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ORIGINAL ARTICLE

Detection of infectious myonecrosis virus in penaeid shrimps using immunoassays: usefulness of monoclonal antibodies directed to the viral major capsid protein

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Abstract Despite the economic impact of the infectious myonecrosis virus (IMNV) on shrimp farms in several countries, no method for immunological detection is currently available. With the aim of developing immunodiagnostic methods for IMNV detection in infected shrimps, a recombinant fragment of the IMNV major capsid protein gene encoding amino acids 105–297 ($rIMNV_{105–297}$) was heterologously expressed in *Escherichia coli* and used to immunize Balb/c mice, generating monoclonal antibodies (MAbs). Six hybridomas were obtained, and four of these recognized the presence of IMNV in tissue homogenates from naturally infected shrimps by immunodot blot assay. Among these MAbs, three were able to detect a ~100-kDa protein, which corresponds to the predicted mass of the IMNV major capsid protein, as well as viral inclusion bodies in muscle fibroses by western blot and immunohistochemistry. Two MAbs showed high specificity and sensitivity, showing no cross-reaction with healthy shrimp tissues in any assays, indicating their usefulness for IMNV detection.

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Introduction

The shrimp farming industry is an important agribusiness worldwide, accounting for annual incomes of over US$ 10 billion due to export of penaeid shrimps, which is one of the most important internationally traded fisheries commodities [1]. However, since 1980 the penaeid shrimp farming industry has been affected by at least four pandemic viruses, incurring major economic losses [2]. Among these, infectious myonecrosis virus (IMNV), also known as penaeid shrimp myonecrosis virus (PsIMNV), was identified most recently and has been shown to have a major economic impact [3]. IMNV is a non-enveloped virus containing a 7560-nucleotide non-segmented double-stranded RNA genome with two open reading frames (ORFs). The viral particles are icosahedral in shape, with a diameter of 40 nm and fiber-like protrusions on the surface [4–6]. ORF1 encodes a putative RNA-binding protein and the 901-aa major capsid protein, while ORF2 encodes a 736-aa protein that presents typical motifs of an RNA-dependent RNA polymerase (RdRp) [4, 5]. Phylogenetic analysis based on the RdRp identified IMNV as a member of the family *Totiviridae* [4]. Most of the viruses from this family cause latent and avirulent infections, with IMNV being the only member to infect a host that is not a fungus or a protozoan and that has been implicated in causing disease [6]. Typical IMNV infection results in clinical signs consisting of extensive necrotic areas in striated muscles with opaque appearance, particularly in the distal abdominal segments and the tail fan [3, 4, 7]. In cultured penaeid shrimp, infection results in a chronic, persistent, and slowly progressive disease that results in cumulative mortality rates of up to 70% [3]. Once confined to a geographic region, global shrimp commerce has favored the spread of IMNV worldwide, which was not followed by the characterization of the

etiological agents [8]. The first report of an IMNV epizootic event was in Brazil in 2002 in a shrimp farm located in the coastal area of the state of Piauí, in the northeastern region [3, 7]. Since then, the virus silently and progressively spread to farms in other Brazilian states [9–11] as well as to other countries such as Indonesia [12], causing a social and economic impact.

Since an efficient method to control the spread of IMNV has not yet been reported, the search for sensitive and specific diagnostic methods that can be employed under field conditions and are suitable to simultaneously analyze a large number of samples is highly desirable. Nowadays, the diagnosis of IMNV infection relies basically on clinical signs [7], histopathological examination [13] and molecular techniques. Compared to the last of these, the first two methods have the disadvantage poor specificity and thus are prone to give false negative results. On the other hand, molecular techniques are usually very sensitive and specific but require trained personnel and specialized equipment, making them expensive and difficult to implement on shrimp farms. Despite these drawbacks, several molecular methodologies have been described recently for IMNV detection, such as RT-PCR [14], nested-PCR [12], real-time qPCR [9] and new alternatives like RT LAMP LFD, which combines nucleic acid amplification under isothermal conditions and chromatographic visualization [15].

