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Short communication

Infectious myonecrosis virus and white spot syndrome virus co-infection in Pacific white shrimp (*Litopenaeus vannamei*) farmed in Brazil

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ABSTRACT

Over the past few years, infectious myonecrosis virus (IMNV) and white spot syndrome virus (WSSV) have caused substantial economic losses to Brazilian shrimp farmers. Initially reported from different geographical locations within the country, these pathogens are becoming increasingly prevalent throughout Brazil. In the present study, 30 diseased shrimp collected from a farm in Northeastern Brazil were screened for viral infection. The shrimp were submitted to histological analysis and quantitative real-time PCR (qPCR). New probes and primers were designed for the qPCR assays, and IMNV detection was based on a modified protocol. Clinical signs of both white spot syndrome and infectious myonecrosis were observed in histological preparations from four of the sampled shrimp. The presence of both IMNV and WSSV was confirmed by qPCR in twelve specimens. Viral co-infection has previously been reported in farmed shrimp. However, this is the first Brazilian report of co-infection with IMNV and WSSV in farmed *Litopenaeus vannamei*.

1. Introduction

Diseases caused by infectious myonecrosis virus (IMNV) and white spot syndrome virus (WSSV) can cause large economic losses to the shrimp farming industry (Lightner, 2011). Over the past few years, both viruses have had a strong impact on shrimp farming in Brazil. IMNV was first identified in specimens of Pacific white shrimp (*Litopenaeus vannamei*) collected in 2002 from farms in the state of Piauí (Brazil), but soon spread to other states in the region (Rio Grande do Norte, Pernambuco, Paraíba, Maranhão and Ceará) (Andrade et al., 2007). WSSV was first detected in Brazil in 2005 and was responsible for the collapse of the shrimp farming industry in Southern Brazil (Muller et al., 2010; OIE, 2005a, 2005b).

IMNV infection causes characteristic necrosis in abdominal striated muscles, cephalothorax muscles and appendages, and disease in the lymphoid organ and gill tissue (Lightner et al., 2004). Muscle lesions manifest as opaque patches, especially in the distal abdomen and tail regions, which evolve from milky white during the early stages to a red tinge and boiled appearance in advanced stages. IMNV is the only totivirid known to infect invertebrates (Poulos et al., 2006). WSSV is currently the most devastating pathogen in farmed shrimp worldwide, causing 90–100% mortality in farmed *L. vannamei* within 3–10 days from the emergence of clinical signs (Sánchez-Martínez et al., 2007). White spot disease has been named after 0.5 to 2 mm spots that may be present within the exoskeleton. WSSV is extremely virulent and may target a number of ectodermal and mesodermal tissues in a wide range of hosts (Lightner, 1996).

Earlier field reports have suggested the presence of clinical signs of both viruses in shrimp farmed in Northeastern Brazil. The purpose of the present study was to confirm the occurrence of IMNV/WSSV co-infection in Brazilian samples of farmed Pacific white shrimp based on histological analysis and quantitative real-time PCR (qPCR).

2. Materials and methods

2.1. Sample collection

In February 2007, thirty specimens of Pacific white shrimp (*L. vannamei*) weighing 10 ± 2 g on the average were collected from a commercial grow-out pond in Ceará (Northeastern Brazil) and transported alive to Centro de Diagnóstico de Enfermidades de Organismos Aquáticos (CEDECAM/UFC) in Fortaleza. The 4th pair of pleopods was removed and stored in 95% ethanol for qPCR analysis. Each shrimp was then fixed for histology by injection of 30 mL

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Davidson's AFA solution (11.5% acetic acid, 22% formalin, 33% ethanol) and immersion in the same solution (Bell and Lightner, 1988).

2.2. Histopathology

Four-micrometer sections were prepared and submitted to routine staining with hematoxylin–eosin (Bell and Lightner, 1988). The histological slides were examined under light microscopy against standard published references of crustacean pathologies (Lightner, 1996; Pereira et al., 2008).

2.3. Total DNA/RNA preparation and first-strand cDNA synthesis

Total DNA was purified from a ~20 mg sample of pleopod muscle tissue using a standard protocol of proteinase K digestion followed by phenol:chloroform:isoamyl alcohol extraction (Sambrook et al., 1989). Total RNA was extracted from a ~50 mg pleopod muscle tissue sample using Trizol® (Invitrogen) according to the manufacturer's instructions, followed by DNase I digestion at 37 °C for 30 min. Prior to cDNA synthesis, the extracted RNA was boiled at 100 °C for 5 min to denature dsRNA and then placed on ice. cDNA was synthesized with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) in a 20-μL reaction volume containing 2.5 μg total RNA and 1 μL 500 mg mL^{-1} random hexamers and reverse transcriptase, as recommended by the manufacturer. DNA and RNA concentrations were determined by fluorimetric quantification using Qubit™ quantification assay kits (Invitrogen) for dsDNA and RNA, respectively.

