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Pathogenicity of white spot syndrome virus on postlarvae and juveniles of *Penaeus* (*Litopenaeus*) *vannamei*

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Abstract

White Spot Syndrome Virus (WSSV) has decimated the shrimp aquaculture around the world. Breeding efforts to generate resistant stocks are necessary but there is a lack of basic information on challenge test strategies focused on genetic selection. Infection routes and developmental stages were evaluated on *Penaeus vannamei* as a first step in a strategy to select white spot virus (WSSV)-resistant stocks. Mortalities could not be induced before the PL30 stage. The impact of infection by immersion and blended tissue was intermediate on mortalities when compared to the minced tissue treatment on PL30. Blended and minced tissue treatments produced the highest mortalities on PL40 while immersion was intermediate. A general tendency towards higher susceptibility associated with older stages was detected. Additionally, juveniles of 1 g average weight from three local breeding programs were challenged. There were no differences in survival between the programs, although two of them derived their progenies from survivors of strong WSSV events. The implications of these results to the WSSV epidemiological characteristics and breeding programs are discussed.

Keywords: Breeding; Challenge test; Larval stages; Survival; *Penaeus vannamei*; WSSV

1. Introduction

Among the multiple viral problems reported on shrimp, White Spot Syndrome Virus (WSSV) is the most devastating and widespread (Wang et al., 1999; Tapay et al., 1999). Since 1992, the disease has caused mass mortalities in Asia. In 1995, the virus was reported in the United States, and in 1999, it was detected in Central and South America where it caused considerable socio-economic losses.

WSSV was detected in Ecuador in May 1999 (Jimenez et al., 2000), yet the disease may have been incubating in wild populations at least since 1996 (Granda et al., 2001). Although no prominent

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white spots are found on the carapace of *Penaeus vannamei* (Penaeidae: Crustacea) affected animals, these shrimp become lethargic, stop feeding and swim erratically around the pond borders where they die. Mass mortalities start 30–40 days after stocking.

Until the start of the WSSV epidemic, the Ecuadorian industry relied on wild-caught larvae and broodstock, which made it highly vulnerable to old and new emerging diseases. The impact of the WSSV revealed the need for breeding programs directed to the production of resistant/tolerant stocks. However, no information regarding the behavior of the disease or the effectiveness of challenge tests under local conditions was available.

Family selection is used when individual performance cannot be measured directly or heritability is low. As shrimp heritability to Taura Syndrome Virus resistance is low (Argue et al., 1999) and if the trend is similar for WSSV, family selection should be the most appropriate breeding scheme. From a family selection program point of view, the earliest the selection of resistant/tolerant families, the sooner families without genetic value can be culled from the breeding program. In this work, we aimed at developing challenge protocols for WSSV on early stages of white shrimp as part of a strategy to select resistant or tolerant animals. We also evaluated the WSSV resistance of juveniles derived from breeding programs using pond survivors as broodstock.

2. Materials and methods

2.1. PL stage and infection route

Challenge tests with postlarvae at stages PL20, PL30 and PL40 were carried out sequentially. Biological material was derived from families that were part of a breeding program aimed at increasing growth. Each family was raised separately from nauplii to PL12 in 50-l tanks. Families were then transferred to individual 1.5-m^3 tanks until they reached the appropriate stage (1500 PL12 per tank). These crosses were kept separate until they reached 1 g average weight (around PL65). At that size, each family could be marked by elastomers and pooled for the final growth out and evaluation in the breeding program. For each PL stage evaluated on our sequential set of experiments, an equal number of animals from 20 families were pooled in order to avoid possible genetic effects.

For the inoculum preparation, 5-g animals were injected with a viral stock derived from pond-infected animals and maintained by CENAIM. All the inoculated animals died within 4 days and were WSSV positive by an in-house nested PCR protocol (http://www.cenaim.espol.edu.ec/publicaciones/quincenal/bquinc41.pdf). The viral stock was prepared by maceration of 10% of whole infected tissue and 90% of PBS solution (weight/weight). The mix was centrifuged at 4000 rpm and the supernatant stored at -20 °C. The inoculum was used directly for the immersion treatment or to infect other animals as needed for the other evaluated treatments.

Three methods of infection were evaluated: immersion, minced tissue and blended tissue. For the immersion treatment, a 10^{-2} dilution of a viral stock was used (30 ml of concentrated viral stock per experimental unit). For the minced treatment, a mixture of 95% of infected muscle tissue and 5% deionized water was blended in a commercial blender for 1 min. Experimental units with this treatment were fed at a 10% weight/weight of the estimated biomass. For the blended tissue treatment, a mixture of 5% muscle tissue with 95% deionized water was blended for 1 min in a commercial blender. Thirty milliliters of blended tissue was added to each experimental unit. A non-infected treatment was included as control. Shrimp in the control experimental units were fed with minced tissue derived from non-infected animals as shown by nested PCR.