In contrast to nucleic acid-based approaches, it is noteworthy to mention that no efforts have been made towards the development of immunological methods to detect IMNV, probably due to limited knowledge of the IMNV protein expression profile and the lack of specific tools like monoclonal antibodies directed against the virus. Diagnostic methods employing MAbs have been used in the diagnosis of other viral infections in farm-reared shrimps [16–23]. Some of these MAbs-based methods, such as ELISA and western blot, are routinely performed in laboratories, but simple, low-cost methods allowing accurate diagnosis by untrained personnel under field conditions, such as latex agglutination and immunochromatographic strip tests, are urgently needed. In this study, we report the generation and characterization of MAbs directed against a recombinant portion of the IMNV major capsid protein (rIMNV$_{105–297}$) that specifically recognize the native protein in naturally infected shrimps, allowing the development of immunological diagnostic methods for IMNV infection.

Materials and methods

Shrimp and sample preparation

Pacific white shrimps, *Litopenaeus vannamei*, that were naturally infected with IMNV were obtained from shrimp farms at Piauí State, Northeastern Brazil. Animals exhibiting clinical signs had IMNV infection confirmed by RT-PCR using specific primers to the IMNV major capsid protein gene as described below. Samples from abdominal muscle of infected shrimps were dissected, preserved in RNAlater™ (Qiagen, Germantown, USA) or Davidson's alcohol–formalin–acetic solution for molecular and immunological assays, respectively, or frozen at −80°C. Tissue samples taken from healthy animals were also analyzed by RT-PCR and used as negative controls in all experiments.

RNA isolation and PCR amplification

Total RNA was extracted from all samples using the TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. RNA concentration and quality were assessed spectrophotometrically at 260 and 280 nm using NanoVue™ equipment (GE-Healthcare, Buckinghamshire, UK). After cDNA synthesis using M-MLV reverse transcriptase, an oligo dT$_{(12)}$ primer (Invitrogen) and 1 μg of total RNA as template, specific primers IMNV$_{105–297}$-F (5′-CAT ATG GGG CAA TTA CGG TTA CAG GG-3′), and IMNV$_{105–297}$-R (5′-CGG GAT CCG TAT ACA TAC CAA ATG GCC-3′) were used for PCR amplification of the region encoding amino acids 105–297 of the hydrophilic region of IMNV major capsid protein (GenBank accession no. AY570982.1) as determined by the Kyte and Doolittle method using the ProtScale software (http://www.expasy.ch/tools/protscale.html). To simplify the cloning procedures, *Nde*I and *Bam*HI restriction sites were added to the 5′ end of the IMNV$_{105–297}$-F and IMNV$_{105–297}$-R primers (underlined nucleotides), respectively. Amplification of the IMNV$_{105–297}$ region by PCR was performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, DE) using 1U of *Taq* DNA polymerase (LGC Biotechnology, Cotia, Brazil) in buffer containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.4 μM of each primer. The following thermal profile was used: 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with a final extension step of 72°C for 10 min. The amplification products were resolved in 1% agarose gels, stained with ethidium bromide, visualized in a Macrovue-UV20 transilluminator (Hoefer, San Francisco, CA, USA), and recorded digitally.

IMNV$_{105–297}$ cloning

The amplification product of 600 bp that was obtained was cloned into pGEM-T Easy Vector (Promega, Madison, USA) and used to transform *Escherichia coli* DH5α (Promega), which was then cultured on LB-agar plates

containing IPTG (1 mM), X-GAL (20 μg/ml) and ampicillin (100 μg/ml). Plasmid DNA was extracted from positive clones by standard methods and were checked by sequencing the inserts in both directions in a MegaBACE 1000® DNA Analysis System (GE-Healthcare) using DYEnamic® ET dye terminators as instructed by the manufacturer. The quality of all sequences thus obtained was assessed using the Phred/Phrap/Consed software (http://www.phrap.org), which allowed the determination of a high-quality consensus sequence [24]. An identity check was performed by similarity analysis using the BlastP algorithm (http://blast.ncbi.nlm.nih.gov/) based on the $IMNV_{105–297}$ deduced amino acid sequence obtained using the Expasy proteomic server (http://ca.expasy.org/). The deduced amino acid sequence was aligned with major capsid protein sequences from Brazilian and Indonesian IMNV isolates (GenBank accession no. AY570982 and EF061744, respectively) using the ClustalX program [25] in an attempt to identify variability within the fragment chosen for expression. Once its identity was confirmed, the $IMNV_{105–297}$ sequence was subcloned into the expression vector pET-14b (Novagen, Gibbstown, USA) by *Bam*HI/*Nde*I digestion followed by ligation with T4 DNA ligase. After transformation of *E. coli* BL21/DE3 competent cells (Novagen) and growth in LB-agar broth containing 100 μg/ml ampicillin, all positive clones were further checked by sequencing as described above to assure identity and the maintenance of the open reading frame.

