start cycle at 94 °C for 1 min, 35 cycles at 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 1 min with a final extension at 72 °C for 5 min. For nested PCR, 2 μL of the one-step reaction mixture was added to the second PCR

mixture. DNA from WSSV-negative PL was used as negative control.

Previously, a 441-bp fragment from a shrimp 18S rRNA DNA sequence (441F, 5′-TTGTAC GAGGATCGAGTGGA-3′; 441R, 5′-ATGCTTT CGCAGTAGGTCGT-3′) (Tang & Lightner 2000) was amplified to determine the quality of total DNA extracted, based on a one-step PCR with the same parameters as for WSSV. PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 mg mL^{-1}) and visualized by ultraviolet transillumination. A molecular weight marker of 1 kb (Promega) was used to estimate the size of obtained amplicons. The gels were observed and photographed using a gel documentation system (Electrophoresis Documentation and Analyses System 120; Eastman Kodak Company, New Haven, CT, USA).

Per os challenge with WSSV

At PL44 stage, 300 PL (mean weight: 0.11 ± 0.1 g) from each treatment were transferred and stocked in 20 trays, each containing 10 glass bottles, with 1.5 L of sterilized, aerated sea water (35‰ and 27 °C) per bottle. Each tray contained one replicate of 10 treatments with 15 shrimp per replicate. PL were acclimatized and starved for 1 day prior the challenge. PL45 were fed with minced WSSV-infected tissue at 12% of their mean body weight divided into two rations over 1 day, followed by pelleted artificial feed twice daily for 10 days post-infection (p.i.). One non-exposed treatment (SPF shrimp) to IHHNV or inactivated WSSV was used as positive control (+). In addition, three WSSV non-infected treatments: naïve shrimp (SPF), shrimp exposed to inactivated WSSV at Z1, Z2, Z3 and M1 (InWSSV,Z1-M1) and shrimp challenged with IHHNV at N5 and Z1 (IHHNV-N5/Z1), were used as non-infected controls during WSSV challenge. The shrimp in these treatments were also fed with pelleted artificial feed twice daily until the challenge test terminated. The challenge test was concluded when mortalities slowed or ended.

Virus detection after *per os* challenge with WSSV

During experimental WSSV infection, moribund shrimp were collected every 4–6 h and fixed for PCR. Later, 30 moribund shrimp from every treatment were randomly chosen in order to test for WSSV. For IHHNV detection, shrimp were taken from the following treatments: challenge with IHHNV at PL22, IHHNV(PL22)-W, exposure to inactivated WSSV at PL35, InWSSV(PL35)-W and challenge with IHHNV at N5, Z1 and PL22, IHHNV(N5/Z1/PL22)-W. Virus tests were performed using commercially available kits (IQ2000 System Kit; Farming Intelligene Technology Corporation Inc., Taipei, Taiwan) according to the manufacturer's instructions. Additionally, pooled samples ($n = 20$) from non-infected controls were tested for WSSV infection.

Real-time PCR for WSSV and IHHNV

Viral loads were quantified by real-time PCR in five moribund and five surviving shrimp from IHHNV(PL22)-W, InWSSV(PL35)-W and IHHNV(N5/Z1/PL22)-W treatments in the Aquaculture Pathology Laboratory (University of Arizona, Tucson, AZ, USA), according to Tang & Lightner (2001) and Durand & Lightner (2002). Briefly, IHHNV primers (IHHN1608F, 5′-TACT CCGGACACCCAACCA-3′; IHHN1688R, 5′-G GCTCTGGCAGCAAAGGTAA-3′) generated an 81-bp amplicon. The TaqMan probe (5′-ACCAG ACATAGAGCTACAATCCTCGCCTATTTG-3′) was synthesized and dual-labelled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5′ end and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) on the 3′ end. Sequences of WSSV primers were selected from a region of WSSV genomic sequence (GenBank U50923). The primers (WSS1011F, 5′-TGGTCCCGTCCTCATCT CAG-3′; WSS1079R, 5′-GCTGCCTTGCC GGAAATTA-3′) generated a 69-bp amplicon. The TaqMan probe (5′-AGCCATGAAGAA TGCCGTCTATCACACA-3′) was also dual-labelled with FAM and TAMRA. The TaqMan assay was carried out with the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA). A sample of 10 ng of DNA, extracted with a High-pure DNA template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), was added to the master mix containing 0.3 μM of each primer and 0.15 μM TaqMan probe in a final volume of 25 μL. Amplification was performed with the following profile: 50 °C for 2 min and 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The quantity of DNA from each sample was determined by GeneAmp 5700 Sequence Detection System software (SDS 1.0, Applied Biosystems). Each sample was run in

