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Purification of infectious myonecrosis virus (IMNV) in species of marine shrimp *Litopenaeus vannamei* in the State of Ceará

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A B S T R A C T

In Brazil, shrimp farming has been developed most intensely in the Northeast Region. Recently, however, exporters have become concerned over the appearance of Infectious Myonecrosis (IMN), the etiological agent of which is a virus called Infectious Myonecrosis Virus (IMNV). Although IMNV has been characterized extensively, purification methods are complicated to reproduce and very expensive. The objective of this study was to purify the IMNV virus using an easy reproductive method and to produce anti-IMNV antibodies to be used in diagnostic methods. Shrimp samples showing symptoms of IMN obtained from two aquaculture farms in Ceará were used for this purpose.

IMNV-positive shrimps were macerated in phosphate buffer, pH 7.5, enriched with antioxidants, clarified with chloroform and the supernatant was submitted to differential centrifugation, precipitated using PEG and NaCl and finally loaded on a discontinuous gradient of sucrose. Purified IMNV was submitted to RT-PCR and electrophoresis either in agarose gel or SDS-PAGE, which revealed RNA and protein bands, characteristic of IMNV. IMNV induced humoral immune response in Swiss mice when administered subcutaneously. Anti-IMNV antibodies were identified by ELISA (enzyme-linked immunosorbent assay) and Western blotting methods and produced a response against purified IMNV and the crude extract obtained from the infected shrimp. However, antibodies specific to the crude extract obtained from uninfected shrimp were not detected. This is the first report of IMNV having been purified in Brazil and the first time that specific antibodies against IMNV proteins have been produced. These results suggest that easy methods can be developed to produce specific antiserum for viral diagnosis on a large scale.

1. Introduction

The Northeast Region is the main producer of shrimp in Brazil and, in fact, has become the leading shrimp aquaculture producer in South America, with *Litopenaeus vannamei* L. (Pacific white shrimp) accounting for 90% of the total output in 2003–2004 (Lightner and Pantoja, 2004).

However, the industry has been affected by the emergence of new viruses, which have spread to other regions of Brazil. Among the virus-related diseases, Infectious Myonecrosis (IMN) has resulted in serious financial losses over the past few years (Nunes et al., 2004a,b). IMN is characterized by the appearance of white spots in the distal abdominal segments due to necrosis of the skeletal muscles. The species of shrimp *L. vannamei*, *Litopenaeus stylirostris* and *Penaeus monodon* are susceptible to IMN and among these species *L. vannamei* is the most vulnerable (Tang et al., 2005).

The first report of Infectious Myonecrosis in cultured shrimp occurred in the State of Piauí in 2002, after a period of intense rainfall.

IMN initially seemed to be limited to Brazil, but shrimp with similar signs have also been reported in other countries where *L. vannamei* is cultured (Lightner and Pantoja, 2004). In Indonesia, the non-native species have been imported and grown successfully on a large scale since 2003. However, in 2006, there were reports of high mortality at *L. vannamei* farms.

The shrimp showed signs of white spots on the muscles, similar to those reported on infected shrimp in Brazil (Senapin et al., 2007). IMN is now a cause of significant losses due to morbidity, mortality, or quality of the final product.

Initially, the etiological agent was unknown and the disease was named Idiopathic Myonecrosis. A series of investigations was con-

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ducted to identify the etiological agent of this malady (Lightner and Pantoja, 2004; Poulos et al., 2006; Tang et al., 2008). Poulos et al. (2006) showed that the agent was a virus which was subsequently named the Infectious Myonecrosis Virus (IMNV).

IMNV is icosahedra-shaped, 40 nm in diameter, has 120 subunits, is not enveloped, and presents protrusions that mediate its extracellular transmission and pathogenesis. Its genome consists of only double-stand RNA (dsRNA), composed of 7560 nucleotides. The sequencing of the viral genome showed two open reading frames (ORFs), designated ORF1 and ORF2. ORF1 encodes a protein of liaison to RNA and a capsidal protein, while ORF2 encodes an RNA-dependent RNA polymerase (Lightner and Pantoja, 2004; Tang et al., 2005, 2008; Poulos et al., 2006; Senapin et al., 2007).

The IMNV was classified as belonging to the *Totiviridae* family of non-segmented dsRNA viruses with isometric capsids and has been placed in the genus *Giardivirus* (Wang et al., 1993; Poulos et al., 2006). Nevertheless, Tang et al. (2008) demonstrated that its capsid is organized much like that of the genus *Totivirus* and proposed the creation of a new monotype genus to accommodate IMNV but retaining this genus in the *Totiviridae* family.

The defense system of shrimp depends mainly on innate immunity, which is formed of humoral defenses (activation of various proteolytic cascades such as the prophenoloxidase [proPO] system, hemolymph clotting mechanism, melanization and antimicrobial immune response) and cellular defenses (phagocytosis, encapsulation, cellular degranulation and release of defense factors) (Jiravanichpaisal et al., 2006 *apud* Han-Ching et al., 2010).

In vertebrates, response to the dsRNA virus is mediated by a class of Toll-like receptors (TLRs) and results in activation of the interferon system. Recently, a receptor structurally related to the Toll receptor of *Drosophila melanogaster* was identified in *L. vannamei* and designated lToll (Labreuche et al., 2009). Nevertheless, Labreuche et al. (2009) demonstrated that IToll did not protect *L. vannamei* against IMNV.

