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Short Communication

Survival in juvenile shrimps (*Penaeus vannamei*) exposed to inactive against active white spot virus: a challenge bioassay perspective

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ABSTRACT. White spot syndrome virus (WSSV) has damaged the Ecuadorian shrimp culture industry severely. The shrimp industry is highly important as it has generated high revenues over the past few years in Ecuador. Therefore, research on preventing devastating viral infections such as WSSV, is of major relevance. This study aimed to evaluate the survival rate in white shrimps (*Penaeus vannamei*) by using a vaccination method of inactive white spot virus (WSV) against an active WSV infection. A bioassay using 120 shrimps, 60 injected with inactive WSV and 60 injected with shrimp tissue without WSV infection, was conducted. Firstly, 30 specimens injected with inactive WSV were challenged against active WSV after 48 h. The remaining 30 specimens were challenged against shrimp tissue without WSV infection, as a negative control. Secondly, 60 specimens injected with the tissue without WSV infection were challenged as follows: 30 against active WSV and 30 against shrimp tissue without WSV infection. Kaplan-Meier analysis ($P \leq 0.961$) of the results showed no significant differences between the groups. Thus, these results showed no decrease in the mortality rate in juvenile shrimps (*P. vannamei*) after treatment.

Keywords: *Penaeus vannamei*, white spot virus, infection, survival rate, aquaculture.

The shrimp culture industry is of major importance to Ecuador and is almost entirely dependent on the white shrimp *Penaeus vannamei* (Lightner, 2011), representing nearly 95% of the Ecuadorian aquaculture. In 2014, *P. vannamei* culture generated an annual revenue of 150 million dollars in Ecuador (FAO, 2012). Moreover, the shrimp culture industry has been affected by several viral and bacterial diseases (Lightner, 2011), most importantly white spot syndrome virus (WSSV) due to its high mortality rate (Rodríguez *et al*., 2003).

The WSSV was first discovered in the 1990s in Taiwan (Wu *et al*., 2005), and is considered one of the most pathogenic viruses in shrimp culture due to its rapid transmission and large specimen mortality (Lo *et al*., 1996). White spot virus (WSV) has a broad host range and also infects other invertebrate aquatic organisms, such as crab and crayfish (Wang *et al*., 1998). Due to the considerable mortality and economic damage to the shrimp farming industry caused by WSSV in the past decade (Witteveldt *et al.*, 2004a), extensive research on virus infection and replication mechanism has been undertaken (Durand *et al*., 1997; Wang *et al*., 2000; Wu *et al*., 2005).

In Ecuador, WSSV was first discovered in May, 1999. Research conducted by Rodríguez *et al.* (2003) suggested an apparent association between lower temperature and increased shrimp mortality rates. Thus, given importance of this industry and the limitations of high-intensity cultivation due to viral infections causing high mortality (Ramos-Carreño *et al*., 2014; Melena *et al.*, 2015), particularly in extreme salinities (50), the development of prevention methods against WSV and other viruses would be necessary (Witteveldt *et al*., 2004a, 2004b).

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Studies have shown that different compounds such as *β*-glucans (Rendón & Balcázar, 2003) and intramuscular injections (Melena *et al.*, 2015) play a protective role against viral diseases. In addition, different findings in shrimp species and other crustaceans, together with *in vivo* WSV challenge infection experiments with *Penaeus japonicas* suggest that a potential adaptive immune defense system may exist (Kurtz & Franz, 2003; Namikoshi *et al*., 2004; Chen *et al.*, 2016). Additionally, results of larvae and post-larvae of *P. vannamei*, challenged with inactivated WSV, suggest a protective role against further exposures, potentially as a result from a non-specific antiviral immune response (Melena *et al*., 2006).

Thus, to investigate whether protection against WSSV and a further increase in the survival rate could be induced by injections of inactive WSV; juvenile shrimps of 1 g were selected because growth stage, feeding and environmental requirements can be easily adapted (Sugathan *et al.*, 2014). Overall, the specimens were injected with a solution containing either macerated shrimp tissue infected with inactive WSV (Stim) or with a control solution without the virus (Non-Stim) WSV, before being exposed to active WSV injections or preserved shrimp tissue without any viral infection (Prior *et al.*, 2003).

