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# Susceptibility of the wild southern brown shrimp (*Farfantepenaeus subtilis*) to infectious hypodermal and hematopoietic necrosis (IHHN) and infectious myonecrosis (IMN)

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ABSTRACT

The highest losses in shrimp farming have been due to viral infections. Among them, the virus of infectious hypodermal and hematopoietic necrosis (IHHNV) affects wild and farmed shrimp at several development stages. The disease is considered to have low impact on cultures of *Litopenaeus vannamei* where it induces a chronic disorder known as the Runt-Deformity Syndrome (RDS). In contrast, the virus of infectious myonecrosis (IMNV) has been associated with mortality rates of up to 60% in shrimp ponds and may affect post-larvae, juveniles and adults. Very little information is available on the impact of IHHN and IMN on wild shrimp from Brazilian coastal waters. The objective of the present study was to assess the susceptibility of native *Farfantepenaeus subtilis* to these infectious diseases. 300 healthy juvenile shrimp, with an average weight of 2.56 g (±0.44 g), were accommodated in individual tanks under physical and chemical conditions similar to those of shrimp ponds. The animals were distributed in two groups, *A* ($n=50$) and *B* ($n=100$), and challenged *per os* over a period of 3 days with IHHNV and IMNV respectively. Control groups received a similar but virus-free diet. The animals were monitored daily for clinical signs or behavior suggestive of viral infection. Biological samples were taken every 5 days, over a period of 30 days, for molecular and histological analyses in order to determine the susceptibility of the species to the viruses. 10% of the wild *F. subtilis* studied were PCR-positive in both cases. The results show that the native *F. subtilis* is susceptible both to IHHNV and IMNV.



**Contents**



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## 1. Introduction

Viral diseases are the main biological factor limiting and impairing shrimp farming worldwide, causing extremely high economic losses (Lightner, 2005). The etiological agent causing infectious hypodermal and hematopoietic necrosis (IHHNV) is an icosahedral, non-enveloped parvovirus containing single-stranded DNA. IHHNV is the smallest (*c.* 22 nm) of the viruses affecting penaeid shrimp (Bonami et al., 1990). Discovered in the Americas in 1981, IHHNV has been responsible for acute disease and staggering mortality rates (over 90%) in blue shrimp (*Litopenaeus stylirostris*) farmed in Hawaii (Lightner et al., 1983). IHHNV often affects wild and farmed juvenile and sub-adult shrimp (Lightner et al., 1983), and may be transmitted either vertically or horizontally (Motte et al., 2003). IHHNV is considered a low-impact virus in *Litopenaeus vannamei* culture where it induces a chronic disorder known as the Runt-Deformity Syndrome (RDS). The virus may infect ectodermal, mesodermal and, more rarely, endodermal tissues (Bell and Lightner, 1984).

The etiological agent inducing infectious myonecrosis (IMNV) is a horizontally transmitted icosahedral, non-enveloped totivirus containing double-stranded RNA, with *c.* 40 nm (Poulos et al., 2006; Andrade et al., 2007). When shrimp are affected by IMN their abdominal muscles lose transparency, starting around the second or third segment and extending towards the telson. As the disease advances, the tail acquires a milky or even pink hue and may eventually putrefy (Nunes et al., 2004). Losses on shrimp farms due to IMN are often considerable, with mortality rates around 40%. However, IMNV takes relatively longer to induce death because the target tissues, the abdominal muscles, can sustain more damage than most other shrimp tissues (Tang et al., 2005). In 2004 and 2005, Brazilian shrimp farming suffered a substantial decrease in productivity, some of which caused by viral diseases, especially IMN (Rodrigues, 2005). The first regional report of IMN came from a shrimp farm in Piauí State in 2003, after which the disease spread to most of Northeastern Brazil (Lightner et al., 2004). More recently, IMN has also been observed in *L. vannamei* from farms in Indonesia (Senapin et al., 2007).