duplicate. Quantitative standard curves for IHHNV and WSSV were dilutions of a cloned PCR fragment containing the target sequence for real-time PCR primers (Tang & Lightner 2001). Concentration of each recombinant plasmid (copy number known) was determined with a spectrophotometer and the copy number for serial dilutions of the standards was estimated for use in quantification. From these copy numbers, final results were expressed as the mean copy number of WSSV or IHHNV μg^{-1} of total DNA.

Statistical analysis

Statistical analysis included all seven WSSV-challenged treatments, but in order to distinguish differences, mortality curves were separated into two graphs, one for IHHNV-infected treatments and other for InWSSV-exposed treatments. The mortality data in graphs are presented as percentage mean values with standard deviation. Final mortality data for all WSSV-challenged treatments were compared using the non-parametric Kruskal–Wallis test (Zar 1999). A non-parametric Tukey-type multiple comparison (Zar 1999) was used to compare mean ranks when significant effects were observed. Differences were considered significant when $P < 0.05$.

Results

Detection of virus by PCR in larvae

Untreated SPF larvae were negative for WSSV and IHHNV. Similarly, larvae exposed to formalin-inactivated WSSV were also negative for both viruses. As expected, larvae infected with IHHNV were positive for IHHNV, but negative for WSSV, at PL10. After PL15, all 10 treatments were WSSV negative, demonstrating that WSSV was not able to replicate after formalin treatment. At PL42, shrimp exposed to IHHNV at PL22 (IHHNV-PL22) and at N5, Z1 and PL22 (IHHNV-N5/Z1/PL22) were positive for IHHNV giving the expected PCR amplified band at 600 bp (Fig. 2). No deformity was observed in these shrimp even after 20 days p.i. with IHHNV.

Kinetics of mortality following *per os* challenge with WSSV

The non-infected controls showed very low mortalities: 9% for SPF shrimp, 15% for InWSSV(Z1-M1) and 14% for IHHNV(N5/Z1) when WSSV challenge was concluded. These low mortalities resulted from cannibalism occurring after individual moulting. In contrast, the positive control displayed 100% mortality at 9 days p.i. (Table 1).

Moreover, shrimp from IHHNV-PL22 had 100% mortality at 8 days p.i., while animals challenged with IHHNV at N5 and Z1 (IHHNV-N5/Z1) exhibited similar survival as did the positive control, i.e. no shrimp survived to day 9 p.i. (Fig. 3a). In addition, all shrimp exposed to inactivated WSSV at Z1, Z2, Z3 and M1 (InWSSV,Z1-M1) and at Z1, Z2, Z3, M1 and PL35 (InWSSV,Z1-M1/PL35) also died by 9 days p.i. (Fig. 3b).

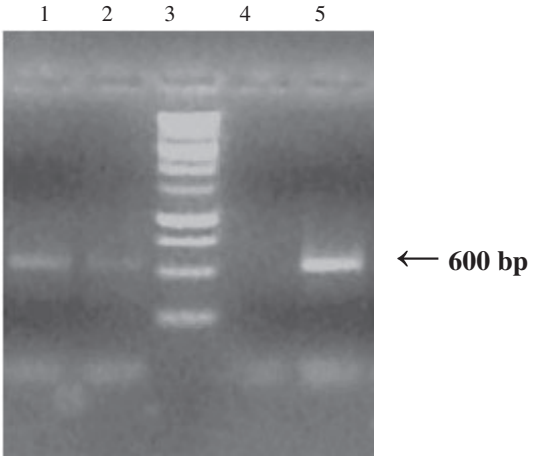

Figure 2 Larval and post-larval pooled samples of *Penaeus vannamei* tested by polymerase chain reaction for infectious hypodermal and haematopoietic necrosis virus (IHHNV). Lane 1: PL10 from IHHNV-infected larval culture. Lane 2: PL42 from IHHNV(PL22) treatment. Lane 3: 1 kb DNA ladder. Lane 4: negative control (IHHNV non-infected PL). Lane 5: PL42 from IHHNV(N5/Z1/PL22) treatment.