The experimental setup consisted of four treatments (immersion, minced, blended and control) distributed in a Completely Randomized Design for each larval stage (PL20, PL30 and PL40). For each treatment, four replicates were used that comprised four 3-l glass flasks (one per replicate). Twenty animals from a balanced pool derived from 20 families as previously explained were used in each replicate. Each flask was kept at a constant temperature (25 °C) using an external water bath able to contain the 16 experimental units and heavy aeration was applied to each replicate. Postlarvae were starved 24 h previous to infection. Infection by three infection methods and the

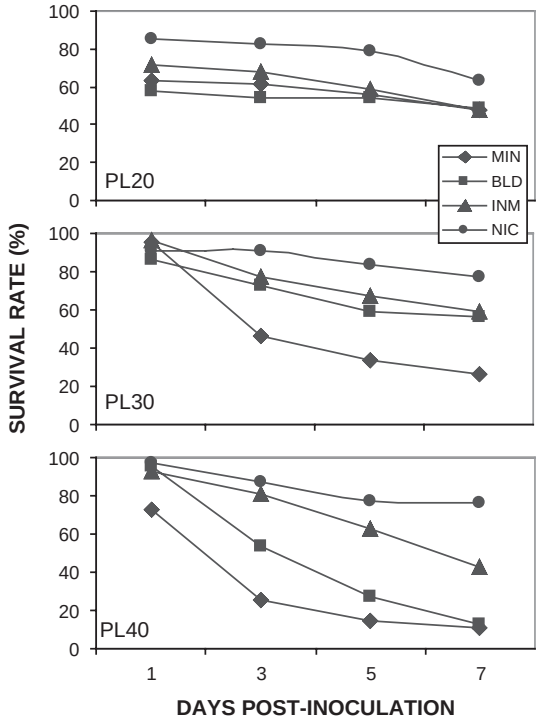

Fig. 1. Survival percentages of *P. vannamei* postlarvae infected by three methods (minced tissue, MIN; blended tissue, BLD; immersion in viral extract, IMM) on PL20, PL30 and PL40 stages. NIC: non-infected control.

control setup as described above were carried out twice for a period of 3 h the same day. After each infection, water was exchanged completely. Before starvation and after infection, animals were fed ad libitum. Water in the experimental units was exchanged 100% daily. Dead animals were removed twice during daily inspections (800 and 2000 h). In many cases, because of cannibalism and the small size of animals, it was not possible to recover whole carcasses. Hence, survival was evaluated every 2 days during the water exchange of each experimental unit by counting of all the survivors up to the seventh day post-infection.

2.2. Resistance to WSSV of selected stocks

In order to test the resistance of animals originating from three local breeding programs, an experiment was set up with juveniles of 1 g average weight. Two of these programs used broodstock selected from ponds that experienced survival rates below 5% as a result of WSSV infection as evaluated by histology. The third program maintained animals under a domestication scheme and this population was not affected by WSSV. Larvae from the three sources were grown from PL12 to 1 g juveniles in 1.5-m^3 plastic tanks over a 9-week period. Three replicates (20 animals per replicate) per breeding program were evaluated using 30-l aquaria as experimental units. Experimental units were infected with minced tissue (10% of estimated biomass) after 1 day of starvation. Two non-infected replicate controls (20 animals pooled from the three breeding lines per replicate) were kept under the same conditions and fed non-infected minced tissue. Replicates were randomly distributed according to a Completely Randomized Design. Management was similar to the earlier experiments except that there was no temperature control in the experimental units. Regular temperatures in the aquaria reached 22 °C with ± 2 °C fluctuations. Data were collected on a daily basis for 11 days.

2.3. Statistical analysis

Statistical analyses for the first set of experiments were carried out under a 4×3 bifactorial model corresponding to four infection modes and three PL stages. Data on survival at the last day of the experiment were normalized with the arcsine of the square root transformation before ANOVA. Statistically significant results were separated with the Tukey Honest Significant Difference HSD. For the selected stocks experiment, a one-way ANOVA was carried out on transformed data (arcsine of the square root) and ranks were obtained with Tukey HSD test. All data analyses

Table 1
Bifactorial ANOVA on WSSV challenge tests for three types of infection (minced, blended tissue and viral extract) plus non-infected control and three developmental stages (PL20, PL30 and PL40) in *P. vannamei*

	SS	DF	MS	*F*	*p*
Infection	5376.5	3	1792.2	23.997	0.000000
Stage	1724.7	2	862.4	11.547	0.000134
Infect*Stage	2118.5	6	353.2	4.728	0.001208
Error	2688.5	36	74.7		