Domestication of wild shrimp species and their successful introduction into the shrimp farming business require in-depth knowledge of the biology of the species, especially with regard to reproduction, nutrition and health (Brock and Main, 1994). However, very little information is available on the impact of IHHN and IMN on wild shrimp from Brazilian coastal waters. The brown shrimp *Farfantepenaeus subtilis* is native to Brazilian waters, and has long been considered a species with a high potential for aquaculture (Nunes et al. 1997). Therefore, the objective of the present study was to assess the susceptibility of native *F. subtilis* to IHHNV and IMNV.

## 2. Materials and methods

### 2.1. Viral challenge

The study included 300 juvenile *F. subtilis* weighing 2.56 ± 0.44 g, of which 50 were challenged with IHHNV (Group A), 100 were challenged with IMNV (Group B) and 150 were assigned as respective controls. The animals were collected in the Estuary of the Pacoti River (Ceará, Brazil), transported to and identified at the Marine Sciences Institute (Labomar/UFC) according to Farfante and Kensley (1997) and acclimated during 7 days in tanks with filtered seawater (salinity 30 ups, pH 7.8 ± 0.5, temperature 27.0 ± 2.0 °C, dissolved oxygen 6.4 ± 0.2 mg/L). Before viral challenge, pleopod (Group A + controls) and gill (Group B + controls) samples were collected from all animals for Polymerase Chain Reaction (PCR) analysis, to make sure that all animals were free of IHHNV and IMNV. The animals were then placed in individual 5-L tanks with chlorinated sea water, biological filtration and continuous aeration.

During the challenge phase, the animals were fed contaminated *L. vannamei* tissue once a day for three consecutive days, in place of regular artificial feed, in an amount equivalent to 3.5% of the body weight. The 50 juveniles in Group A were fed with IHHNV-infected tissue and the 100 juveniles in Group B were fed with IMNV-infected tissue. Control groups received a similar but virus-free diet. The animals were monitored daily for 30 days to check for clinical signs or behavior suggestive these viral infections.

A random sample of 10 animals was taken from Group A and from the respective control group on days 5, 10, 15, 20 and 30 following exposure, totalizing 100 sampled individuals. Samples taken from Group B and its control followed the same procedure but were twice the size, totalizing 200 animals. Pleopods and gills were used for the molecular analysis of IHHNV and IMNV, respectively. After retrieving tissues for analysis, the animals were labeled and preserved in Davidson's AFA fixative for 24 h for subsequent histological analyses.

### 2.2. Nucleic acid extraction

The extraction of total DNA from pleopod for IHHNV detection was performed using DNazol (Invitrogen®, Life Technologies), following the manufacturer recommendations. The extraction of total RNA from gill tissue for IMNV detection was done with the IQ2000 kit (Farming IntelliGene Technology Corporation, Taiwan) and followed the protocol recommended by the manufacturer.

### 2.3. PCR amplification for IHHNV

PCR testing for IHHNV followed the protocols recommended by the World Organisation for Animal Health (OIE, 2006). The reaction contained 0.4 µm of each primer 389F (5′-CGG-AAC-ACA-ACC-CGA-CTT-TA-3′) and 389R (5′-GGC-CAA-GAC-CAA-AAT-ACG-AA-3′) (GenBank accession no. AF218266), 1× PCR buffer (Invitrogen®), 200 µM of dATP, dTTP, dGTP and dCTP each, 2 mM $MgCl_2$, 0.04 U/µL Taq (Invitrogen®), and 1 µL of DNA template in addition to ultrapure water adjusted to a total volume of 25 µL. PCR was performed using a thermo cycler (Techne-Flexigene®), starting with a denaturation cycle at 95 °C/5 min, followed by 35 cycles of 95 °C/30 s (denaturation), 55 °C/30 s (primer annealing) and 72 °C/1 min (DNA extension), followed by a final extension at 72 °C/7 min.