Table 1 Time-course mortality (expressed as percentage) of *Penaeus vannamei* post-larvae during *per os* challenge with white spot syndrome virus

	Days post-infection			
Treatments	3	5	7	9
Specific pathogen-free	3	3	5	8
Control (+)	42	82	96	100
IHHNV(PL22)-W	46	91	99	100
InWSSV(PL35)-W	36	74	87	94
InWSSV(Z1-M1)	2	4	7	12
InWSSV(Z1-M1)-W	48	86	99	100
InWSSV(Z1-M1/PL35)-W	51	82	96	100
IHHNV(N5/Z1)	4	6	10	12
IHHNV(N5/Z1)-W	44	86	97	100
IHHNV(N5/Z1/PL22)-W	40	75	89	95

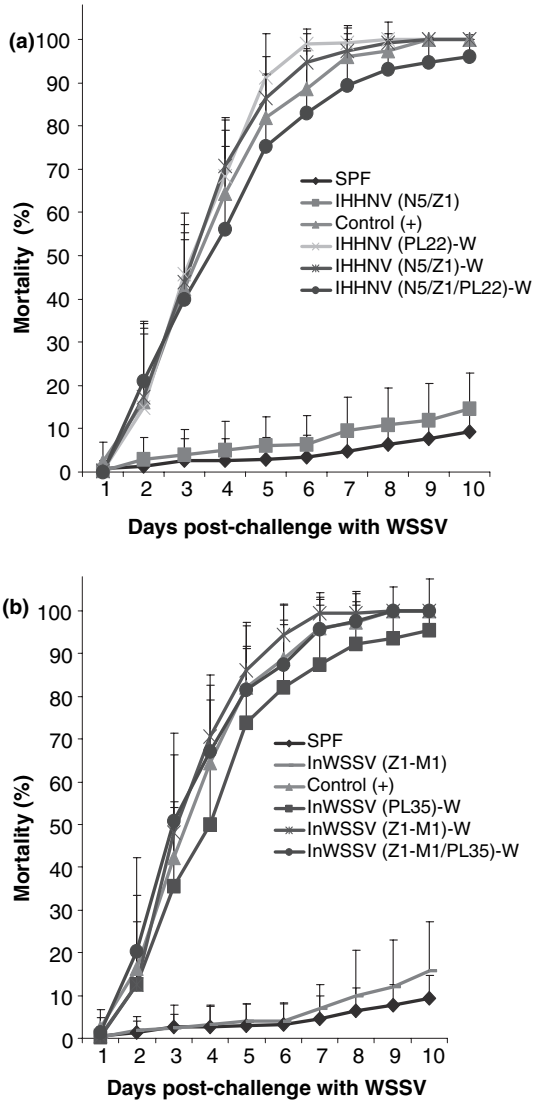

Figure 3 Kinetics of cumulative mortalities expressed as percentage after *per os* white spot syndrome virus (WSSV) challenge at PL45. Each point represents the mean value of 20 replicates with standard deviation (bar). (a) Treatments performed on shrimp previously challenged with infectious hypodermal and haematopoietic necrosis virus. (b) Treatments performed on shrimp previously exposed to inactivated WSSV. Non-infected and positive controls are included in both graphs.