Heterologous expression and purification of $rIMNV_{105–297}$

The clones containing the $IMNV_{105–297}$ fragment were grown in LB broth containing 100 μg/ml of ampicillin and 1 mM isopropyl-D-thiogalacto-pyranoside (IPTG) (Sigma-Aldrich, St. Louis, USA) at different temperatures (37, 27, 18°C) and with different induction periods (1.5, 3 and 15 h) to achieve the best recombinant expression levels. The bacterial cultures were then centrifuged (5,000×*g* for 10 min at 4°C), and the pellet was suspended in 20 mM Tris–HCl (pH 7.0) and sonicated three times for 30 s at 12 W, followed by another centrifugation step (10,000×*g* for 20 min) to obtain the soluble and insoluble fractions. Protein profiles of both fractions were resolved by 15% SDS-PAGE at 25 mA for 2 h and, after staining the gel with 0.1% Coomassie Brilliant Blue R-250 for 2 h, the results were recorded digitally. Since the majority of $rIMNV_{105–297}$ was detected in the insoluble fraction, bacterial pellets were treated with a denaturing lysis buffer (8 M $CO(NH_2)_2$, 100 mM NaH_2PO_4, 100 mM Tris–HCl, pH 8.0) at 60°C for 1 h, with agitation. After centrifugation (12,000×*g* for 30 min at 4°C), the supernatant fraction containing the N-terminal 6-histidine-tagged $rIMNV_{105–297}$ was purified using Ni-NTA Agarose (Qiagen) according to manufacturer's protocol. After four cycles of dialysis for 12 h each in dialysis buffer (100 mM Tris–HCl, 500 mM NaCl, 0.5 mM EDTA, 10% glycerol, pH 8.5), the protein was quantified [26]. The purified protein was resolved by15% SDS-PAGE as described above and electroblotted onto a nitrocellulose membrane (GE-Healthcare) soaked with blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Western blot assays were carried out to assess the expression of $rIMNV_{105–297}$. For this, membranes were blocked with 5% non-fat milk for 90 min, incubated for 1 h with anti-HisTag MAb (Sigma-Aldrich) at 25°C in blotting buffer, washed 5 times in TBS buffer with 0.1% Tween and then incubated with HRP-conjugated goat anti-mouse total immunoglobulin (Sigma-Aldrich) for 1 h at 25°C. Detection was carried out using ECL reagent (GE-Healthcare) and exposure to radiographic films (GE-Healthcare), which were developed using an automated SRX-101A film processor (Konica-Minolta, Tokyo, Japan).

Immunization and MAb production

MAbs were prepared based on procedures described by Köhler and Milstein [27] and Yokoyama et al. [28] with minor modifications. All procedures involving experimental animals were approved in advance by the UFSC Ethics Committee for Animal Care. 5–6- to 8-week-old Balb/c mice from breeding stocks maintained at the animal facility of the Departamento de Microbiologia, Imunologia e Parasitologia, UFSC, were immunized subcutaneously with 50 μg of purified $rIMNV_{105–297}$ mixed with complete Freund's adjuvant (Sigma-Aldrich) at a 1:1 ratio. Ten days later, a second subcutaneous immunization was performed using the same amount of antigen mixed with incomplete Freund's adjuvant (Sigma-Aldrich). Two additional doses of 50 μg $rIMNV_{105–297}$ were administered without any adjuvant by the intraperitoneal route at 10-day intervals. Four days after the last immunization, mouse sera were obtained from blood collected from the retro-orbital plexus to assess antibody production by ELISA as described below. Spleens from the mice with the highest antibody titers against $rIMNV_{105–297}$ were removed under aseptic conditions, and splenocytes were fused with P3X63Ag8.653 myeloma cells (ATCC® number CRL-1580) at a ratio of 5:1 using 50% polyethyleneglycol-4000 (Sigma-Aldrich) as fusion reagent. The resulting hybridomas were grown in RPMI-1640 medium (Cultilab, Campinas, Brazil) containing 20% fetal calf serum (Cultilab), and a 1% antibiotic and antimycotic cocktail (Sigma-Aldrich), supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Sigma-Aldrich). Two weeks after fusion, hybridomas were screened by indirect ELISA for antibody secretion and recognition of purified $rIMNV_{105–297}$ as described below.