Tissue samples for injections, containing both, inactive WSV and non-infected WSV, were processed and sterilized with 5% formalin solution to inactivate the virus (Hiatt, 1964). TN buffer was replaced by 20 mM Tris-HCL, 0.4M NaCl, pH 7.4 (Prior *et al*., 2003). Also, formalin was used to preserve the DNA quality (García *et al*., 2006) of the WSV within the samples, and to avoid tissue putrefaction by killing bacteria and fungi. To prepare the Stim solution, a 10 min incubation of different macerated tissue (in 0.5% formalin) dilutions were done. Once prepared, the lethal dose was tested by injecting one gram specimens through bioassay. The amount of formalin-treated WSV inactive inoculum was the necessary to obtain a final lethal dose of 100% (Labreuche, *pers. comm.*). The same procedure was done to obtain the Non-Stim solution. The amount of non-infected tissue, was equivalent to the macerated infected tissue able to produce a 10% mortality. Once the formalin treatment was completed, both inoculate (infected and non-infected) dilutions were centrifuged at 30,000x *g* for 1 h at 4ºC. After centrifuging, the supernatant was discarded and the "pellets" were recovered in 10 mL of saline buffer. Subsequently, samples were centrifuged once again at 30,000x *g* for 1 h at 4ºC, to be then resuspended in 5 mL of saline buffer prior to use.

Subsequently, a bioassay following the Prior *et al.* (2003) but with only one dilution of the virus inoculum was done. Mortality, after challenges, was measured to infer the effect of inactive WSV exposure on the survival rate of juvenile shrimps. To do this, 120 specimens were allocated to individual containers (one animal per container) and separated into two groups, each group having specimens exposed to Stim and Non-Stim solutions. 100% water exchange was performed daily and the specimens were fed with a small piece of the pellet (commercially available), once per day.

Sixty juvenile shrimps were injected, in the last abdominal segment, with 20 μL of Stim solution. From them, 30 shrimps were injected in the last abdominal segment after 48 h with 20 μL of a solution containing a concentration of shrimp tissue equivalent to a DL50, whilst the remaining 30 specimens were injected with a solution of active WSV. The same experiment and protocol (20 μL injection after 48 h) were followed in parallel for the Non-Stim group. The concentration of tissue equivalent to a DL50 was used to match the initial killing effect of the vaccination process.

Mortality rate was estimated using the Kaplan-Meier analysis; a non-parametric maximum likelihood estimate (MLE) of the survival function. The estimation of significant differences in the survival rate among groups was done using three different Kaplan-Meier comparison tests. The *P*-value was calculated: 1) using the log-rank test, 2) with the Wilcoxon method, and 3) using the Tarone-Ware test. RStudio software for Macintosh (Version 0.99.891-© 2009-2016 RStudio, Inc), was used to perform the analysis and to plot the Kaplan-Meier survival curves.

The experimental results showed a mortality of 20 shrimps (Table 1), which was equivalent to a 67% mortality of the specimens firstly exposed to Stim solution and subsequently challenged against the active DL50 WSV-tissue dilution.

Shrimp tissue without WSV infection was implemented as a negative control, considering the potential toxicity effect of the tissue, particularly by basic proteins such as histones (Reiner *et al*., 1942; Marks *et al.*, 2000). Furthermore, the specimens first injected with Non-Stim solution and subsequently challenged against the active DL50 WSV-tissue dilution resulted in a mortality of 18 shrimps (Table 1), which was equivalent to a 60% mortality. The analysis of the controls of infection first injected with Stim or Non-Stim solutions and further challenged against non-infected virus tissue capable of causing a mortality of 50%, showed that no mortality was exhibited (Table 1).

Kaplan-Meier estimate showed that no differences in survival were exhibited in the challenge bioassay (Fig. 1). Log-rank test to evaluate the survival rate

Table 1. Daily mortality. Detailed mortality results per hour and days during the assay. Daily mortality results measured during the challenge bioassays (infection with a solution capable of causing a 50% mortality) in shrimps injected with formalin-inactive WSV or non-infected shrimp tissue (organisms 1 g).