Conversely, shrimp from IHHNV(N5/Z1/PL22)-W and InWSSV(PL35)-W treatments survived (4% and 4.7%, respectively) to day 10 p.i. Survival at day 10 was significantly associated ($P < 0.05$) with these treatments. Each of the survivals for IHHNV(N5/Z1/PL22)-W and InWSSV(PL35)-W showed significant differences ($P < 0.05$) compared with the other infected treatments at day 10, but survival in both

Table 2 Non-parametric Tukey-type multiple comparison on survival rates (mean ± standard deviation) of white spot syndrome virus-challenged *Penaeus vannamei* post-larvae at day 10 post-infection

Treatment	% Survival	Ranks sums	Mean ranks
Control (+)	0	1260	63 b
IHHNV(PL22)-W	0	1260	63 b
InWSSV(PL35)-W	4.7 ± 12.1	1682	84 a
InWSSV(Z1-M1)-W	0	1260	63 b
InWSSV(Z1-M1/PL35)-W	0	1260	63 b
IHHNV(N5/Z1)-W	0	1260	63 b
IHHNV(N5/Z1/PL22)-W	4.0 ± 5.0	1888	94 a

Letters a and b refer to significant differences.

treatments was not significantly different ($P > 0.05$) from each other at day 10 (Table 2).

Furthermore, a delay in shrimp mortality was observed in these treatments from day 4 p.i. at least until the end of the challenge, indicating that the infection with WSSV could be reduced by the exposure to IHHNV or to inactivated WSSV.

Virus detections after *per os* challenge with WSSV

A kit based on competitive PCR to grade viral infections, from severe to light infections, was used to check for WSSV in individual shrimp. PCR analyses of 30 randomly selected moribund shrimp (72 h p.i.) from each challenged treatment confirmed the presence of WSSV. All samples exhibited severe infection according to the observed pattern of bands (296, 550 bp and other longer bands) in an agarose gel (Fig. 4). In contrast, no band was observed from all the non-infected controls, confirming the absence of WSSV.

In addition, the presence of IHHNV was checked in 30 moribund shrimp from IHHNV(PL22)-W, InWSSV(PL35)-W and IHHNV(N5/Z1/PL22)-W treatments. Among these shrimp the presence of the typical band at 600 bp characteristic of IHHNV was observed in five animals, all from IHHNV(N5/Z1/PL22)-W treatment (Fig. 5). No amplicon was obtained from moribund shrimp from IHHNV(PL22)-W and InWSSV(PL35)-W treatments.

Quantification of viruses

Viral loads were quantified by real-time PCR in moribund and in surviving shrimp from IHHNV(PL22)-W, InWSSV(PL35)-W and

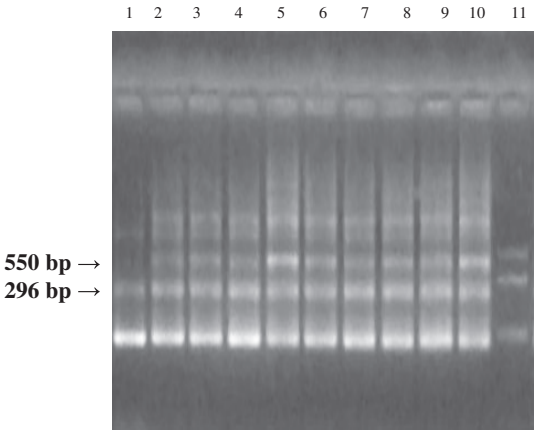

Figure 4 Moribund shrimp from a representative challenged treatment IHHNV(PL22)-W individually tested by polymerase chain reaction for white spot syndrome virus. Lanes 1–10: individual PL from IHHNV(PL22)-W. Lane 11: DNA markers (848, 630 and 333 bp).

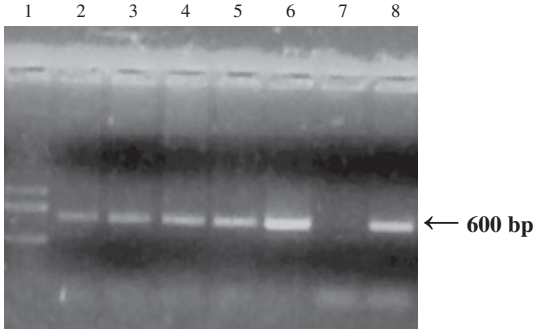

Figure 5 Moribund post-larvae from IHHNV(N5/Z1/PL22)-W treatment individually tested by polymerase chain reaction (PCR) for infectious hypodermal and haematopoietic necrosis virus (IHHNV). Amplified products correspond to PCR by using 600F and 600R primers. Lane 1: DNA markers (848, 630 and 333 bp). Lanes 2–6: individual PL from IHHNV(N5/Z1/PL22)-W. Lane 7: negative control (IHHNV non-infected PL). Lane 8: positive control (IHHNV-infected PL).