### 2.4. RT-PCR amplification for IMNV

RT-PCR for IMNV detection was performed according to the guidelines provided in the IQ2000 IMNV Detection and Prevention System kit (Farming IntelliGene Technology Corporation, Taiwan).

### 2.5. Detection of PCR and RT-PCR products

Ten microlitres of the PCR or RT-PCR products were analyzed by electrophoresis in 2% agarose gels containing 1× Tris-Acetate-EDTA buffer and stained with ethidium bromide. Electrophoresis was run in a FisherBiotech® tray (Electrophoresis Systems FB-SBR-1316) for 60 min with a 60-W continuous current. The gel was visualized with a Spectroline® UV transluminator and recorded with a Kodak EDAS 290 system.

### 2.6. Histology

The shrimp preserved in Davidson's AFA fixative were sectioned into three parts (cephalothorax and third and sixth abdominal segments) which were rinsed in tap water and preserved in 70% alcohol. The classic histology method was employed, including dehydration in increasing alcohol concentration, clarification in xylene and paraffin embedding at 60 °C. Samples were cut in 4-µm

**Table 1**
Distribution of challenged specimens of wild *F. subtilis* testing positive on PCR for IHHNV and on RT-PCR for IMNV, over 30 days of experiment.

| Days of experiment | Group A | | | | Group B | | | |
|---|---|---|---|---|---|---|---|---|
| | Number of individuals | Dead | Sampled | +IHHNV (dead/sampled) | Number of individuals | Dead | Sampled | +IMNV (dead/sampled) |
| 0 day | 50 | – | 0 | 0 (− / −) | 100 | – | – | 0 (− / −) |
| 5th day | 40 | 3 | 7 | 3 (1 / 2) | 80 | 2 | 18 | 2 (1 / 1) |
| 10th day | 30 | 3 | 7 | 1 ( 1 / 0) | 60 | 2 | 18 | 1 (1 / 0) |
| 15th day | 20 | 0 | 10 | 1 ( 0 / 1) | 40 | 0 | 20 | 2 (0 / 2) |
| 20th day | 10 | 0 | 10 | 0 (0 / 0) | 20 | 1 | 19 | 1 (0 / 1) |
| 30th day | 0 | 0 | 10 | 0 (0 / 0) | 0 | 0 | 20 | 4 (0 / 4) |
| | Total | 6 | 44 | 5 (2 / 3) | Total | 5 | 95 | 10 (2 / 8) |

sections for staining by hematoxylin-eosin for light microscopy analysis (Bell and Lightner, 1988).

## 3. Results and discussion

Viral challenge with IHHNV and IMNV resulted in successful infection of wild *F. subtilis per os* (Table 1). 10% of the challenged animals were positive after molecular diagnosis involving PCR, in both cases. However, only IMNV-infected specimens showed signs of tissue alteration (Fig. 1).

### 3.1. Mortality rate and gross signs of IHHNV and IMNV

Six animals from Group A, challenged with IHHNV, died during the first two weeks, but only two of them were PCR-positive (Table 1). None of the clinical signs of IHHNV infection described for *L. vannamei* by Kalagayan et al. (1991), including bent rostrum, curly antennae, blistered carapace, cuticle deformity and stunting, were observed for *F. subtilis* during our experiment. Experimentally infected *L. vannamei* have shown run-deformity only after 30 days infection (Singhapan et al. 2004). According to Lightner (2003), economic and production impacts of IHHNV infection in *L. vannamei* are mainly due to reduced and irregular growth and small size shrimp at harvest, and not to high mortalities.

Only five out of 100 animals died in Group B, challenged with IMNV, two of which in the first week, two in the second week and one in the fourth week. However, only two of these tested positive for the virus on RT-PCR (Table 1). None of the known clinical signs of IMNV infection, such as opacity of the abdominal muscles (Nunes et al., 2004), were observed in our experiment. Since no other clinical sign was observed, the cause of death in Group A as well as in Group B is uncertain, but may include experimental stress.

