

Advances in biology through agronomy, aquaculture, coastal and environmental sciences

Leandris Argentel Martínez
Ofelda Peñuelas Rubio

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**Leandris Argentel Martínez
Ofelda Peñuelas Rubio**

**Advances in biology through
agronomy, aquaculture, coastal and
environmental sciences**



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Prologue

Advances in biology through agronomy, aquaculture, coastal and environmental sciences is an electronic book, edited by Pantanal Editora, based on the compilation of research papers where the authors of the different chapters have used highly current scientific methodologies and research equipment.

The biological sciences as the main object of research in agriculture, aquaculture, coastal and environmental sciences generate every day an understanding of knowledge that allows raising the scientific level of society as part of universal access to knowledge.

This book mainly addresses issues related to the use of plants extracts as sustainable alternatives for biocontrol of pests and bacterial diseases. It also brings together information on viruses and other diseases in aquatic organisms. In addition, studies of mangroves structure and their contribution to carbon sinks in experimental sites in northwestern Mexico are presented. Finally, an analysis on educational strategies for environmental education based on plant biology is carried out.

Editors appreciate the participation of the authors who have come from higher education institutions and research centers of great scientific prestige in Mexico. The majority of them are members of the National Research System of CONACyT, Mexico.

The authors


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Intracellular Holosporaceae pathogen intensifies the susceptibility of shrimp (*Litopenaeus vannamei*) to the white spot syndrome virus (WSSV): a preliminary approach

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ABSTRACT

The susceptibility to white spot syndrome virus (WSSV) in the white shrimp *Litopenaeus vannamei* previously infected with an intracellular Holosporaceae pathogen known as necrotizing hepatopancreatitis bacteria (NHPB) was evaluated. Coinfected shrimp mortality was recorded over 20 days and compared with those infected with WSSV or NHPB alone. NHPB-WSSV-co-infected shrimp reached 100% mortality after 15 days of challenge, whereas shrimp infected with WSSV alone reached the same mortality level after 19 days. Control shrimp and those infected only with NHPB did not show mortality during the trial. These results suggest that NHPB may not be an aggressive pathogen causing mortality but can trigger the susceptibility of shrimp to WSSV.

Keywords: Coinfection; White spot syndrome virus; Necrotizing hepatopancreatitis bacterium; Holosporaceae

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INTRODUCTION

Infectious diseases are still one of the major challenges for shrimp aquaculture in the 2020 decade (Gallaga-Maldonado et al., 2020; Patil et al., 2021). Stressful conditions make aquatic organisms prone to diseases; in this regard, various biotic and abiotic factors may favor opportunistic infections.

In shrimp, intracellular pathogens are considered opportunistic agents that may go unnoticed for long periods unless molecular detection tests are used (Vincent; Lotz, 2005). The necrotizing hepatopancreatitis bacterium (NHPB) is perhaps the most important intracellular pathogen for penaeid shrimp. This bacterium was formerly considered a rickettsia-like pathogen due to its intracellular nature (Gollas-Galván et al., 2014); however, recent evidence derived from multilocus sequence analysis demonstrated that the intracellular agent belonged to the Holosporaceae family, similar to some species considered pathogens of other arthropods (Leyva et al., 2018).

The NHPB resides and multiplies exclusively in the tubular epithelial cells of penaeids but commonly does not cause high mortality rates except in extreme cases involving highly stressful conditions, and the first dead organisms could be detected as long as three weeks after the initial infection (Ávila-Villa et al., 2012; Figueroa-Pizano et al., 2014). Moreover, the immune system is highly activated weeks postinfection; for instance, Figueroa-Pizano et al. (2014) reported the participation of the proPO system and the clotting reaction against NHPB, mostly on days ¹² and 18 postinfection. The evidence suggests a prolonged latent phase of this pathogen that could go unnoticed in nonacute phases. However, this pathogen has physiological implications in shrimp, including immunodepression, leading to susceptibility to other pathogens or coinfections.

Coinfection involves the simultaneous infection of a host by multiple pathogen species; in this regard, the primary infection reduces the immune resistance, leading to second or more concurrent infections and aggravating the scenario for shrimp health. White spot syndrome virus, the most severe virus for white shrimp, is perhaps the most common in coinfections with other viruses or bacteria (Macías-Rodríguez et al., 2014; Pang et al., 2019; Rubio-Castro et al., 2016). In this sense, the problem with the late detection of NHPB due to its intracellular nature and its prolonged latent phase, as well as the greater susceptibility of shrimp to be coinfecting with WSSV, is a latent risk; however, to date, there is no evidence of concurrent infections between these two pathogens, and one of the reasons is that NHPB is still an unculturable bacterium. Therefore, this work evaluated the survival of shrimp (*L. vannamei*) previously infected with necrotizing hepatopancreatitis bacteria (NHPB) to white spot syndrome virus (WSSV).

MATERIALS AND METHODS

Animals.