IHHNV(N5/Z1/PL22)-W treatments, and are presented as mean ± standard deviation (Table 3). Surviving shrimp from the IHHNV(N5/Z1/PL22)-W treatment showed two different pathological conditions. The first subgroup (a) had a high mean load of IHHNV (6.6×10^8 copies μg^{-1} of DNA; $n = 3$), whereas WSSV was undetectable. The second subgroup (b) had a low mean load of both IHHNV (9.0×10 copies μg^{-1} of DNA; $n = 5$) and WSSV (4.4×10^2 copies μg^{-1} of DNA). Furthermore, surviving shrimp from InWSSV-PL35 had a low mean load of WSSV (4.4×10^2 copies μg^{-1} of DNA; $n = 5$). Conversely, there were no surviving shrimp in IHHNV(PL22)-W. In addition, moribund shrimp from IHHNV(N5/Z1/PL22)-W ($n = 5$), InWSSV(PL35)-W ($n = 5$) and IHHNV(PL22)-W ($n = 5$) treatments, showed a high mean load of WSSV ranging from 1.9 to 2.4×10^9 copies μg^{-1} of DNA, and a low mean load of IHHNV ranging from 1.3×10^3 to 1.5×10^4 copies μg^{-1} of DNA.

Our data indicated that a higher level of WSSV was detected in the moribund shrimp (10^9 copies μg^{-1} of DNA) compared with surviving shrimp, which showed a range of $0–1 \times 10^3$ copies μg^{-1} of DNA for IHHNV(N5/Z1/PL22)-W and $0–2 \times 10^3$ copies μg^{-1} of DNA for InWSSV(PL35)-W.

The load of WSSV (1.4×10^8 copies μg^{-1} of DNA) and IHHNV (2.5×10^7 copies μg^{-1} of DNA) were also quantified from infected tissue stocks used to prepare the viral suspensions, and were similar to viral loads quantified in experimentally infected shrimp from previous studies (Durand & Lightner 2002; Tang *et al.* 2003).

Discussion

The occurrence of simultaneous viral infections is frequent in invertebrates. This state, also called viral co-infection, is characterized by the presence

Table 3 Quantification of infectious hypodermal and haematopoietic necrosis virus (IHHNV) and white spot syndrome virus (WSSV) loads through real-time polymerase chain reaction in DNA samples extracted from moribund and surviving *Penaeus vannamei* post-larvae after *per os* challenge with WSSV

Treatment	Shrimp status (tested shrimp)	IHHNV (copies μg^{-1} DNA)	WSSV (copies μg^{-1} DNA)
IHHNV(PL22)-W	Moribund (5)	$4.2 \times 10^3 \pm 6.7 \times 10^3$	$2.4 \times 10^9 \pm 1.1 \times 10^9$
InWSSV(PL35)-W	Surviving (5)	$5.8 \times 10^2 \pm 6.5 \times 10^2$	$4.4 \times 10^2 \pm 9.8 \times 10^2$
InWSSV(PL35)-W	Moribund (5)	$1.3 \times 10^3 \pm 1.5 \times 10^3$	$1.9 \times 10^9 \pm 2.0 \times 10^8$
IHHNV(N5/Z1/PL22)-W(a)	Surviving (3)	$6.6 \times 10^8 \pm 6.8 \times 10^8$	Undetectable
IHHNV(N5/Z1/PL22)-W(b)	Surviving (5)	$9.0 \times 10 \pm 1.2 \times 10^2$	$4.4 \times 10^2 \pm 6.0 \times 10^2$
IHHNV(N5/Z1/PL22)-W	Moribund (5)	$1.5 \times 10^4 \pm 2.3 \times 10^4$	$2.2 \times 10^9 \pm 9.0 \times 10^8$

Data are presented as mean ± standard deviation.

of two or more viruses in the same host, infecting the same tissue or cell (Harper 1986). Several investigations have revealed this condition in shrimp (Krol, Hawkins & Overstreet 1990; Madhavi, Janakiram, Jayasree & Murthy 2002; Manivannan, Otta, Karunasagar & Karunasagar 2002; Otta, Karunasagar & Karunasagar 2003; Umesha, Uma, Otta, Karunasagar & Karunasagar 2003; Flegel, Nielsen, Thamavit, Kongtim & Pasharawipas 2004).