Hybridomas with the highest titers against the recombinant protein in their supernatants were further subjected to two rounds of cloning by the limiting dilution method and stored in liquid nitrogen. The immunoglobulin isotype of the MAbs was determined by sandwich ELISA using an SBA Clonotyping System/HRP (Southern Biotech, Birmingham, USA), following the manufacturer's instructions.

Indirect ELISA

Ninety-six-well microplates (Costar, Lowell, USA) were coated for 3 h with 50 ng/well of $rIMNV_{105–297}$. After blocking at 4°C for 12 h with 3% bovine serum albumin in PBS-T (PBS supplemented with Tween-20 0.05%), incubation of each hybridoma supernatant was carried out for 1 h at 37°C. As positive and negative controls, sera from mice immunized with $rIMNV_{105–297}$ and RPMI-1640 medium, respectively, were used. After three wash cycles with PBS-T, incubation with HRP-conjugated goat anti-mouse immunoglobulin (Sigma-Aldrich) was performed for 1 h at 37°C, followed by five washes with PBS-T and addition of the substrate *o*-phenylendiamine (0.4%; Sigma-Aldrich) and 0.03% H_2O_2 in 0.1 M phosphate-citrate buffer (pH 5.6). Absorbance was measured by spectrophotometry at 495 nm in a Sunrise™ Basic device (Tecan, Männedorf, Sweden).

Detection of IMNV in tissues from naturally infected shrimps

Frozen striate muscle from IMNV-infected and uninfected shrimps were macerated using sterile pestles for 5 min in lysis buffer (0.2 mM Tris–HCl, 400 mM NaCl, pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was subjected to three cycles of sonication for 30 s at 12 W and were stored at −20°C for immunodot blot and western blot assays. For immunodot blot, 100 ng of purified $rIMNV_{105–297}$, 1 μg of IMNV-infected, and 1 μg of uninfected striated muscle homogenates were spotted onto nitrocellulose membranes. These same samples were also used for western blot assays along with homogenates of *E. coli* BL21/DE3 expressing $rIMNV_{105–297}$ or not, which were resolved and transferred as described above. After incubation of the membranes at 25°C for 1 h with each MAb generated in this study as well as with anti-rabies virus MAb, which was used as a control, both assays were carried out and developed as described above.

Immunohistochemical analysis

MAbs that recognized the IMNV major capsid protein in western blot assays were further tested using immunohistochemical assays. For this, healthy and IMNV-infected *L. vannamei* tissues were fixed for 48 h with Davidson's solution, followed by washes with 70% ethanol. Sections measuring 3 μm were taken from paraffin-embedded tissues, and antigen retrieval was performed by immersion of the slides in 10 mM trisodium citrate-buffer (pH 6.0) at 95–98°C for 45 min. After blocking nonspecific binding with normal goat serum, tissue sections were incubated with anti-$IMNV_{105–297}$ MAbs for 15 h at 4°C using an anti-rabies virus MAb as a negative control. Following washes of the tissue sections with PBS, development was performed using an Envision™ plus horseradish peroxidase kit (Dako Cytomation, Glostrup, Denmark). Slides were then counterstained with Harris's hematoxylin solution, and images were acquired and digitally recorded using an Eclipse 50i microscope (Nikon, Tokyo, Japan) coupled to a DS-5M-L1 camera (Nikon).