Table 2
Tukey HSD range test on WSSV challenged *P. vannamei* postlarvae (mean survival ± S.E. in percentage)

Infection					Infection*stage					
MIN	28.3 ± 5.3	A			MIN*PL40	11.3 ± 6.3	A			
BLD	39.2 ± 6.0	A	B		BLD*PL40	12.5 ± 4.3	A			
IMM	49.6 ± 4.7		B		MIN*PL30	26.3 ± 6.3	A	B		
NIC	72.5 ± 4.2			C	IMM*PL40	42.5 ± 7.2		B	C	
					IMM*PL20	47.5 ± 10.2		B	C	D
					MIN*PL20	47.5 ± 1.4		B	C	D
					BLD*PL20	48.8 ± 3.8		B	C	D
					BLD*PL30	56.3 ± 1.3		B	C	D
Stage					IMM*PL30	58.8 ± 5.5		B	C	D
PL40	35.6 ± 7.3	A			NIC*PL20	63.8 ± 11.3			C	D
PL20	51.9 ± 4.0		B		NIC*PL40	76.3 ± 2.4			C	D
PL30	54.7 ± 5.3		B		NIC*PL30	77.5 ± 4.3				D

MIN=minced tissue; BLD=blended tissue; IMM=immersion in viral extract; NIC=non-infected control. The capital letters (A–D) refer to significant differences.

were carried out with the Statistica Software (StatSoft Inc., 2001).

3. Results

Survival rates of *P. vannamei* varied with the infection routes and PL stages and decreased steadily over a period of 7 days (Fig. 1). Infection route, PL stage and their interactions were statistically significant as determined by the bifactorial ANOVA (Table 1).

The infection route by minced tissue gave the lowest survival rate (28.3%) followed by the blended tissue (39.2%) and immersion (49.6%) treatments. The non-infected control survival (72.5%) was the highest and statistically different from the three infection treatments. Ranking of the results showed that the minced and blended tissue were the most effective ways to infect the animals. Immersion in viral extract was less effective than the other two evaluated routes, although it produced mortalities since it was statistically different from the control (Table 2).

Survival of the PL40 stage was lower (35.6%) than and statistically different from PL20 (51.9%) and PL30 (54.7%). PL40 was distinct from PL20 and PL30 according to the Tukey HSD test (Table 2).

Interactions between stage and infection route showed that minced tissue on PL40 (11.3% survival) and PL30 (26.3%), together with blended tissue on PL40 (12.5%), grouped at a single rank and were successful in inducing WSSV mortalities. A second rank grouped all other interactions that included infection treatments. However, two other ranks grouped part of these interactions with the non-infected controls, which indicates low efficacy of infection induction on PL20 and PL30 (Table 2).

The survival rate of the juveniles from the three breeding programs dropped within days and did not differ between stocks (Fig. 2). ANOVA of the experiment showed significant differences ($P<0.001$). The Tukey HSD test ranked the three stocks together and different from the non-infected control (Table 3).

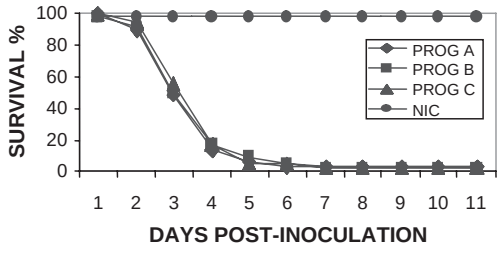

Fig. 2. Survival percentages of *P. vannamei* 1 g juveniles derived from three breeding programs (A, B and C). Infection was carried out by minced tissue. NIC: non-infected control.

Table 3
Tukey HSD range test in survival rates 7 days post-infection to WSSV infection in of *P. vannamei* by minced tissue of 1 g average weight animals from three breeding programs and non-infected control (mean survival ± S.E.)

Treatment	% Survival
Non-infected control	97.5 ± 2.5 A
Program A	3.3 ± 3.3 B
Program B	1.7 ± 1.7 B
Program C	1.7 ± 1.7 B

4. Discussion

Survival of PL20 and PL30 was higher and statistically different from the PL40 stage. WSSV infection of PL20 did not show mass mortalities in either infection route. Pilot tests on PL12 using immersion in viral extract gave similar results (data not shown). The earliest stage where mass mortalities could be detected was PL30. Our data support the idea of a higher resistance to WSSV in early larval stages of *P.*

vannamei and might indicate that the susceptibility to WSSV depends on size and physiological state.