The experimental approach was carried out with healthy adult shrimp (*Litopenaeus vannamei*) weighing 25-30 g, acclimated to optimal conditions in artificial seawater with salinity at 30 PSU,

temperature 28 °C, constant aeration, photoperiod 12:12, and 10% water change per day. Shrimp were fed *ad libitum* twice a day with commercial food pellets (Camaronina, Purina™) containing 35% crude protein. Aquariums were cleaned daily to remove feces, food debris, and dead organisms.

Preparation of NHPB inoculum.

Because the Holosporaceae bacterium NHPB is unculturable, the inoculum consisted of a homogenate of hepatopancreatic tissue from shrimp positive for NHPB and negative for WSSV, IHNV, TSV, and *Vibrio parahaemolyticus* after employing molecular detection through PCR. The dissected hepatopancreas was homogenized and suspended in 0.9% saline solution with NaCl in pyrogen-free water.

Genomic DNA was extracted from the hepatopancreas using the Gene Clean commercial kit (BioQ® Inc., 1101-601) following the manufacturer's specifications. The presence of NHPB was determined by PCR through amplification of the ribosomal subunit 16S gene-specific for NHPB using the primers forward: 5' - CGT TGG AGG TTC GTC CTT CAG T - 3' and reverse: 5' - GCC ATG AGG ACC TGA CAT CAT C - 3' (Nunan et al., 2008). Reactions were performed in 25 µL using a gradient Px2 Thermal Cycler (Thermo, USA) under the following conditions: Step 1: 95 °C/2 min; Step 2: 25 cycles of 95 °C/30 sec, 60 °C/30 sec and 70 °C/30 sec; Step 3: 60 °C/1 min and 72 °C in 2 min. The PCR products were visualized in agarose gel (1.2%) under UV light using the KODAK Imaging System 4.0. Samples positive for NHP-B were homogenized at 4 °C, and 300 µL of glycerol was added and stored at -20 °C until use as inoculum.

Preparation of viral inoculum

Virions of the white spot syndrome virus (WSSV) were obtained from the muscle of experimentally infected shrimp, according to Gracia-Valenzuela et al. (2009). One gram of tissue was diluted in 4 mL of 150 mM nuclease-free sterile saline solution, homogenized, centrifuged at 3000 x g, and filtered through 0.45 µm (MF-Millipore Membrane Filters, Millipore™), followed by 0.22 µm. The filtered solution was placed in a 100 kDa cutoff microfilter (Amicon Ultra15, Millipore™) and centrifuged at 1000 × g for 30 min at 4 °C. The microfilter material retained and containing WSSV virions was recovered and placed in 1 mL of 150 mM saline, sterile, and nuclease-free solution. The filtered suspension was stored without additional buffer at -20 °C and used as viral inoculum. The presence of WSSV in the filtered suspension was confirmed by PCR based on Gracia-Valenzuela et al. (2009).

The quantification of WSSV virions was estimated by following the instructions of the IQREAL kit in a 15 µL volume reaction. The quantification was determined by calibration curve construction using 10¹, 10², 10³, 10⁴ and 10⁵ WSSV copy/µL solutions included in the i-screen kit (GeneReach™ Biotechnol Corp., Taiwan) based on the method of Nunan et al. (2004).

Experimental infections

Eighty shrimp were infected with NHPB by oral supplementation with infective inoculum. Forty other shrimp were inoculated with NHPB-free hepatopancreas. NHPB was detected in the feces and hepatopancreas of randomly sampled shrimp to confirm the infection, while no detection was expected for the control shrimp. Thirty NHPB-positive shrimp were selected for the NHPB group, 30 for the NHPB-WSSV coinfection group, and 30 of the NHPB-negative shrimp for the NHPB control.

Infection with WSSV was performed via intramuscular injection to the 30 shrimp of the NHPB-WSSV coinfection group and an additional 30 of the WSSV group. A WSSV-free control group of shrimp was also injected with saline solution. Each group was stocked in a 60 L aquarium. Finally, the survival was evaluated for each group, and WSSV and NHPB were monitored in each dead and living shrimp at the end of the trial.

RESULTS AND DISCUSSION

Except for the control group, all shrimp were positive for their corresponding infection and coinfection (Figure 1). No mortalities were detected for any of the control groups (NHPB-free and WSSV-free) or the NHPB-infected group; however, mortalities were recorded in the WSSV-NHPB coinfection and WSSV infection groups.