2.4. qPCR

Construction and cloning of plasmid positive controls was accomplished by the use of 908 bp IMNV (GenBank accession no. AY570982.1, nt 4274–5181) and 1447 bp WSSV (GenBank accession no. AF332093.1, nt 224261–225708) amplicons obtained by RT-PCR and semi-quantitative PCR, respectively, of shrimp samples known to be contaminated. The amplicons were purified with the illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) and then cloned into pCR® 2.1-TOPO vector TOPO TA Cloning® (Invitrogen). The positive clones, designated as pTOPO-IMNV and pTOPO-WSSV, were purified with the illustra plasmidPrep Mini Spin Kit (GE Healthcare) and confirmed by DNA sequencing (data not shown).

The qPCR primers and TaqMan probes for IMNV and WSSV detection and quantification were designed using the software Primer Express v. 2.0 (Applied Biosystems) (Table 1). TaqMan probes were labeled at the 5′ end with 5-carboxyfluorescein (FAM) and at the 3′ end with N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) (Invitrogen).

All qPCR reactions for detection of IMNV and WSSV were performed in a final volume of 10 μL containing 5 μL Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.3 μL (10 μM) of each primer, 0.15 μL (10 μM) TaqMan probe, 0.2 μL (50×) Rox reference dye, 1 μL (5–15 ng) DNA (for WSSV detection) or 1 μL (10–25 ng) cDNA (for IMNV detection) and 3.05 μL ddH_2O. Negative controls (containing all reagents except target DNA or cDNA) and 10-fold serial dilutions of the positive control (pTOPO-IMNV or pTOPO-WSSV), equivalent to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies of IMNV or WSSV, were included in each run for standard curve construction.

Detection and quantification of IMNV and WSSV were done with an ABI 7300 Real-time PCR system (Applied Biosystems). Amplification included the following steps: UDG incubation at 50 °C for 2 min, followed by activation of Platinum®Taq DNA polymerase at 95 °C for 2 min, and 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Data were collected during the 60 °C extension step. The viral amount of each sample was determined with the software SDS 1.3.1 (supplied with the ABI 7300 Real-time PCR system, Applied Biosystems). Each sample was tested in duplicate. The result was considered positive only if both replicates were positive.

Table 1
Sequences of primers and TaqMan probes used for the detection of infectious myonecrosis virus (IMNV) and white spot syndrome virus (WSSV).

Oligo ID	Sequence 5′–3′	Amplicon
IMNV(qPCR)Forward	ATGGACGCAATCACAAAGTTGT	58 bp
IMNV(qPCR)Reverse	TGGAACGGGCATTCATAAAAC	
TaqMan probe (IMNV)	*FAM*-TGCACGAAAGTCG-*TAMRA*	
WSSV(qPCR)Forward	GCCCTCTCGCCTTTGATTTC	61 bp
WSSV(qPCR)Reverse	TCGGCGTTCTTTTCTTCGA	
TaqMan probe (WSSV)	*FAM*-TTCTGTCAAAGGGAGATAC-*TAMRA*	

3. Results and discussion

3.1. Histopathological analysis

Sixteen of the 30 shrimp specimens (*L. vannamei*) examined displayed skeletal muscle damage in the abdomen and cephalothorax. Damage was characterized by focal hemocyte infiltration, coagulative necrosis, liquefactive necrosis and fibrosis (Fig. 1A, B and C). Nine shrimp displayed lymphoid organ hypertrophy and spheroids were evident (Fig. 1D). Six shrimp presented ectopic spheroids, especially near the antennal gland and in the skeletal muscle (Fig. 1E and F).

A frequent manifestation in IMNV-infected shrimp during the more advanced stages of the disease, ectopic spheroids are commonly detected in the hemocoel and connective tissue, especially in the lumen of the heart and around the antennal gland (Lightner et al., 2004). Lymphoid organ spheroids (LOS) appear to be a generic defense response to a variety of viruses. In *L. vannamei* infected with Taura syndrome virus (TSV), lymphoid organ hypertrophy has been directly associated with rapid spheroid morphogenesis and proliferation (Hasson et al., 1995).