Time		Non-Stim + tissue	Non-Stim + WSSV	Stim + tissue	Stim + WSSV
13/04-24 h	8 h	0	0	0	0
14/04-08 h	16 h	0	0	0	0
14/04-16 h	24 h	0	0	0	0
14/04-24 h	32 h	0	0	0	0
15/04-08 h	40 h	0	1	0	1
15/04-16 h	48 h	0	2	0	4
15/04-24 h	56 h	0	0	0	0
16/04-08 h	64 h	0	0	0	3
16/04-16 h	72 h	0	3	0	1
16/04-24 h	80 h	0	2	0	0
17/04-08 h	88 h	0	0	0	0
17/04-16 h	96 h	0	3	0	3
17/04-24 h	104 h	0	0	0	0
18/04-08 h	112 h	0	3	0	4
18/04-16 h	120 h	0	2	0	3
18/04-24 h	128 h	0	1	0	1
19/04-08 h	136 h	0	1	0	0
19/04-16 h	144 h	0	0	0	0
19/04-24 h	152 h	0	0	0	0
20/04-08 h	168 h	0	0	0	0
20/04-16 h	176 h	0	0	0	0
	Total	0	18	0	20

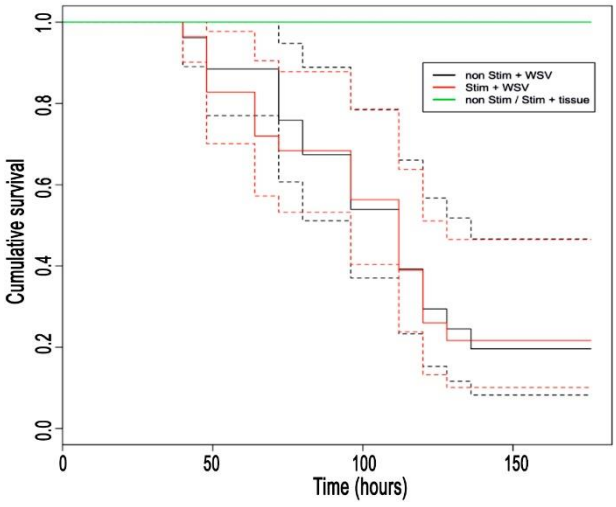

Figure 1. Kaplan-Meier survival plot. Kaplan-Meier survival analysis plot (solid line), and confidence intervals (dashed lines) of the experimental (stim + WSV; red line), placebo (non-Stim + WSV; black line) and controls (non-Stim and Stim + tissue; green line) groups.

across the assay, considering that the ratio of hazard functions is the same at all time points, showed no significant differences (P = 0.961). Wilcoxon method was used to assign more weight to deaths at early time points, in order to account for the higher risk of death in the Non-Stim group over the Stim group. Results showed no significant differences between the two groups (P = 0.805). Similarly, the Tarone-Ware test, considering early and late deaths due to manipulation and bioassay conditions, showed no significant differences (P = 0.749). Of relevance, as no confirmatory tissue test to determine whether mortality was specifically due to WSV infection; 100% survival results in Stim and Non-Stim + noninfected tissue, were considered as sufficient information to infer that mortality was due to WSV infection yet not to the injection method.

Moreover, our results contrast findings in *Penaeus japonicas* and *Penaeus monodon* challenge infection experiments (Namikoshi *et al.*, 2004; Witteveldt *et al.*, 2004a), as well as larvae and post-larvae of *P. vannamei*, challenged with inactivated WSV, and juvenile *P. vannamei* exposed to WSV and IHHNV via intramuscular injection (Melena *et al.*, 2006, 2015). These results exhibited a delayed mortality in the specimens, suggesting a protective role against further viral exposures injection (Melena *et al.*, 2006, 2015). Nevertheless, our results are in line with findings by

Prior *et al*. (2003) showing that no increase in the survival rate was given by injection of inactive virus, under these experimental conditions. Thus, further analysis should be conducted to elucidate whether 1) WSV amount which is available to enter the specimen's system differs among injection methods or specimens, 2) strain virulence might differ among isolation sites, favouring a higher/lower mortality rate, and 3) growth stage and shrimp species would play an important role by influencing the adaptation to stress stimuli and the prevention of further infections (Witteveldt *et al*., 2004a, 2004b; Vaseeharan *et al*., 2006; Melena *et al*., 2015; Chen *et al*., 2016).

