Journal of Virological Methods xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

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*Article history:*
Received 7 June 2010
Received in revised form 18 May 2011
Accepted 25 May 2011
Available online xxx

*Keywords:*
*Litopenaeus vannamei*
IMNV
Purification
Polyclonal antibodies

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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doi:10.1016/j.jviromet.2011.05.032

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 lToll did not protect *L. vannamei* against IMNV.

Recently, Han-Ching et al. (2010) showed that the lToll 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 lToll 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).

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### 2.3. Purification and characterization of IMNV

IMNV was purified according to the method described by Florindo et al. (2002), with some modifications. Approximately 500 g of IMNV-positive *Litopenaeus vannemei* shrimp were placed in 0.1 M phosphate buffer, pH 7.5 plus 0.5% of $Na_2SO$ (w/v), ground in a mixer, and then filtered through a double layer of cheesecloth.

The homogenate was clarified with 10% chlorophorm (v/v) and centrifuged at 8000 × *g* for 15 min. The supernatant was again centrifuged at 8000 × *g* for 15 min. The supernatant was then precipitated by adding 10% PEG (w/v) and 4% NaCl (w/v), stirred at 24 °C for 1 h, and centrifuged at 8000 × *g*, at 4 °C for 20 min. The pellet was resuspended in the same extraction buffer and loaded onto a discontinuous sucrose gradient comprised of four layers containing 10, 20, 30 and 40% of sucrose, respectively, and then centrifuged at 20,000 × *g* for 30 min. After being carefully removed from the cushion, the pellet present on the layer containing 40% of sucrose was diluted in 0.1 M phosphate buffer, pH 7.5.

An aliquot of the sample was used for the RT-PCR and the proteins were quantified according to Bradford (1976). The sample was then stored at −20 °C for future assays.

### 2.4. SDS-PAGE

After the IMNV had been purified, electrophoresis (SDS-PAGE) was performed on polyacrylamide gels according to the method described by Laemmli (1970). The concentration of the stacking gel was 4% in 1.0 M Tris–HCl, pH 6.8 and of the main gel was 12% in 1.5 M Tris–HCl, pH 8.8, containing 10% SDS.

The samples were diluted in Tris/HCl buffer containing 10% SDS, 14 mM ß-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue and then heated at 100 °C for 3 min. The mass of the protein bands was estimated using a molecular mass marker.

Electrophoresis was performed at a constant voltage of 120 V and an initial amperage of 27 mA. The gel was stained in 0.05% blue Cromassie R250 (dissolved in a mixture of 40% ethanol and 10% acetic acid) for 30 min, and then placed in decolorizing solution (30% ethanol and 7% acetic acid), and left overnight. After the gel had been washed with distilled water, it was dried by wrapping it in cellophane paper.

### 2.5. Immunization by subcutaneous via

A group of ten two-month-old female Swiss mice (provided by the Central Animal House of UFC) subcutaneously received doses of purified IMNV preparation containing 200 μg of viral capsid protein and Freund's incomplete adjuvant. Boosters (200 μg viral capside protein) were given subcutaneously 21 and 35 days after the start of the immunization. The mice were bled from the retro orbital plexus to obtain antiserum 7, 21, 28, 35 and 42 days after starting the immunization.

### 2.6. Enzyme-linked immunosorbent assay

The immune response induced by IMNV was evaluated by the enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with purified IMNV (3 μg of protein) or crude extracts from IMNV-infected shrimp (3 μg of protein) or crude extracts of IMNV – uninfected shrimps (3 μg of protein) diluted in 50 mM potassium phosphate buffer, pH 9.6, at a final volume of 100 μL. After being incubated at 4 °C overnight, the plates were blocked with 5% nonfat milk in 10 mM potassium phosphate buffer, pH 7.2, with 0.9% NaCl (PBS) at room temperature for 2 h. The plates were washed once, 100 μL of the appropriate serum diluted in PBS was added, and they were reincubated at 4 °C overnight. The plates were washed five times with 0.05% PBS-Tween 20 and treated with peroxidase-conjugated rabbit antimouse IgG (100 μL/well, 1:1000 final dilution) at room temperature for 2 h and then washed five times with PBS-Tween 20. The reaction was initiated by adding orthophenylenediamine. The mixture was incubated at 37 °C for 20 min, and the reaction was then stopped by adding 20 μL of 2.5 N $H_2SO_4$. The resulting color was read at 492 nm. The results were reported as the mean values of the absorbance obtained for the sera at different dilutions.

