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Short Communication

Molecular characterization of infectious myonecrosis virus (IMNV) isolated from the shrimp *Litopenaeus vannamei* farmed in Ceará State, Brazil

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ABSTRACT. The shrimp *Litopenaeus vannamei*, one of the most important species in world aquaculture, has seriously affected by infectious myonecrosis virus (IMNV) that causes up to 70% mortalities. With the aim of improving the development of new strategies for rapid and reliable diagnosis, we isolated IMNV, from *L. vannamei* farmed in Brazil, through a discontinuous sucrose gradient, and sequenced cDNA fragment encoding the major capsid protein from this virus. Nucleotides sequences corresponding to the viral capsid protein was obtained by RT-PCR and confirmed by automatic sequencing. Comparison with sequences which encode the capsid protein obtained from Indonesia isolates showed a high identity.

Keywords: *Litopenaeus vannamei*, IMNV, RNA, RT-PCR, sequencing, aquaculture, Brazil.

Caracterización molecular del virus de la mionecrosis infecciosa (IMNV) aislado de camarones *Litopenaeus vannamei* cultivados en el estado de Ceará, Brasil

RESUMEN. El camarón *Litopenaeus vannamei*, una de las especies más importante para la acuicultura a escala mundial, es seriamente afectado por el virus de la mionecrosis infecciosa (IMNV) que causa hasta un 70% de mortalidad. Para mejorar el desarrollo de nuevas estrategias para un diagnóstico rápido y fiable, se aisló el IMNV a partir de *L. vannamei* cultivado en Brasil, a través de un gradiente discontinuo de sacarosa. Se secuenció un fragmento de cDNA que codifica la principal proteína del capsidio de este virus. El fragmento fue amplificado por RT-PCR y sometido a secuenciamiento automático. La comparación con secuencias obtenidas en bancos de datos consiguió establecer una alta identidad con secuencias provenientes de Indonesia.

Palabras clave: *Litopenaeus vannamei*, IMNV, RNA, RT-PCR, secuenciamiento, acuicultura, Brasil.

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Viral illnesses constitute the main problem faced by penaeid shrimp farms worldwide. Among the diseases of viral origin which affect the shrimp cultivation farms in the northeast region of Brazil, the infectious myonecrosis virus (IMNV) has caused significant losses in the sector. Since its initial sudden appearance in 2002, the IMNV has spread through the northeast states of Brazil (Andrade *et al*., 2006). In this context, in order to inhibit the expansion and control the disease there is a need for studies aiming to improve our understanding of this virus and to develop methods for rapid and sensitive detection.

One of the shrimp species which inhabit the Brazilian coast is *Litopenaeus vannamei*, known as Pacific white or gray shrimp, a species of great economic importance. The introduction of this species in the mid-1980s consolidated shrimp farming in Brazil, obtaining high levels of production and profitability. However, during the past decade there has been a decline in production and consequently a significant economic loss for the sector due to the appearance of IMNV (Pinheiro *et al*., 2007).

The manifestation of symptoms in shrimps affected by IMNV is characterized by an extensive area of necrosis of the skeletal muscle, mainly in the abdominal and distal segments, with a loss of the tail transparency, with some areas having a milky aspect (Lightner & Pantoja, 2004; Poulos & Lightner, 2006). In the chronic stage, lesions are accompanied by liquefying of the necrotic fibrotic muscles. The muscles and appendices

affected exhibit a redish coloration, giving the appearance of cooked shrimps (Nunes *et al.*, 2004).