Results and discussion

Amplification, cloning, and sequence alignment of $IMNV_{105–297}$

The nucleotide sequence encoding a hydrophilic region of the IMNV major capsid protein, between residues Ala-105 and Tyr-297 ($IMNV_{105–297}$), was successfully amplified by PCR using the specific primers described herein. The cDNA template was obtained from IMNV-infected animals collected between 2008 and 2009 in the same geographic region where the first IMNV outbreak occurred in Brazil in 2002. The expected PCR product of 600 bp was ultimately cloned into an expression vector in the correct frame, as assessed by sequencing both strands from four distinct clones. The sequences obtained were of high quality (phred $\geq$20), and all were used to obtain a consensus sequence that revealed a high degree of nucleotide identity with IMNV genome sequences available in the GenBank database, confirming the identity of the clone fragment. ClustalX alignment showed that the $IMNV_{105–297}$ deduced amino acid sequence was 99% identical to a previously reported IMNV sequence from Brazil [4], with a single amino acid change at position 294 (F–C). Moreover, 98% identity was found when $IMNV_{105–297}$ was compared to sequences of IMNV isolated in Indonesia [12], with amino acid changes at positions 223 (A–V) and 294 (F–C). The sequence data were submitted to GenBank with the accession number HM357803. Considering the limited nucleotide variability in the capsid region between amino acids 105 and 297, resulting in only two amino acid differences due to non-synonymous substitutions, it is possible to infer that epitopes localized in this portion are likely to be highly similar among different IMNV isolates, regardless of the geographic region considered.

Expression and characterization of rIMNV$_{105–297}$

Being one of the less hydrophobic regions of the major capsid protein, the IMNV$_{105–297}$ portion was then selected for recombinant expression (rIMNV$_{105–297}$) (Fig. 1) because epitopes might potentially be exposed on the virion surface, allowing their recognition by MAbs. The optimal expression of rIMNV$_{105–297}$ was achieved after 15 h of induction with 1 mM IPTG at 27°C (Fig. 2a), although expression was detected by SDS-PAGE under all conditions tested (data not shown). Comparative analysis of soluble and insoluble fractions of bacterial cultures expressing rIMNV$_{105–297}$ by SDS-PAGE showed a prominent and exclusive expression of a ~25-kDa protein in the insoluble fraction, which was absent in bacteria transformed with mock vector control (Fig. 2a). Western blot analysis showed that anti-HisTag antibody specifically recognized a single band of ~25 kDa that was present exclusively in lysates of rIMNV$_{105–297}$-expressing bacteria and did not recognize any proteins in *E. coli* mock control lysates (Fig. 2a).

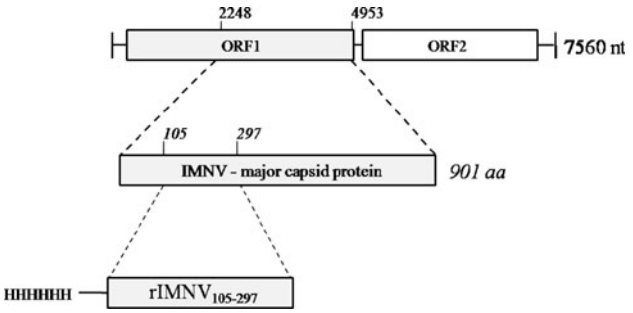

Fig. 1 Schematic representation of rIMNV$_{105–297}$. The N-terminal 6-histidine-tagged rIMNV105–297 encompasses the amino acids 105–297 of the 901-aa major capsid protein encoded by nucleotides 2248–4953 of IMNV ORF1

Production and characterization of anti-rIMNV$_{105–297}$ MAbs

Eighteen of the 219 hybridomas that were generated (8.2%) were positive for anti-rIMNV$_{105–297}$ antibody secretion, as revealed by indirect ELISA on an initial screening. After 2 weeks under selective culture, six hybridomas that remained stable in terms of growth and antibody secretion were selected and cloned, allowing the establishment of monoclonal cell lines named 1.3H, 1.1D, 3.3G, 3.9G, 4.6C, and 5.4H. Isotyping carried out by sandwich ELISA identified MAbs 1.3H and 4.6C as belonging to the IgG1 subclass and MAbs 1.1D, 3.3G, 3.9G, and 5.4H as IgM. MAbs 1.3H, 1.1D, 3.3G, 4.6C, and 5.4H were able to recognize the expected ~25-kDa protein in pET-IMNV$_{105–297}$-transformed *E. coli* lysates in western blot assays (Fig. 2b). These MAbs have also recognized a protein of ~50 kDa, which could be a dimeric form of rIMNV$_{105–297}$ due to disulphide bonds between cysteine residues of two monomeric units of the recombinant protein (Fig. 2b). Interestingly, western blot using anti-HisTag MAb did not recognize the ~50-kDa band (Fig. 2a), which can be explained by conformational changes in the dimeric form that might be blocking the HisTag tail. The 3.9G MAb did not recognize the purified rIMNV$_{105–297}$ or any protein from control bacterial extracts. The MAbs described in the present study did not recognize any proteins in normal *E. coli* lysates nor in lysates from bacteria transformed with mock plasmids, confirming the specificity of these antibodies to the IMNV$_{105–297}$. The anti-rabies-virus