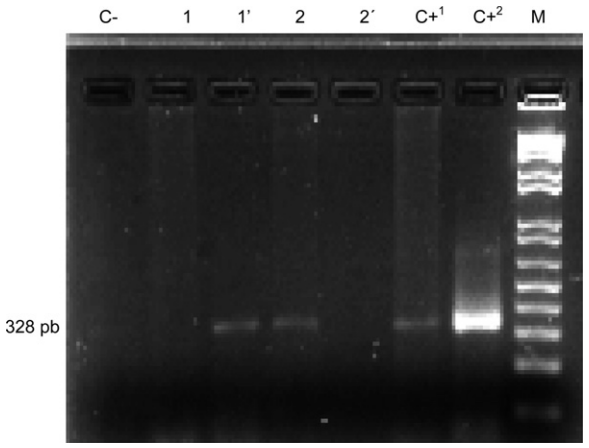


**Fig. 1.** Electrophoresis in 2% agarose gel of products from RT-PCR using Forward Primer IMNV4586-F and Reverse Primer IMNV4914-R, and staining with ethidium bromide. Lanes 1 and 2 represent amplicons obtained from the crude extract of infected shrimp and the virus purified by sucrose gradient, respectively. C− is a negative control in which ultrapure water was used and C+ means a positive control in which the crude extract from infected shrimp was used to extract RNA. M, molecular weight marker.

### 2.7. Western blotting

After the SDS-PAGE, the protein transfer was carried out according to Towbin et al. (1979).

An aliquot containing 20 μL of purified IMNV, crude extract from infected shrimp and crude extract from uninfected shrimps was loaded onto wells of polyacrilamyde gels. After electrophoresis, proteins on the gel were transferred to a nitrocellulose membrane and then immunodetected with the antiserum produced against purified IMNV. The membrane was first blocked for 2 h with phosphate buffered saline (PBS) containing 5% skimmed milk and reacted for 2 h with anti-IMNV antibodies diluted 1000-fold in PBS. The membrane was washed three times with PBS containing 0.1% Tween 20, each time for 15 min. The membrane was then incubated with anti-rabbit IgG antibody conjugated to peroxidase diluted 5000-fold in PBS containing 0.1% Tween 20. After washing, blots were revealed by color development reagents diaminobenzidine tetrahydrochloride/$NiCl_2$/$H_2O_2$.

## 3. Results

### 3.1. Detection of IMNV in L. vannamei

IMNV infection was detected in shrimps from two aquaculture farms after performing RT-PCR. Positive samples showed a single band on 2% agarose gel, estimated at 328 bp representing the amplicon for IMNV (Fig. 1).

### 3.2. Purification of IMNV

Crude extract of IMNV-positive shrimp was used by IMNV purification. The process necessitated submitting the samples to a discontinuous sucrose gradient. An opaque band was located in

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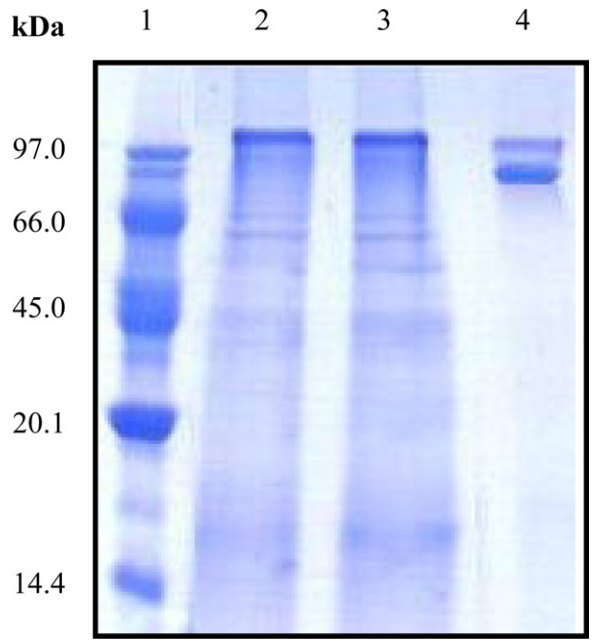


**Fig. 2.** Electrophoresis in denaturing polyacrylamide gel (SDS-PAGE). Lane 1: molecular weight markers, lanes 2 and 3: crude extract from whole infected shimp (10 μg protein) and lane 4: purified IMNV (2 μg protein).


**Fig. 4.** IMNV-specific antibodies produced in mice immunized subcutaneously with purified IMNV obtained from infected shrimp were detected by Western blotting after SDS-PAGE and transfer of purified IMNV (lanes 1 and 2), crude extract from whole infected shrimp (lane 3) and crude extract from whole uninfected shrimp (lane 4). The blots were visualized with peroxidase-conjugated rabbit antiserum to mouse IgG, and color development reagents diaminobenzidine tetrahydrochloride/$NiCl_2$/$H_2O_2$.

a layer of 40% sucrose. SDS-PAGE revealed two bands of protein, whose molecular masses were estimated as 106 and 96 kDa (Fig. 2). The band corresponding to 96 kDa represented a major band and that of 106 kDa appeared as a thin spot on the gel. The purified virus was submitted to RT-PCR as described above for infected shrimp and revealed an amplicon with 328 pb indicating the presence of IMNV in the sample.