### 3.2. Histopathological analysis

Tissue stained by H&E did not exhibit the presence of pathognomonic Cowdry A type-inclusions characteristic of IHHNV infection (Bell and Lightner, 1984), possibly because of the short infection time. However, Tang and Lightner (2001) found that histological grading does not always correlate with the level of IHHNV infection. Among animals challenged with IMNV, 10% presented low levels of hemocyte infiltration and a light coagulation (Fig. 1). All animals that presented histological signs of tissue alteration here tested positive for molecular diagnosis of IMNV.

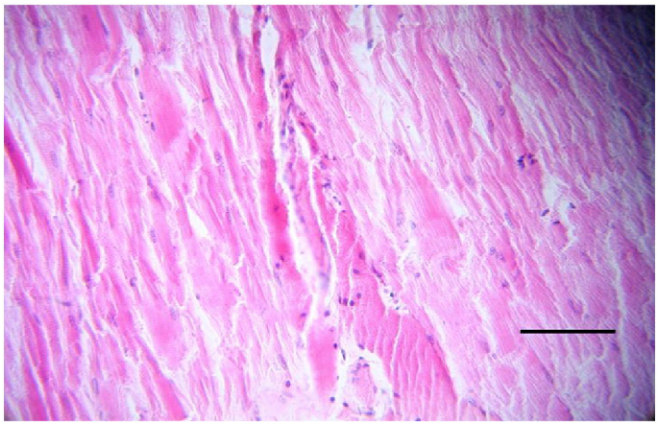

**Fig.1.** Histopathological aspect of wild *F. subtilis* challenged with IMNV, stained by hematoxylin and eosin (H & E). Bar: 50 µm.

### 3.3. Viral detection by PCR/RT-PCR

Although shrimps in Group A were asymptomatic to IHHNV and no histopathological evidence of infection was found, five out of 50 animals in Group A (10%) were susceptible to IHHNV, as evidenced by PCR. Among these, three were registered on the 5th day, one on the 10th day and one on the 15th day following exposure. No infection was observed after this period (Table 1). In *L. vannamei* IHHNV DNA has been detected in the pleopods 21 and 30 days post injection of inoculums prepared from chicken or seagull feces (Vanpatten et al., 2004).

Ten out of 100 animals in Group B (10%) were positive to IMNV by RT-PCR, producing a 284 pb amplicon compatible with positive controls demonstrating that *Farfantepenaeus subtilis* is susceptible to IMNV. Among the samples testing positive for IMNV, two were registered on the 5th day, one on the 10th day, two on the 15th day, one on the 20th day and four on the 30th day following exposure (Table 1). IMNV infecting cultured *L. vannamei* in Brazil causes a slowly progressive disease with cumulative mortalities of up to 70% (Poulos and Lightner, 2006). Nunes et al. (2004) reported that mortality of *L. vannamei* from IMN seems to be higher at the end of the shrimp culture cycle. IMNV infections have been reported to present a relatively long delay in mortalities, possibly because skeletal muscle, the primary target tissue of IMNV infection, can withstand more damage than the other tissues before survival is threatened (Tang et al., 2005).

The present study shows that native wild specimens of *F. subtilis* collected in the Estuary of the Pacoti River on the coast of Northeastern Brazil are susceptible to IHHN and IMN. The mortality rates and overall impact of these infections in *F. subtilis* are yet to be approached. Earlier studies found the three most frequently farmed penaeid species (*L. vanamei*, *L. stylirostris* and *P. monodon*) to be susceptible to IMN, although the infection reported was not as virulent as that caused by TSV, YHV and WSSV (Tang et al., 2005). Even though *F. subtilis* farming has yet to achieve industrial level in the region, it seems that the Southern brown shrimp could represent a native alternative for sustainable shrimp farming in Northeastern Brazil.

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