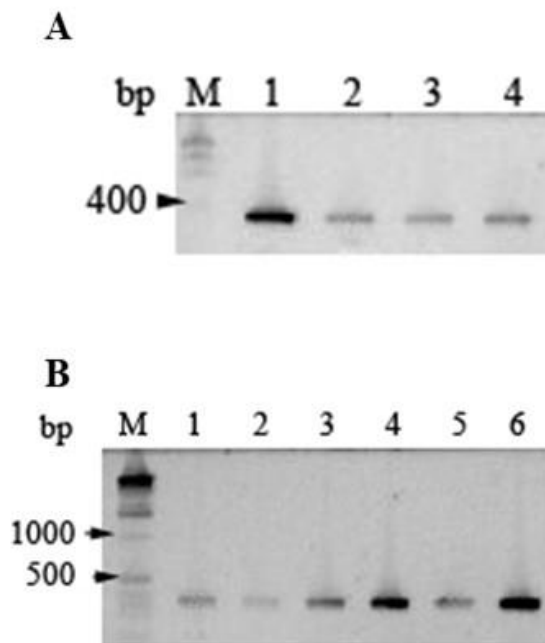


Figure 1. A. Electrophoresis gel with PCR products for the detection of NHP-B in shrimp. Lane M, molecular mass marker. Lane 1, control positive of NHPB. Lanes 2 to 4, hepatopancreas of shrimp samples fed with infective NHPB inoculum. **B.** Electrophoresis gel with PCR products to detect WSSV in shrimp. Lane M, molecular mass marker. Lanes 1 to 5, shrimp inoculated with WSSV. Lane 7, positive control of WSSV. bp= DNA base pairs.

Survival started to decline on the 9th day of the trial; however, mortality occurrence was more pronounced in coinfecting shrimp, reaching 100% on the 15th day, whereas 100% mortality was detected on the 19th day in shrimp infected with WSSV alone (Figure 2).

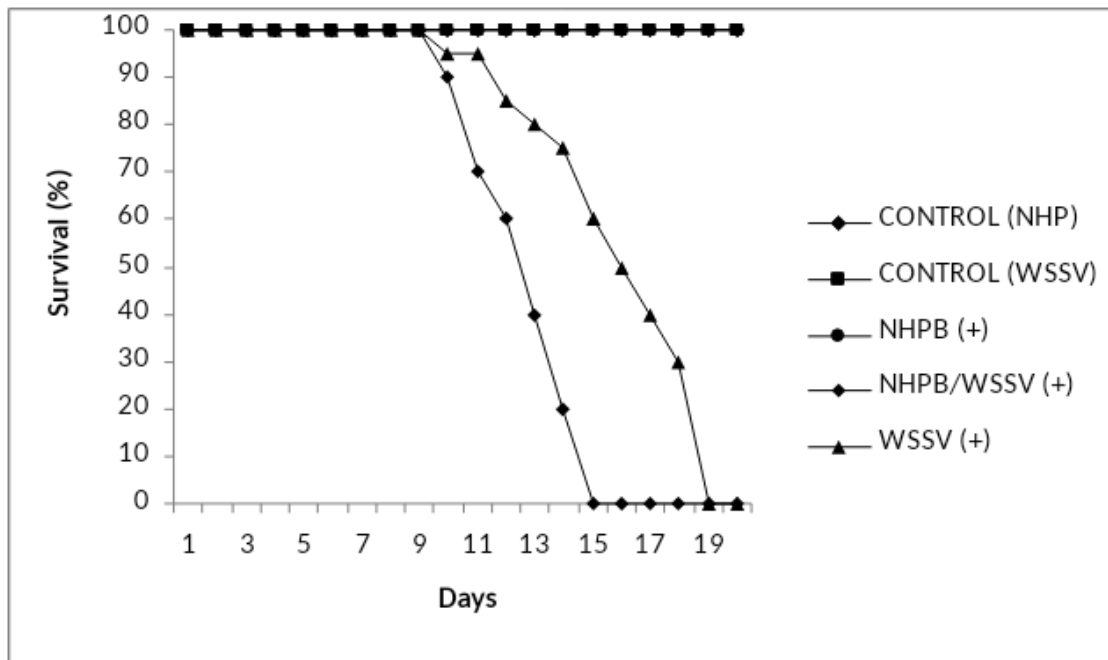


Figure 2. Mortality curves for experimentally infected shrimp with NHPB, WSSV, and NHPB-WSSV. Two controls free of NHPB and WSSV were also considered.

Coinfection of individual hosts by multiple species is a typical pattern in nature and could be even more common than usual in aquaculture given the high stocking densities favoring all kinds of pathogen transmissions.

The results suggest that although NHPB may not cause mortality during a considerable period, this intracellular pathogen can accentuate the mortality caused by WSSV. Previous evidence has documented hepatopancreatic malfunction when infected with NHPB, leading to physiological and behavioral consequences derived from tissue necrosis (Figueroa-Pizano et al., 2014). In this regard, WSSV infection alone was severe, leading to 100% mortality in 19 days; however, the presence of NHPB accelerated the process.

Taking this evidence together with previous reports (Ávila-Villa et al., 2012; Martínez-Córdova et al., 2016), we can conclude that NHPB infections may go unnoticed for long periods but pose a risk of causing severe mortalities if another viral pathogen establishes a clinical coinfection. Although no mortalities were observed in the NHPB group, the infection alone can cause severe mortalities after long periods if no antibiotics are used (Martínez-Córdova et al., 2016), causing chronic detriment that can lead to disastrous results for a farm. Whether this preliminary approach evaluating the shrimp survival

response is evaluated for the first time suggests the severity of NHPB-WSSV coinfection and highlights the relevance of studying coinfections that constitute a realistic scenario in aquaculture.

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