In addition, a peculiar relationship between viruses, known as viral interference, has also been reported in vertebrates and invertebrates. This phenomenon occurs when a cell population that was previously infected with a virus subsequently becomes resistant to a challenge with the same or a different virus (Fenner, Gibbs, Murphy, Rott, Studdert & White 1993). Among hosts involved are fish (Chinchar, Logue, Antao & Chinchar 1998) and chicken (Ashraf, Abdel-Alim, Al-Natour & Saif 2005). Interestingly, a viral interference in crustaceans was reported in juvenile *P. stylirostris* conferring resistance to WSSV after an IHHNV primary infection (Tang *et al.* 2003).

Evidence concerning specific pathogen recognition in invertebrates (Choe, Werner, Stoven, Hultmark & Anderson 2002; Kurtz & Franz 2003; Little, O'Connor, Colegrave, Watt & Read 2003) supports results already known in shellfish aquaculture obtained from experimental bioassays. 'Vaccination' has been used with inactivated *Vibrio* spp. to enhance the resistance to vibriosis in *P. monodon* (Teunissen, Faber, Booms, Latscha & Boon 1998). In addition, inactivated *Aerococcus viridans* (var. *homari*) has been used to induce resistance to gaffkaemia in *Homarus americanus* (Stewart, Arie & Marks 2004). Similar investigations have been conducted to confer protection through 'vaccination' with inactivated WSSV and WSSV recombinant proteins to prevent infections in *P. japonicus* (Namikoshi *et al.* 2004), in *P. monodon* (Witteveldt *et al.* 2004a,b) and in *P. indicus* (Bright Singh *et al.* 2005). However, as vaccination implies a long-lasting protection through immunological memory, requiring primary challenge with antigen and clonally derived lymphocyte subsets, in shrimp this terminology could lead to confusion. In contrast, 'immunostimulant' refers to any substance which boosts reactivity and improves resistance to, or survival after exposure to harmful micro-organisms (Smith, Brown & Hauton 2003). In this context, the term 'immunostimulation' is more appropriate in discussion of our results.

Based on real-time PCR, surviving shrimp from the IHHNV(N5/Z1/PL22)-W treatment were composed of a subgroup with a high load of IHHNV and undetected WSSV, and a second group with a low load of both IHHNV and WSSV. Consequently, the viral loads in survivors from the first subgroup suggests a case of interference with WSSV infection, with IHHNV playing a role as interfering virus in suppressing WSSV replication. Probably primary infection with IHHNV at early larval stages, mimicking a vertical transmission, followed by a booster inoculation at PL22 stage, established the IHHNV infection more effectively in the experimental shrimp. This represented a total incubation period of 7 weeks before the challenge with WSSV. Similarly, the low load of WSSV in survivors from the second group supports the idea that a viral attachment interference mediated by IHHNV was induced in these shrimp. In this situation, the interfering virus destroys or blocks the receptors for the subsequent virus (Fenner, McAuslan, Mims, Sambrook & White 1974). This is possible, considering that interfering virus does not necessarily have to replicate to induce interference, and the ability of the challenge virus to replicate may be completely or partially inhibited (Fenner *et al.* 1993). In many respects, competition between viruses is a competition for host resources (Hackett, Boore, Deming, Buckley, Camp & Shapiro 2000), including cellular receptors. A competition between IHHNV and WSSV for receptor sites on shrimp could exist based on their affinity for target tissues of the same origin, i.e. ectodermal and mesodermal tissues (Tang *et al.* 2003).

Viral interference was previously reported in *P. stylirostris*, with survivals that ranged from 28% to 91%, with higher levels of IHHNV (10^8–10^9 copies μg^{-1} of DNA) and lower levels of WSSV (10^3 copies μg^{-1} of DNA) in surviving shrimp (Tang *et al.* 2003). In contrast, juvenile *P. vannamei*, previously infected with IHHNV, were not protected against subsequent WSSV challenge resulting in 100% mortality at day 5 p.i. Low survivals obtained in our experiments with *P. vannamei* suggest that this viral interaction could be influenced by the relative susceptibility of host species. In this context, reported differences concerning the susceptibility of *P. stylirostris* and *P. vannamei* to IHHNV infection could be related.