Our findings agree with the related shrimp *Penaeus japonicus*, where mass mortalities could not be detected before PL10. Moreover, mortality of PL20-infected animals was lower than animals infected at PL37, demonstrating that susceptibility increases with the postlarval age (Venegas et al., 1998). Differences to WSSV susceptibility at the interspecific level have been demonstrated previously between *Penaeus* (*Litopenaeus*), *Macrobrachium* and *Farfantopenaeus* species (Hameed et al., 2000; Wang et al., 1998, 1999). In another study (Lightner et al., 1998), 50% of the postlarvae of *Penaeus duorarum* (PL19) survived compared to 100% mortality for *Litopenaeus aztecus* (PL19), *Litopenaeus setiferus* (PL13) and *P. vannamei* (PL30). Higher mortalities of *P. vannamei* might be due to higher inoculation levels and longer evaluation times. Similarly, differential survival rates of larval substages in challenge tests with bacteria have been reported previously for *P. vannamei.* Different from this study, early larval substages are more sensitive than later stages, depending on the bacterial species and dosage (Aguirre-Guzman et al., 2001).

Minced and blended tissue treatments were equivalent as infection routes, whereas immersion in viral extract was less effective. However, the effectiveness of the inoculation route varied with the PL stage interaction. Minced tissue was the most effective inoculation pathway in both, PL30 and PL40. Blended tissue and immersion were statistically similar and induced lower mortalities than the minced tissue in PL30. On the other hand, at PL40 blended and minced tissue routes were both statistically similar and gave the highest mortalities. Mortality rates of the blended tissue treatment changed from being intermediate and similar to immersion in PL30 to produce extreme mortalities in PL40. This treatment might be considered as an attenuated immersion and minced tissue treatment, both happening simultaneously. As susceptibility increases in PL40, the amount of viral particles might be sufficient to induce mortalities similar to the minced tissue treatment. In this case, the increased susceptibility to the few free particles on the blended tissue treatments added to the ingestion of infected tissue could account for the observed results.

In *P. japonicus* (Venegas et al., 1998) and our experiment, immersion in virus extracts (10_v^{-2} dilutions) were used, but in neither case was the virus titer determined. This information is important for the standardization of challenge tests dealing with resistance where threshold levels might be present. The development of bioassays using DL50 for shrimp viruses (Prior et al., 2003) might be highly valuable for shrimp breeding and pathogenesis studies in the future. Competitive PCR (Tang and Lightner, 2000) or real-time PCR (Tang and Lightner, 2001) could be used to quantify viral loads.

Our data might explain mass mortalities in *P. vannamei* commercial ponds where postlarvae are traditionally stocked as PL12. Moribund animals are detected after 30–40 days, corresponding to the PL42 to PL52 stages. The lagging period could be explained as the time necessary to amplify the disease in a cascade-like fashion. Around PL30, the first moribund animals appear which are cannibalized. A second mortality peak is reached a few days after the initial moribund animals appeared in the pond. Two to three successive waves might account for the observed mass mortalities. Stocking of infected seed could be the initial source of viral loads, although increased amplification of the disease or even the original viral source might be due to positive carriers that are part of the habitat or of the normal shrimp diet. Krill *Acetes* sp., sand and mud crabs, copepods, Palaemonid prawn and Ephydrid insect larvae have been reported as WSSV carriers (Supamattaya et al., 1998; Lo et al., 1996). Since cohabitation seems not to be an effective infection route in *P. vannamei* (Soto and Lotz, 2001), WSSV transmission in the pond should be mainly caused by ingestion of infected tissue from positive zooplankton or from infected shrimp carcasses.

Use of pond survivors as a means of obtaining WSSV-resistant stocks has been a practice followed by producers in Ecuador and elsewhere. In our experiment, the three evaluated stocks showed similar survival rates, although two of them were derived from animals previously exposed to mass mortalities related to WSSV. Shrimp resistance to Taura Syndrome Virus has been reported as a low heritability character (Argue et al., 1999). Genetically, the use of pond survivors can be seen as a mass selection process on a binary character. Mass selection is not an effective procedure with low heritabilities, since environmental and stochastic factors might be responsible for the

death or survival of an individual, rather than its genetic complement. Differential survival in controlled WSSV challenge tests has been explained by assuming that a proportion of the susceptible shrimps are non-aggressive feeders, and that the inoculum (various parts of the carcass) has not the same viral loads (Soto et al., 2001). Behavioral factors rather than resistance to the virus might play an important role in individual survival in a pond where food competition could be strong. Animals that do not enter into contact with the virus either because of their lack of aggressive feeding behavior or that never ingested critical viral loads might be the survivors used in local breeding programs.

This work supports four main points on *P. vannamei*: (a) WSSV does not produce mass mortalities before PL30 postlarval stages under our experimental conditions; (b) effective challenge test in *P. vannamei* can be done from PL30 on with minced tissue; (c) susceptibility to the virus increases as the shrimp grows and might be related to genetical, physiological or ethological characteristics and; (d) use of pond survivors might not be an efficient strategy to develop WSSV-resistant stocks.

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