Cowdry type A inclusion (CAI) bodies were observed in seven specimens, predominantly in the subcuticular epithelium of the stomach and occasionally in the gills (Fig. 1H). WSSV-infected shrimp usually show CAI bodies in the cells of the cuticular epithelium, connective tissues of the general body cuticle and appendages and, less frequently, in antennal gland epithelium, lymphoid organ membranes and hematopoietic tissues (Durand et al., 2002). As WSD develops, CAI bodies may initially appear eosinophilic, with marginalized chromatin, and thus be mistaken for CAI bodies induced by infectious hypodermal and hematopoietic virus (IHHNV). However, at more advanced stages, the inclusion bodies are characteristically larger and devoid of marginal halo. The inclusion bodies observed in our specimens were typical of advanced WSD (Fig. 1G). Semi-quantitative PCR assays were negative for IHHNV infection (data not shown).

3.2. qPCR assay

qPCR tests confirmed the presence of IMNV and WSSV in 27 and 13 specimens, respectively, of which twelve were qPCR-positive for both viruses. The standard curves generated from serial dilutions of the recombinant plasmids pTOPO-IMNV and pTOPO-WSSV presented a strong linear relation: [IMNV: $r^2 = 0.9964$; $y = (-3.142 \pm 0.05)x + (39.84 \pm 0.30)$] and [WSSV: $r^2 = 0.9956$; $y = (-3.158 \pm 0.08)x + (37.25 \pm 0.51)$] (Fig. 2). IMNV and WSSV copy number quantification in unknown samples was carried out by measuring Ct (threshold cycle) and using the standard curves to infer the initial copy number. The final results were expressed as the mean copy number of WSSV per microgram of DNA or mean copy number of IMNV per microgram of RNA (Table 2).

Dual or multiple infections have already been reported in cultured shrimp. However, cellular and molecular information on the pathogenicity, virulence, susceptibility and immune response of farmed shrimp affected with simultaneous infections remains scarce. IHHNV has been

shown to reduce WSSV pathogenicity in two species of penaeid shrimp pre-infected with WSSV, suggesting that infection by one agent may have a protective effect against other pathogens (Bonnichon et al., 2006; Tang et al., 2003). However, Yeh et al. (2009) observed no significant differences in the gene expression of the immune-related genes pro-phenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and peroxinectin (PE) in hemocytes from *L. vannamei* experimentally infected with both WSSV and IHHNV, as compared to shrimp infected solely with WSSV. In the present study we found no evidence of interference interactions between WSSV and IMNV, as reflected by viral copy numbers in co-infected shrimp (Table 2).

3.3. Histopathology and qPCR

The use of simultaneous methodologies for disease detection in shrimp is strongly recommended and diagnosis usually involves the combination of techniques based on molecular, histopathological, immunological and chemical principles (Andrade and Lightner, 2009; Dhar et al., 2001; Hossain et al., 2004; Phalitakul et al., 2006). In shrimp disease investigation, the sensitivity and specificity of the diagnostic methods employed are of crucial importance, especially for the detection of viral infection in asymptomatic individuals (Quéré et al., 2002).

In the present study, no histological evidence of IMN and WSD was found in, respectively, 11 and 6 of our qPCR-positive specimens (Table 2). For some diseases, the presence of asymptomatic shrimp in *L. vannamei*-based cropping systems may result from tolerance or resistance to the disease agent (Boada et al., 2008). However, tolerant shrimp can only be distinguished from resistant shrimp with specific experimental bioassays, especially in view of the need to determine the level of exposure to the pathogen investigated (Flegel, 2001). No relationship between viral copy numbers and histopathological severity of IMNV or WSSV was found in the present study. However, histological grading and disease severity are not necessarily correlated (Tang and Lightner, 2001).