### 3.3. Production of polyclonal antibodies

The purified virus was used to produce antibodies, which may lead to diagnostic techniques using immunological methods. The results obtained by ELISA (Fig. 3) confirmed the production of specific antibodies against IMNV. When the antigen consisted of crude extract obtained from infected shrimp, the titers of specific antibodies were as high as those obtained when the purified IMNV was used as the antigen. However, the antibodies did not react with the crude extract obtained from uninfected shrimp.

Western blotting revealed spots with molecular masses similar to those obtained by electrophoresis for the purified IMNV and the crude extract obtained from infected shrimp (Fig. 4). Nevertheless, there was no spot formation for the crude extract obtained from uninfected shrimp.

## 4. Discussion

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. In this study, a simple and rapid method of purifying IMNV from infected shrimp is described.

Purified IMNV showed a protein band with a relative molecular mass of 96 kDa and another minor band with a molecular mass of 106 kDa when submitted to denaturing polyacrylamide gels (SDS-

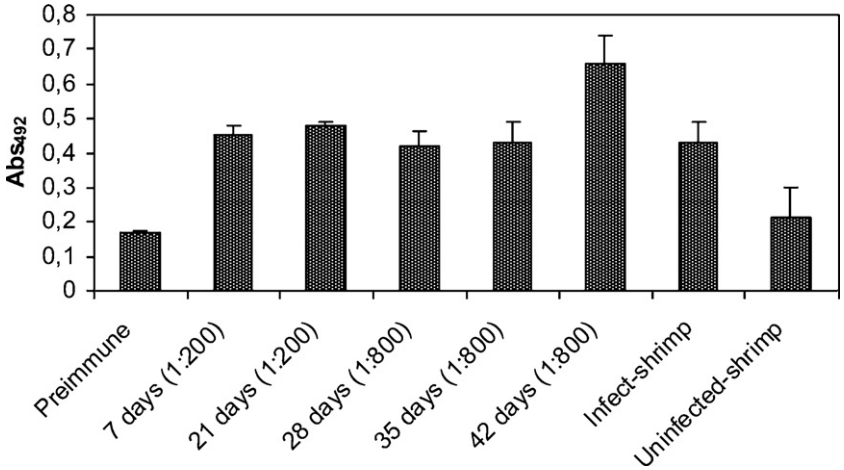


**Fig. 3.** IMNV-specific serum antibody response of mice immunized subcutaneously with purified IMNV from infected shrimp, detected by ELISA. Purified virus, and crude extracts from infected shrimp and uninfected shrimp were used as antigens. Kinetics of IMNV-specific serum antibody production in immunized mice measured by ELISA showed high titers up to 42nd day.

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PAGE). The results reported in this paper indicate that the band of molecular mass equivalent to 96 kDa corresponds to major capsid protein (MCP). In relation to the band of 106 kDa, the results were consistent with those obtained by Tang et al. (2008) and Poulos et al. (2006). These authors suggest that the band represents fusion of MCP/RdRp or fusion of MCP and other fragments from capsid virion.

IMNV was purified by differential centrifugation using a sucrose gradient, in contrast to the study by Poulos et al. (2006) who achieved purified IMNV after ultracentrifugation using a CsCl gradient.

Furthermore, in this study whole shrimp was used, which resulted in a high concentration of purified virus as seen using the Bradford method (2.03 mg of protein $mL^{-1}$), suggesting that the IMNV can infect other organs.

Specific antibodies against IMNV will enable fast and cheap diagnosis to be performed on a large scale at aquaculture farms in the Northeast Region of Brazil as well as at other locations around the world.

The production of specific antibodies against IMNV was monitored for 42 days after the start of the immunization. Specific antibody production increased considerably 21 days after the beginning of immunization and was maintained up to the 42nd day, with slight increases after the boosters at days 21 and 35 (Fig. 3).

Western blotting confirmed the specificity of antibodies toward IMNV, since they reacted with the purified IMNV and the crude extract obtained from infected shrimp but no reaction with the crude extract obtained from uninfected shrimp was detected (Fig. 4).

Although innate immunity has shown efficacy against several bacteria, there is not yet direct evidence of a response triggered when this virus infects shrimps. Therefore, the development of precise and rapid diagnosis is necessary to control the infection at an early stage.

The methods currently used to diagnose IMNV in cultures are RT-PCR (Senapin et al., 2007), real-time RT-PCR (Andrade et al., 2007) and *in situ* hybridization (Tang et al., 2005). Although these methods are quite accurate, they are too expensive to be used viably by small farmers. Thus, the use of immunological methods would enable the diagnosis to be made more accessible on a large scale without losing reliability. In this way, antibodies could be used conjugated to enzymes for viral detection by the ELISA method or linked to fluorescent reagents, which represent important tools for future studies on the infection mechanism and the establishment of IMNV in shrimp. Furthermore, antibodies could be associated with nanoparticles and used as immunosensors, which would provide a very sensitive method.

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