On the other hand, nutritional compounds such as β-glucans (β-1,3-Glucan Binding Protein (BGBP) & Lipopolysaccharide Binding Protein (LPSBP9)), obtained from yeast and fungi cell walls (Raa, 1996), have been associated with a response system against pathogens by stimulating haemocytes phagocytosis and inducing degranulation, as well as activating the prophenoloxidase system (Vargas *et al*., 1996; Vargas-Albores & Yepiz-Plascencia, 2000). In this context, research conducted using probiotics, β-1,3/1,6-glucans, and bacteria (*Vibrio alginolyticus* and *Bacillus subtilis*), challenged against WSV, showed a higher survival rate compared to controls (Rodríguez *et al.*, 2007; Chen *et al.*, 2016; Pham *et al.*, 2017). Thus, hemocytes count, together with transcriptional expression of immune system genes (*e.g.*, hemocytes) analysis in *P. vannamei*, is necessary to substantiate whether species and habitat characteristics might play a role in the survival rate or if shrimps are in the capacity of developing a potential adaptive immune response system to prevent further infections.

To conclude, based on present-day results among shrimp species infections to prevent mortality due to further viral infections; considering Rodríguez *et al.* (2000) suggestion -to activate the immune response without depleting the specimen's energy as an alternative to limit the WSV proliferation in shrimps- and with evidence of the immune system responding efficiently after multiple vaccinations (Fonseca-Moreno *et al*., 2013; Lin *et al*., 2013); an adequate immune system activation in conjunction with the fulfilment of the necessary nutritional requirements could be conceived as "state of the art" to increase the survival rate in shrimp farms.

REFERENCES

Chen, Y.Y., S. Kitikiew, S.T. Yeh & J.C. Chen. 2016. White shrimp *Litopenaeus vannamei* that have received fucoidan exhibit a defense against *Vibrio alginolyticus* and WSSV despite their recovery of immune parameters to background levels. Fish Shellfish Immunol., 59: 414-426.

Durand, S., D.V. Lightner, R.M. Redman & J.R. Bonami. 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). Dis. Aquat. Org., 29(3): 205-211.

Food and Agriculture Organization (FAO). 2012. El estado mundial de la pesca y la agricultura. FAO, Roma, 250 pp.

Fonseca-Moreno, E., R. González-Salas & R. Rico-Gutiérrez. 2013. Sistema inmune de los camarones. Rev. Aquat., (38): 68-84.

García, M.P., M.F. Benavente, A.A. Melo, E.I. Roa & S.J.C. Roa. 2006. Efecto de la fijación en la calidad del ADN: estudio controlado con cinco fijadores. Rev. Esp. Patol., 39(3): 175-179.

Hiatt, C.W. 1964. Kinetics of the inactivation of viruses. Bacteriol. Rev., 28(2): 150.

Kurtz, J. & K. Franz. 2003. Innate defense: evidence for memory in invertebrate immunity. Nature, 425(6953): 37-38.

Lightner, D.V. 2011. Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. J. Invertebr. Pathol., 106(1): 110-130.

Lin, Y.C., J.C. Chen, W.Z.W. Morni, D.F. Putra, C.L. Huang, C.C. Li & J. F. Hsieh. 2013. Vaccination enhances early immune responses in white shrimp *Litopenaeus vannamei* after secondary exposure to *Vibrio alginolyticus*. PloS ONE, 8(7): e69722.

Lo, C.F., C.H. Ho, S.E. Peng, C. Chen, H.C. Hsu, Y.L. Chiu & G.H. Kou. 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org., 27(3): 215-225.

Marks, P.A., V.M. Richon & R.A. Rifkind. 2000. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst., 92(15): 1210-1216.

Melena, J., F. Echeverría, I. Betancourt, R. Santander, J. Candell & J.R. Bonami. 2015. Delayed mortality of juvenile shrimp *Penaeus vannamei* challenged to white spot syndrome virus (WSSV) previously exposed to infectious hypodermal and hematopoietic necrosis virus (IHHNV) or inactivated WSSV. Braz. J. Vet. Pathol., 8(2): 51-57.

Melena, J., B. Bayot, I. Betancourt, Y. Amano, F. Panchana, V. Alday & J.R. Bonami. 2006. Pre-exposure to infectious hypodermal and hematopoietic necrosis virus or to inactivated white spot syndrome virus (WSSV) confers protection against WSSV in *Penaeus vannamei* (Boone) post-larvae. J. Fish. Dis., 29(10): 589-600.