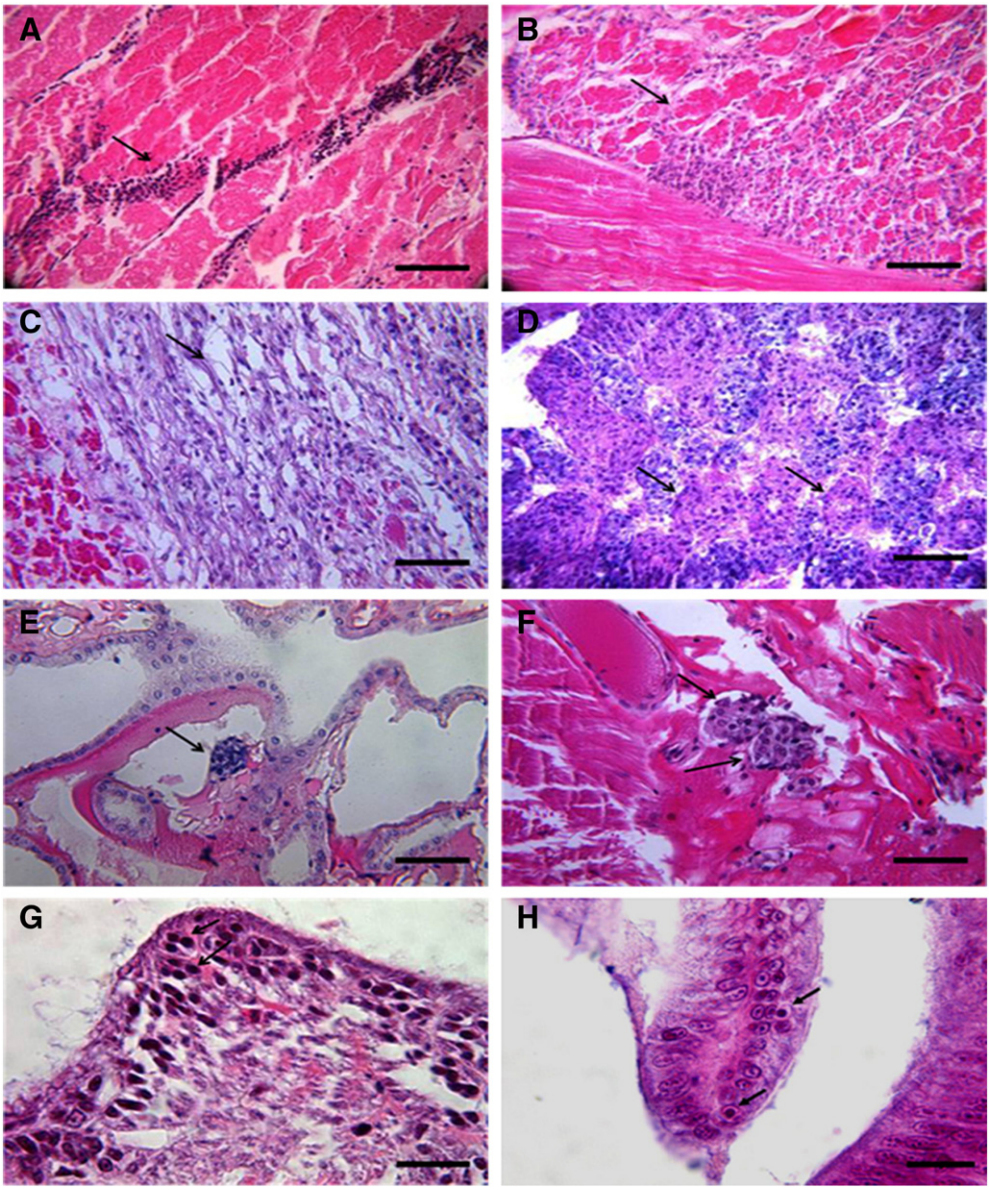

Fig. 1. Photomicrographs of tissue sections from *L. vannamei* examined for IMNV (A, B, C, D, E and F) and WSSV (G and H) lesions: (A) focal hemocytic infiltration in muscle tissue; (B) muscle coagulation necrosis accompanied by infiltration of hemocytes; (C) muscle liquefactive necrosis and fibrosis; (D) lymphoid organ spheroids; (E) ectopic lymphoid organ spheroids near the antennal gland (arrow); (F) ectopic lymphoid organ spheroids in muscle tissue (arrows). Scale bar = 50 μm (to A, B, C, D, E and F); (G) inclusion bodies associated to WSD in stomach subcuticular epithelium; (H) Cowdry type A inclusion (CAI) bodies in gills (arrow). Scale bar = 20 μm (to G and H).