The virus responsible for infectious myonecrosis (IMNV) has an icosahedral symmetry with a diameter of around 40 nm, and its capsid is composed of 120 subunits with a floating density of 1.366 g mL^{-1} in cesium chloride (CsCl). The genome comprises a double strand of RNA, composed of 7,560 bp. There are two open reading frames (ORF1 and ORF2) in different regions of the genome. The 5' end (ORF 1, nt 136-4953) encodes a protein of 179 kDa, which includes the N terminal sequence of the capsid protein and the RNA-binding, while the ORF of the 3' end (ORF 2, nt 5241-7451) encodes an RNA-dependent RNA polymerase (RdRp) (Lightner & Pantoja, 2004; Poulos, 2006; Nibert, 2007). Phylogenic analysis of the RdRp region of IMNV with other viral sequences allowed its classification as a member of the family *Totiviridae* and its similarity with the sequence of the *Giardia lamblia* virus (GLV) indicates that the IMNV is a member of this family, able to infect invertebrates (Poulos, 2006; Nibert, 2007).

Considering the impact of this disease on Brazilian shrimp farming, in this paper we describe the isolation and characterization through sequencing of the infectious myonecrosis virus (IMNV) obtained from tissues of infected *L. vannamei* shrimps in cultivation farms in Ceará State, Brazil (northeast region of Brazil). Adult shrimps showing signs of infectious myonecrosis were collected and sent to the Center for the Diagnosis of Marine Shrimp Diseases Centro de Diagnóstico de Doenças do Camarão Marinho (CEDECAM), Labomar Sciences Institute (Instituto de Ciências do Labomar) of the Universidade Federal do Ceará for examination.

The IMNV was purified according to the method described by Mello *et al.* (2011). Around 500 g of *Litopenaeus vannamei* shrimp which tested positive for IMNV were processed in 0.1 M phosphate buffer, pH 7.5, with the addition of 0.5% of Na_2SO (w/v). The macerated sample was filtered through double-faced gauze to remove the fragments and then clarified with N-butanol or 10% chloroform (v/v). The material was then centrifuged twice at 8,000 g for 15 min at ambient temperature (a.t.) and the supernatant obtained containing the IMNV was precipitated through the addition of buffer containing 10% of polyethylene glycol (PEG) and 4% of NaCl for 1 h at a.t. After this period the material was centrifuged as described above and the precipitate resuspended in 0.1 M phosphate buffer, pH 7.5 with the addition of 0.5% of sodium sulphite, being submitted to a discontinuous sucrose gradient at concentrations of 10 to 40%, and then centrifuged at 20,000 g for 30 min a.t. The fraction containing the virus, formed at the interface of the 40% sucrose layer, was removed and stored at -20ºC for use in the RT-PCR test or analysis in 12% SDS-PAGE gel. The total RNA was isolated from samples of shrimp tissue after purification using the method of acid guanidinium thiocyanate-phenol-chloroform extraction with TRIzol reagent (Invitrogen), according to the manufacturer´s recommendations. The reverse transcripttase reaction of the total RNA extracted from infected shrimp was carried out using the SuperScript® first-strand synthesis system (Invitrogen). The amplification was performed using 2 µL of cDNA, and the reaction was prepared with standard reagents for PCR with the addition of primers: IMNV99372G09- F (5' CGACGCTCTAACCATACAA 3'), IMNV 99372 G10-R (5' ACTCGCCTGTTCGATCAAGT 3') (Pinheiro *et al.*, 2007). A total of 40 cycles was carried out under the following conditions: 94ºC, 20 s, 62ºC, 20 s, 72ºC, 30 min, 72ºC, and 30 s to produce an amplicon of 372 bp.

The product of the PCR reaction was submitted to electrophoresis in 2% agarose gel and stained with ethidium bromide. The PCR product was visualized under an ultraviolet transilluminator (Transilluminator-FBTI 88; FisherBiotech®). The automatic sequencing of double-stranded DNA was accomplished using the BigDye® Terminator Cycle Sequencing kit in an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems®) sequencer. The above-mentioned DNA and amino acid sequences were analyzed using the DNASTAR program, version 5.00 (DNASTAR, Inc).

In Brazil, among the viral illnesses which can cause significant problems in shrimp cultivation farms, the infectious myonecrosis virus (IMNV) found in farms in the northeast region has been identified as one of the causes of reduced national production. With the aim of inhibiting the expansion of infectious myonecrosis at shrimp farms in Brazil, new investments are required in the search for control strategies and information on this disease. Based on such information, the main aim of this study was to isolate and characterize through sequencing the infectious myonecrosis virus (IMNV) in *Litopenaeus vannamei*.