Namikoshi, A., J.L. Wu, T. Yamashita, T. Nishizawa, T. Nishioka, M. Arimoto & K. Muroga. 2004. Vacci-

nation trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture, 229(1): 25-35.

Pham, K.C., H.T.T. Tran, C. Van Doan, P.H. Le, A.T. Van Nguyen, H.A. Nguyen & T.N. Phan. 2017. Protection of *Penaeus monodon* against white spot syndrome by continuous oral administration of a low concentration of *Bacillus subtilis* spores expressing the VP28 antigen. Lett. Appl. Microbiol., 64(3): 184-191.

Prior, S., C.L. Browdy, E.F. Shepard, R. Laramore & P.G. Parnell. 2003. Controlled bioassay systems for determination of lethal infective doses of tissue homogenates containing Taura syndrome or white spot syndrome virus. Dis. Aquat. Organ., 54(2): 89-96.

Raa, J. 1996. The use of immunostimulatory substances in fish and shellfish farming. Rev. Fish. Sci., 4(3): 229-288.

Ramos-Carreño, S., R. Valencia-Yáñez, F. Correa-Sandoval, N. Ruíz-García, F. Díaz-Herrera & I. Giffard-Mena. 2014. White spot syndrome virus (WSSV) infection in shrimp (*Litopenaeus vannamei*) exposed to low and high salinity. Arch. Virol., 159(9): 2213-2222.

Reiner, L., E.J. De Beer & M. Green. 1942. Toxic effects of some basic proteins. Exp. Biol. Med., 50(1): 70-74.

Rendón, L. & J.L. Balcázar. 2003. Inmunología de camarones: conceptos básicos y recientes avances. Rev. Aquat., 19: 27-33.

Rodríguez, J., R. Cedeño, C. Molina, V. Otero, E. Valenzuela & M.A. Sotomayor. 2000. Efecto de la calidad de la dieta sobre la respuesta inmune del camarón *Penaeus vannamei.* Avances en Nutrición Acuícola. Memorias del V Simposium Internacional de Nutrición Acuícola, pp. 19-22.

Rodríguez, J., Y. Espinosa, F. Echeverría, G. Cárdenas, R. Román & S. Stern. 2007. Exposure to probiotics and β-1, 3/1, 6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to white spot syndrome virus challenge and pond culture. Aquaculture, 273(4): 405-415.

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Rodríguez, J., B. Bayot, Y. Amano, F. Panchana, I. De Blas, V. Alday & J. Calderón. 2003. White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure. J. Fish. Dis., 26(8): 439-450.

Sugathan, S., C. Shakir, A.P. Lipton, A. Manilal & J. Selvin. 2014. Effect of stocking density on the survival rate and growth performance in *Penaeus monodon*. J. Basic Appl. Sci., 10: 231 pp.

Vargas, F., I. Higueras, F. Jiménez, J. Hernández, T. Gollas & G. Yepiz. 1996. Posibilidades de inmunestimulación del camarón a través del alimento. Avances en Nutrición Acuícola III. Memorias del Tercer Simposium Internacional de Nutrición Acuícola, pp. 433-439.

Vargas-Albores, F. & G. Yepiz-Plascencia. 2000. Beta-glucan binding protein and its role in shrimp immune response. Aquaculture, 191(1): 13-21.

Vaseeharan, B., T. Prem-Anand, T. Murugan & J.C. Chen. 2006. Shrimp vaccination trials with the VP292 protein of white spot syndrome virus. Lett. Appl. Microbiol., 43(2): 137-142.

Wang, Y.C., C.F. Lo, P.S. Chang & G.H. Kou. 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture, 164(1): 221-231.

Wang, C.H., H.N. Yang, C.Y. Tang, C.H. Lu, G.H. Kou & C.F. Lo. 2000. Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis. Aquat. Organ., 41(2): 91-104.

Witteveldt, J., C.C. Cifuentes, J.M. Vlak & M.C. van Hulten. 2004a. Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. J. Virol., 78(4): 2057-2061.

Witteveldt, J., J.M. Vlak & M.C.W.V. Hulten. 2004b. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish Shellfish Immunol., 16: 571-579.

Wu, W., L. Wang & X. Zhang. 2005. Identification of white spot syndrome virus (WSSV) envelope proteins involved in shrimp infection. Virology, 332(2): 578-583.