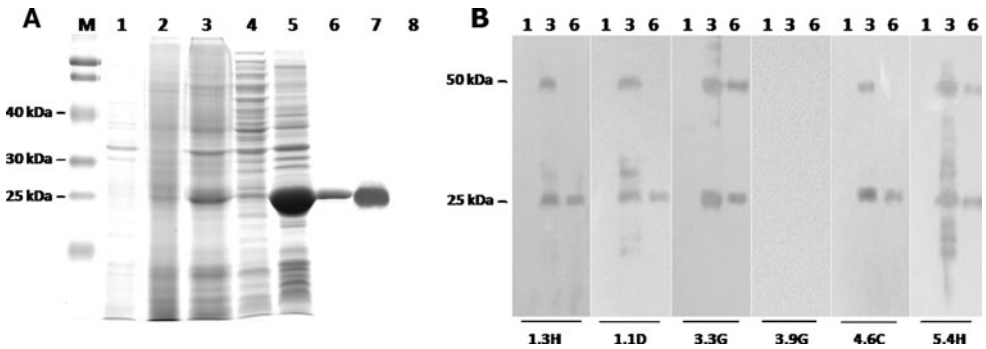

Fig. 2 Heterologous expression and purification of a recombinant fragment of the IMNV major capsid protein (rIMNV$_{105–297}$) and reactivity of anti- rIMNV$_{105–297}$ MAbs against *E. coli* homogenates by western blot. **a** 15% SDS-PAGE gel stained with Coomassie blue showing the protein profile of *E. coli* BL21/DE3 expressing rIMNV$_{105–297}$ and western blot using anti-HisTag antibody. **b** Western blot of the same samples resolved by SDS-PAGE using the MAbs produced in this work, showing their ability to bind to rIMNV$_{105–297}$. *M*, BenchMark™ protein ladder (Invitrogen); *1*, *E. coli* mock control, transfected with pET-14b; *2*, *E. coli* transfected with pET-IMNV$_{105–297}$ before IPTG induction; *3*, *E. coli* transfected with pET-IMNV$_{105–297}$ induced with IPTG; *4*, soluble and *5*, insoluble fractions of the *E. coli* expressing rIMNV$_{105–297}$ after IPTG induction; *6*, purified rIMNV$_{105–297}$; *7*, western blot using anti-HisTag antibody against purified rIMNV$_{105–297}$; *8*, western blot using anti-HisTag antibody against *E. coli* mock control