On the other hand, a low load of WSSV in surviving shrimp from the InWSSV-PL35 treatment suggests induced resistance to WSSV infection through immunostimulation by inactivated WSSV. Interestingly, this response was achieved by supplying formalin-inactivated viral particles to experimental shrimp 10 days before WSSV challenge and it was different from an 'active viral accommodation' (Flegel & Pasharawipas 1998) because survivors did not contain high loads of WSSV. Similar anti-WSSV responses have been observed after 'vaccination' in *P. monodon* (Witteveldt *et al.* 2004a,b), in *P. japonicus* (Namikoshi *et al.* 2004) and in *P. indicus* (Bright Singh *et al.* 2005). Hence, we hypothesize that *P. vannamei* is also able to recognize WSSV structural proteins in order to produce an antiviral immune response.

A delay in the progression of mortality was also observed in the IHHNV(N5/Z1/PL22)-W and InWSSV(PL35)-W treatments, from day 4 p.i. until the end of the challenge. In addition, most of the surviving shrimp (10/13), analysed by real-time PCR, did not contain WSSV. Thus, absence or lower levels of WSSV in these shrimp showed that both treatments affected its replication rate, resulting in protection against WSSV.

In the IHHNV-PL22 treatment, although the shrimp were infected once with IHHNV at PL22 and confirmed to be infected by PCR at PL42, sampled moribund shrimp were negative for IHHNV. This result could be a consequence of sampling at 72 h p.i., suggesting that all prematurely dead shrimp (at this time) had a non-detectable load of IHHNV. Moreover, an incubation period of 3 weeks with IHHNV may not be enough to allow a higher degree of infection in these shrimp, resulting in 100% mortality by subsequent challenge with WSSV. Thus, the boosted IHHNV infection in the IHHNV(N5/Z1/PL22)-W treatment was a suitable strategy to induce protection.

The load of WSSV in individual moribund shrimp from IHHNV(PL22)-W, InWSSV(PL35)-W and IHHNV(N5/Z1/PL22)-W averaged 2.2×10^9 copies μg^{-1} of DNA ($n = 15$), similar to viral levels in moribund shrimp from several species experimentally infected with WSSV (Durand & Lightner 2002).

As expected, there was 100% mortality in the positive control. In addition, a similar mortality in the InWSSV(Z1-M1)-W treatment demonstrates that doses of inactivated WSSV administered at early larval stages are not enough to confer protection against WSSV infection.

The absence of surviving shrimp in the InWSSV(Z1-M1/PL35)-W treatment could be related to variation in conditions among the post-larval tanks or to differences in levels of inactivated particles among shrimp. Similar variations in response have been reported previously (Tang *et al.* 2003). In the IHHNV(N5/Z1)-W treatment, 100% mortality could be the consequence of the lack of IHHNV booster during PL culture, confirming that infections at both early and late stages would be necessary to establish IHHNV infection.

In conclusion, survival to day 10 p.i. was significantly associated with both InWSSV(PL35)-W and IHHNV(N5/Z1/PL22)-W treatments, and their survivals were significantly different at day 10 p.i. compared with the remaining WSSV-challenged treatments, suggesting that it is possible to induce some protection in *P. vannamei* against WSSV infection by a pre-exposure to IHHNV at N5, Z1 and PL22 or to inactivated WSSV at PL35. IHHNV suppresses WSSV replication partially or completely, while inactivated WSSV induces a non-specific antiviral immune response. These two effects delay the mortality rate due to WSSV. However, this protection appears to be dependent on the shrimp species and on the time of application of the competing agents.

Further studies must be developed to evaluate the scope of these protections through experimental re-challenges with WSSV, the immune response of co-infected shrimp by characterizing activities related to innate immunity, together with cross-infection bioassays with other shrimp viruses to determine whether the observed effect is WSSV specific.

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