Recently, Han-Ching et al. (2010) showed that the IToll receptor is an important factor in the shrimp innate immune response to acute *Vibrio harveyi* infection, but not to the white spot syndrome virus (WSSV), which confirmed the results encountered by Labreuche et al. (2009) who demonstrated that IToll receptors were not involved in the response of *L. vannamei* to IMNV.

Current methods of purifying IMNV, using ultracentrifugation and gradients of CsCl, have proved to be expensive (Poulos et al., 2006; Tang et al., 2008). Thus, more practical and less expensive methods which are able to be used on a large scale need to be developed.

In this study, IMNV was purified by differential centrifugation and sucrose gradient, and then used to produce antibodies, which can be used for immunological diagnosis.

Therefore, considering that the innate immune system of *L. vannamei* seems incapable of conferring substantial protective immunity against IMNV, the results of this study are encouraging since they may enable early and precise diagnosis of this viral infection.

2. Materials and methods

2.1. Infected shrimp

IMNV was isolated from shrimp cultured at two farms located in the State of Ceará, Northeast Brazil. Shrimp showing symptoms of Infectious Myonecrosis were first collected and then sent to the Center for Diagnosis of Marine Shrimp Diseases-CEDECAM, Institute of Marine Science – Labomar – Federal University of Ceará (UFC), to be diagnosed. The biological material was stored at −40 °C and the diagnosis was performed by RT-PCR following the extraction of total RNA, using primers specific to IMNV, according to Vila Nova et al. (2008).

2.2. RT-PCR

2.2.1. RNA extraction.

Total RNA was extracted using the Trizol® method according to the instructions of *Invitrogen Life Technologies*. Seven hundred and fifty microliters of a crude extract obtained from infected shrimp (sample 1) or purified IMNV (sample 2) were placed in micro-tubes containing 500 μL of Trizol and left at room temperature for 10 min. Two hundred microliters of chloroform were then added to each tube and shaken vigorously for 15 s. The mixture was again left at room temperature for 5 min and then centrifuged at 12,000 × *g* at 4 °C for 15 min. RNA precipitation was performed by transferring 450 μL of supernatant to a microtube containing 500 μL of isopropyl alcohol. The mixture was incubated at room temperature for 15 min and centrifuged at 12,000 × *g* at 4 °C for 15 min. The precipitate was suspended in 1 mL of 75% ethanol, shaken gently in a vortex and centrifuged at 6600 × *g* at 4 °C for 5 min. The supernatant was discarded and the tubes were gently inverted and placed on filter paper to dry. The precipitate was then dissolved in 50 μL of DEPC-treated water and stored at −20 °C for later use. The extracted RNA was quantified by spectrophotometry using wavelengths of 260 nm and 280 nm. The integrity of the RNA was assessed by electrophoresis in 2% agarose using TAE as the running buffer. The gel was then stained with ethidium bromide and bands were observed through translumination of ultraviolet light.

2.2.2. cDNA synthesis.

cDNA was synthesized from the total RNA of either sample 1 or sample 2 using the SuperScript First Strand Synthesis Systems Kit for RT-PCR (Invitrogen). RT was performed in duplicate using 2.5 μg of total RNA together with 10 mM dNTPs, 150 ng of primers and DEPC-treated water to give a volume of 10 μL. Samples were incubated at 65 °C and immediately placed in glass for 1 min. A solution of 2.0 μL of RT buffer (10×), 4.0 μL of 25 mM $MgCl_2$, 2.0 μL of 0.1 mM DTT and 1.0 μL of *RNaseOUTTM Recombinant RNase Inhibitor* was prepared and 9.0 μL was added to each sample. The mixture was shaken in a vortex, centrifuged at 950 × *g* for 5 min and incubated at 42 °C for 2 min. Fifty units of the enzyme *SuperScriptTM* II RT were placed in the tubes and left at 42 °C for 50 min, and cDNA synthesis was performed at 70 °C for 15 min. cDNA was quantified by spectrophotometry through readings at wavelengths of 260 nm and 280 nm. The efficiency of the synthesis was evaluated by amplifying the gene obtained from ß-actin using RT-PCR and the *primers* ß-AC *Forward* (5′-ACHAACTGGGAYGAYATGG-3′) and ß-AC *Reverse* (5′TAGATGGGBACDGTGTGGG-3′).

2.2.3. PCR technique

PCR was performed using 2 μL of cDNA (300 ng) obtained as previously described.

The mixture contained 50 mM KCL, 10 mM Tris pH 9, 0.1% Triton X-100, 200 μM dNTPs, 0.4 μM Forward Primer IMNV4586-F (CGACGCTGCTAACCATACAA), 0.4 μM Reverse Primer IMNV4914-R (ACTCGGCTGTTCGATCAAGT), 0.4 μM $MgCl_2$, 1U of Taq Polymerase and 25 μL H_2O qsp. A total of 40 cycles were performed, all completed in the following order: 94 °C, 20 s, 62 °C, 20 s, 72 °C, 30 min, 72 °C, 30 s to produce an amplicon of 328 pb. The amplified products were subjected to electrophoresis in 2% agarose and then stained with ethidium bromide. The length of the fragments was determined using the standard marker *1Kb plus DNA Ladder* (INVITROGEN).