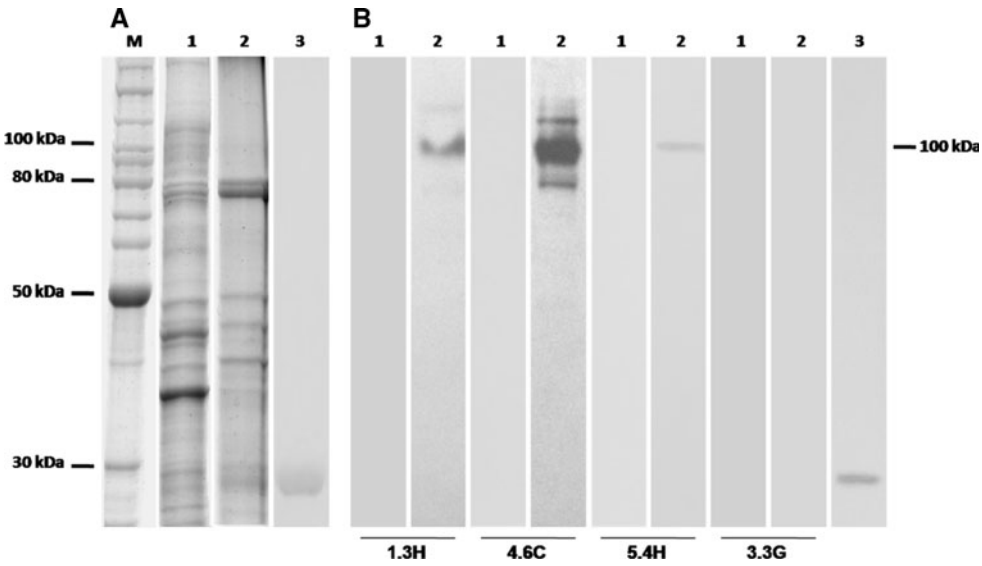

Fig. 3 Detection of IMNV in naturally infected *L. vannamei* tissue homogenate by western blot using anti-rIMNV$_{105–297}$ MAbs. **a** 15% SDS-PAGE Coomassie-blue -tained gel; **b** western blot of the same SDS-PAGE using MAbs anti-rIMNV$_{105–297}$ 1.3H, 4.6C, 5.4H or 3.3G, as indicated; in *lane 3*, MAb 1.3H was used. *M*, BenchMark™ protein ladder (Invitrogen); *1*, striated muscle homogenate from healthy shrimp; *2*, striated muscle homogenate from IMNV-infected shrimp; *3*, purified rIMNV$_{105–297}$

MAb used as negative control did not recognize any proteins in any of the assays (data not shown).

Specific detection of IMNV in tissues of naturally infected shrimps

In order to identify MAbs that are able to recognize the native IMNV major capsid protein, immunodot blot assays using anti-rIMNV$_{105–297}$ MAbs were carried out with homogenates of skeletal muscle from naturally infected and uninfected *L. vannamei* shrimps. MAb 1.1D showed no reaction, but MAbs 1.3H, 3.3G, 4.6C, and 5.4H were able to recognize the presence of IMNV in infected shrimp tissues and did not show any reaction against uninfected shrimps (data not shown). MAbs 1.3H, 3.3G, 4.6C, and 5.4H were further screened by western blot against the same homogenates of IMNV-infected and uninfected *L. vannamei* tissues. A protein of ~100 kDa could be recognized by MAbs 1.3H, 4.6C, and 5.4H, but not by MAb 3.3G, reinforcing their usefulness for the specific detection of the IMNV major capsid protein (Fig. 3b). None of the MAbs tested showed cross-reactivity with uninfected shrimp tissues in this assay (Fig. 3b), as was also observed for the anti-rabies virus MAb used as a negative control (data not shown).

Detection and localization of IMNV in infected skeletal tissues by immunohistochemistry

The three MAbs showing specific recognition of the major capsid protein of IMNV in western blot assays (1.3H, 4.6C, and 5.4H) were tested by immunohistochemistry for the detection of IMNV in abdominal skeletal muscle of infected *L. vannamei*, since replication of the virus is prominent in this tissue [4, 13, 29]. MAbs 1.3H and 4.6C bound to viral inclusions in myonecrosis foci in muscle samples from IMNV-infected shrimps, as clearly revealed by the brownish color observed in Fig. 4a–d, corresponding to areas of muscle fibrosis. MAbs 1.3H and 4.6C showed no cross-reaction with uninfected *L. vannamei* tissues by immunohistochemistry (Fig. 4e–f). However, MAb 5.4H demonstrated nonspecific binding to shrimp striated muscle (data not shown). This result indicates that MAbs 1.3H and 4.6C are promising tools for histopathology, allowing assessment of viral distribution and cytolocation. No cross-reactivity with *L. vannamei* tissues was observed while using the unrelated anti-rabies-virus MAb (Fig. 4g). The specific recognition by MAbs 1.3H and 4.6C was not affected by putative conformational changes of the target epitope, showing specific binding to both native and denatured forms of the protein. Similar characteristics have already been reported for MAbs produced against others shrimp viruses, reinforcing their importance for development of immunoassays [16–18].