The IMNV was isolated from whole shrimps of the species *L. vannamei* after confirmation of infection by RT-PCR, through a discontinuous sucrose gradient. The presence of two protein bands can be observed with apparent molecular masses of 96 and 106 kDa, corresponding to the major capsid protein (MCP) and the protein corresponding to the fusion of the MCP with RdRp or with other viral capsid fragments (data not shown) in accord with Poulos *et al.* (2006) and Tang *et al.* (2008).

In order to characterize the genetic conservation of the MCP in the viral isolates from Ceará State, a fragment of 372 bp corresponding to the IMNV was previously obtained by amplification (RT-PCR) of cDNA from the total RNA extracted from the viral suspension (Fig. 1). The negative and positive controls in this experiment were represented by absence and presence of cDNA from crude extract of infected shrimps respectively. In this study the amplification of the fragment corresponding to the viral capsid confirmed the presence of the virus at cultivation farms of the *L. vannamei* species in Ceará State.

Comparative analysis of the nucleotide sequence of the fragment of 372 bp with the gene sequences which encode the capsid of IMNV (AY570982.2 and EF061744.1) deposited in the databank revealed the high degree of identity with sequences previously described originating from Brazil and Indonesia (Senapin *et al*., 2007) (Fig. 2a). Our results confirm those described by Senapin *et al*. (2007) which demonstrate the occurrence of substitutions in the nucleotide sequence of the Indonesian isolates when compared with those obtained for the Brazilian isolates, verifying the high level of conservation.

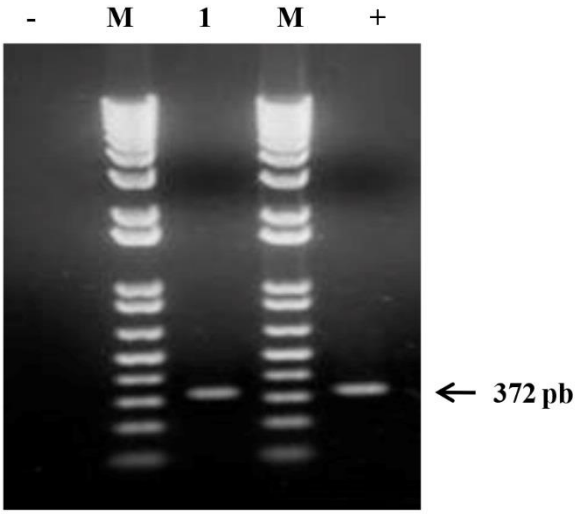

Figure 1. RT-PCR of IMNV from viral isolates from Ceará State. M: molecular mass marker (kb); 1- 372 bp amplicon from sucrose gradient purified virus. The symbols (-) and (+) indicate negative and positive control respectively.

Figure 2. Multiple alignments of sequences of nucleotides a) and b) amino acids of IMNV amplicon from the viral isolate originating from Ceará State with sequences deposited in the GenBank (AY570982.2 and EF061744.1, respectively).

As shown in Fig. 2b, the alignment of the above-mentioned sequence of the capsid protein of IMNV showed a high degree of similarity with the sequences of the viral isolates originating from Brazil and Indonesia (99% and 98%, respectively).

In recent years, several illnesses, particularly of viral origin, have been responsible for significant losses threatening the sustainability of shrimp farming in Brazil and around the world. One important strategy in the control of mortality caused by infectious myonecrosis is the rapid and sensitive detection of the pathogen.

Considered together, our results constitute the first study on the molecular characterization through automatic sequencing of a fragment (372 bp) of the isolated infectious myonecrosis virus from the Ceará State in which the absence of polymorphism of the capsid protein was demonstrated. The findings reported herein suggest that this protein could be used as an alternative system of early diagnosis of this disease.

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