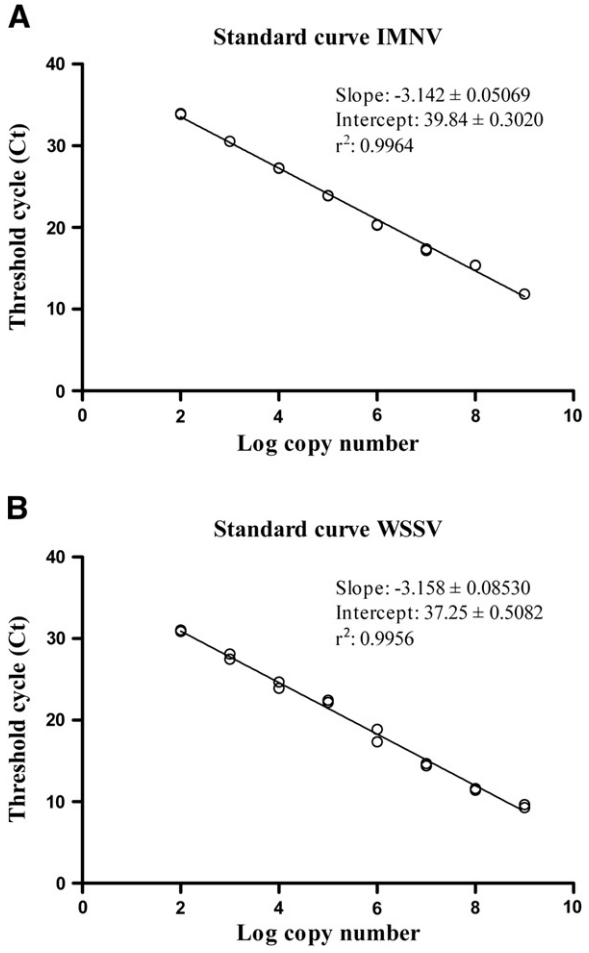

Fig. 2. Standard curves of IMNV and WSSV copy numbers versus threshold cycle (Ct) values. The resulting Ct values were plotted against the logarithm of their respective copy numbers.

Unlike WSD, muscle necrosis symptoms consistent with IMNV infection have been reported a number of times in Northeastern Brazil. It remains to be determined whether the low prevalence of WSD symptoms in our samples was due to disease tolerance/resistance (Boada et al., 2008), low pathogenicity of the specific viral strain involved (John et al., 2010) or specific environmental conditions (Granja et al., 2006). In addition, an interaction between WSSV and IMNV might conceivably affect the pathogenicity of either virus in *L. vannamei*. Genotyping of Brazilian WSSV isolates detected in shrimp from the states of Bahia and Santa Catarina have revealed differences between Brazilian isolates and isolates from other regions of the Americas (USA, Panama, Honduras, Mexico and Nicaragua) (Muller et al., 2010). However, the pathogenicity of the Brazilian WSSV isolates has yet to be assessed. Economic losses to the shrimp farming industry resulting from multiple infections are difficult to quantify and little is known about the importance of environment–pathogen and pathogen–pathogen interactions in such circumstances.

4. Conclusions

In the present work we were able to confirm the co-infection of *L. vannamei* by IMNV and WSSV, in a commercial farm from Ceará, Brazil, through histological and molecular analysis. To our knowledge, this study provides the first evidence of IMNV/WSSV co-infection in Pacific white shrimp farmed in Brazil.

Table 2
Quantitative analysis of IMNV and WSSV according to qPCR and histopathological status.

Shrimp no.	qPCR		Histology	
	(IMNV copies/μg RNA)	(WSSV copies/μg DNA)	IMNV	WSSV
1	8.59E + 06	3.22E + 05	Positive	Negative
2	5.81E + 05	Nd[a]	Positive	Negative
3	2.79E + 07	1.99E + 03	Positive	Positive
4	1.19E + 06	3.12E + 05	Positive	Negative
5	3.41E + 06	1.46E + 03	Positive	Positive
6	1.23E + 06	Nd	Positive	Negative
7	3.57E + 05	Nd	Positive	Negative
8	1.69E + 08	Nd	Positive	Negative
9	6.79E + 04	4.37E + 05	Positive	Positive
10	9.44E + 07	Nd	Positive	Negative
11	3.53E + 05	7.53E + 03	Negative	Negative
12	1.70E + 06	Nd	Negative	Negative
13	1.71E + 05	6.32E + 03	Negative	Positive
14	Nd	4.30E + 05	Negative	Negative
15	1.79E + 04	Nd	Negative	Negative
16	Nd	Nd	Negative	Negative
17	1.80E + 05	2.77E + 05	Negative	Positive
18	1.98E + 06	Nd	Negative	Negative
19	3.48E + 08	Nd	Positive	Negative
20	Nd	Nd	Negative	Negative
21	1.61E + 05	3.67E + 05	Positive	Negative
22	1.88E + 06	Nd	Positive	Negative
23	1.02E + 05	Nd	Positive	Negative
24	1.84E + 04	2.69E + 04	Negative	Negative
25	1.20E + 09	Nd	Positive	Negative
26	6.27E + 06	Nd	Negative	Negative
27	1.80E + 05	Nd	Negative	Negative
28	3.86E + 05	Nd	Negative	Negative
29	4.71E + 06	5.32E + 04	Positive	Positive
30	6.51E + 05	4.17E + 05	Negative	Positive

[a] Nd: not detected.

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