In summary, this present study describes the generation and characterization of two MAbs that bind with high specificity to the major capsid protein of IMNV, since they do not show any nonspecific recognition of proteins from healthy shrimp tissues in immunodot blot, western blot, or immunohistochemical analysis. Ongoing studies employing MAbs 1.3H and 4.6C are in progress for the purpose of developing a fast and simple immunodiagnostic assays such as immunochromatographic and agglutination tests. Such development based on the MAbs described in this study will allow early and specific diagnosis of IMNV

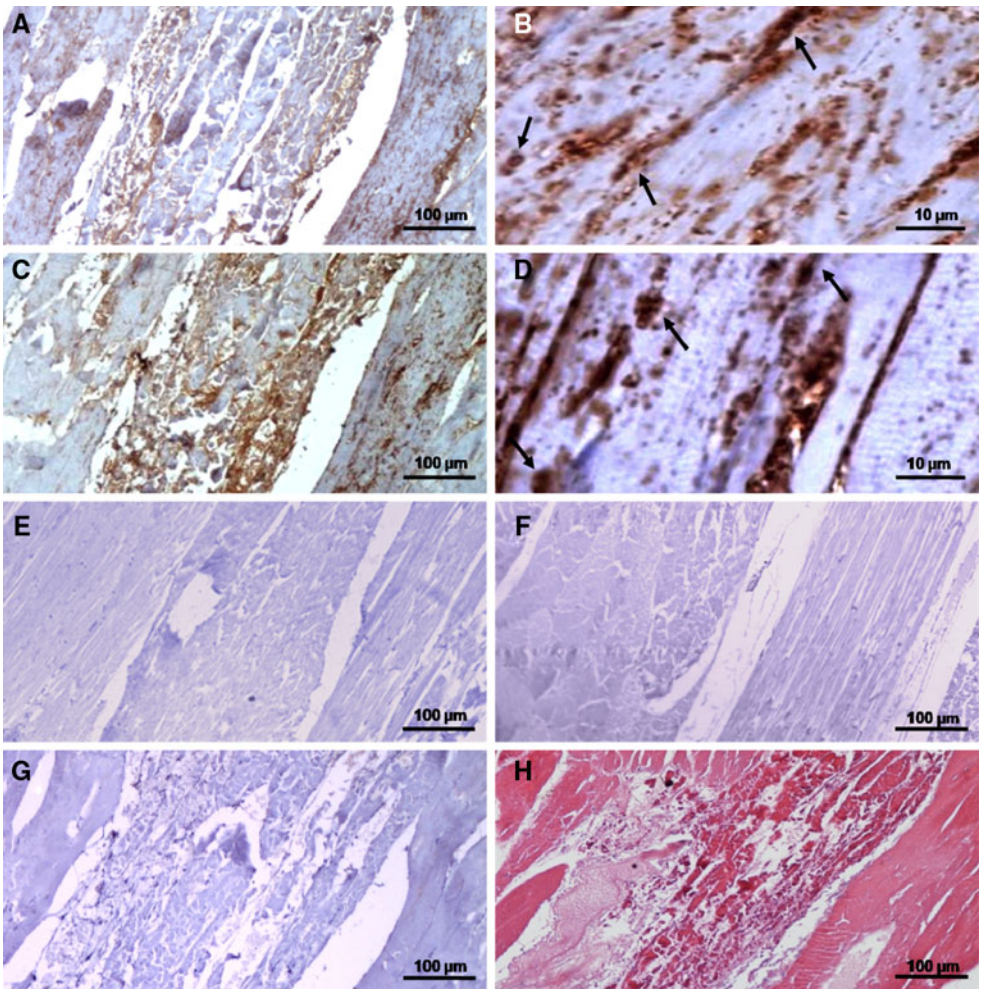

Fig. 4 Immunohistochemical analysis of IMNV-infected shrimp skeletal tissues using MAbs 1.3H and 4.6C. IMNV-infected (**a**, **b**, **c**, **d** and **g**) and uninfected (**e**, **f**) skeletal tissues were fixed with Davidson's solution, embedded in paraffin, sectioned at 3 µm, and incubated with the MAbs 1.3H (**a**, **b** and **e**), 4.6C (**c**, **d** and **f**), or an unrelated MAb against rabies virus used as negative control (**g**), followed by HRP-conjugated antibody. Slides were colored with 3,3-diaminobenzidine and counterstained with Harris's hematoxylin solution. **h** Infected tissue stained with hematoxylin and eosin. Arrows indicate viral inclusions

infection, contributing to minimizing the major economic impact of this virus on shrimp farming and commerce.

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Conflict of interest The authors declare no conflict of interest.

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