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Short communication

Quantitation of infectious myonecrosis virus in different tissues of naturally infected Pacific white shrimp, *Litopenaeus vannamei*, using real-time PCR with SYBR Green chemistry

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A B S T R A C T

The Pacific white shrimp, *Litopenaeus vannamei*, is the most important shrimp species in volume in world aquaculture. However, in recent decades, outbreaks of diseases, especially viral diseases, have led to significant economic losses, threatening the sustainability of shrimp farming worldwide. In 2004, Brazilian shrimp farming was seriously affected by a new disease caused by the Infectious myonecrosis virus (IMNV). Thus, disease control based on rapid and sensitive pathogen detection methods has become a priority. In this study, a specific quantitation method for IMNV was developed using real-time PCR with SYBR Green chemistry and viral load of the principal target tissues of chronically infected animals was quantified. The quantitative analysis revealed that mean viral load ranged from 5.08×10^8 to 1.33×10^6 copies/μg of total RNA in the hemolymph, 5.096×10^5 to 1.26×10^3 copies/μg in the pleopods, 6.85×10^8 to 3.09×10^4 copies/μg in muscle and 8.15×10^6 to 3.90×10^3 copies/μg in gills. Different viral loads of IMNV were found with greater values in the hemolymph and muscle, followed by the pleopods and gills.

The global shrimp farming industry has grown rapidly over the last 30 years (Lightner, 2005), accounting for as much as 70% of penaeids produced worldwide in 2006 (FAO, 2008). The Pacific white shrimp, *Litopenaeus vannamei*, is one of the predominant species produced in aquaculture (Burge et al., 2007), with a total global production of 2,296,630 tons in 2007 (FAO, 2008).

Since the intensification of shrimp farming due to technological advances in the industry, problems related to environmental degradation and disease have become increasingly commonplace (Bachère, 2000). The economic impact of viral pandemics, such as White spot syndrome virus (WSSV), Taura syndrome virus (TSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Yellow-head virus (YHV), has cost the shrimp industry billions of dollars in lost product, jobs and export revenue (Lightner, 2005).

In Brazil, the outbreak of Infectious myonecrosis virus (IMNV) was one of the main causes of the decline in the national production of *L. vannamei* in 2004 (Madrid, 2005). Infectious myonecrosis disease is characterized by extensive necrosis of skeletal muscle, especially in the distal abdominal segments and tail fan. This disease resulted in cumulative mortality rates throughout the production cycle of up to 70% and economic losses estimated at US$20 million in 2003 (Nunes et al., 2004).

Disease control has become a worldwide priority for achieving sustainable shrimp production. According to Bachère (2000), shrimp production is dependent on the balance between three factors: environmental quality; disease prevention through diagnosis and epidemiological surveys of pathogens; and the evaluation of shrimp health.

The current methods for the monitoring, detection and diagnosis of Infectious myonecrosis are clinical signs, direct light microscopy, histopathology, *in situ* hybridization and polymerase chain reaction (PCR). In all these tests, the adequacy of diagnostic specificity and sensitivity is related to the sampling (life stage, sufficient sample material, preservation of samples and target tissue) and the purpose of the diagnosis (presumptive or confirmatory diagnosis) (OIE, 2009). The World Organization for Animal Health (OIE) recommends the sampling of striated muscle as the target tissue for the diagnosis of IMNV in acute or chronic

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stages and the hemolymph or pleopods for non-lethal testing (OIE, 2009).

Although conventional PCR is a sensitive diagnostic method, it is not able to detect the viral load in the infected tissue (Dhar et al., 2001). The determination of viral load in infected animals has become of the most important aspect of the monitoring of shrimp diseases, especially with regard to the detection of viral infection in asymptomatic individuals (Tang and Lightner, 2001), for the development of specific pathogen-free stocks and monitoring shrimp transport from country to country (Dhar et al., 2002).

However, progress in the quantitation of viruses in shrimp has been hampered due to the lack of shrimp cell culture systems (Tang and Lightner, 2001). Consequently, direct detection methods able to measure low numbers of viral copies in infected tissue without the use of cell cultures for the propagation of viruses are crucial (Bowers et al., 2008). Real-time PCR has been used for just such a purpose (Tang and Lightner, 2001). This method is based on the monitoring of the target gene amplification by the fluorescence emitted from the SYBR Green dye that binds to double-stranded DNA or by the hydrolysis of a specific TaqMan probe (Swillens et al., 2008). Real-time PCR has been used to detect and quantify viral pathogens in fish and shellfish aquaculture (Bowers et al., 2008).

Andrade et al. (2007) developed a method for the detection and quantitation of IMNV in *L. vannamei* based on real-time PCR using a TaqMan probe. However, TaqMan real-time PCR is more expensive than SYBR Green-based real-time PCR due to the need to design a specific probe for virus detection (Dhar et al., 2008). In addition, information on viral load in different tissues of *L. vannamei* in the chronic phase of IMNV remains unavailable.

The aim of the present study was to quantify the viral load in tissues commonly sampled for the diagnosis of IMNV using real-time PCR and the SYBR Green method during the chronic phase in naturally infected shrimp.

Fifteen adult specimens of *L. vannamei* naturally infected with IMNV were collected during an outbreak in a shrimp farm in the state of Pernambuco (northeastern Brazil) in October 2009. Specimens (mean weight: ~11.6 g) exhibiting clinical signs of the chronic stage of IMNV infection, with necrotic and reddened muscles (Nunes et al., 2004), were selected for IMNV quantitation. After collecting the hemolymph, the shrimp were killed by thermal shock and tissues (muscle, pleopods and gills) were removed and preserved at −80 °C. RNA extraction was carried out following the method described by Chomczynski and Sacchi (1987), with modifications. Samples (60 mg) from each animal were ground in 1 ml of Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and incubated at room temperature for 5 min for digestion. The aqueous phase was transferred to a fresh tube and RNA was precipitated with isopropyl alcohol (1:1). Pellets were washed in 75% ethanol, dried and dissolved in DEPC water. The presence of RNA was assessed by electrophoresis in 1% formaldehyde agarose gel following the standard protocol (Sambrook et al., 1989). The concentration and quality of extracted RNA were determined by spectrophotometry.

cDNA was synthesized using the Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA), with 0.5 μg of oligo(dT)$_{15}$ and 2 μl of extracted total RNA in a 20-μl reaction volume, following the manufacturer's protocol. cDNA was used either directly in a real-time PCR and PCR analyses or stored at −20 °C until further use.

Before quantitative analysis, all hemolymph samples were submitted to conventional PCR amplification for the confirmation of IMNV infection. The PCR amplification was carried out in a final volume of 25 μl, containing 1 μl of cDNA, 1 U of Taq polymerase, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 5 pmol of each IMNV-specific primer (IMNV-F 5′-CGA-CGC-TGC-TAA-CCA-TAC-AA-3′ and IMNV-R 5′-ACT-CGC-CTG-TTC-GAT-CAA-GT-3′) and 1× PCR buffer, as described by Pinheiro et al. (2007). PCR products were visualized after electrophoresis in 2% agarose gels stained with ethidium bromide and the amplified fragment of 328 bp was determined using a 100-bp standard ladder (Invitrogen, Carlsbad, CA, USA).

In order to construct a standard curve for real-time PCR, an amplicon of 464 bp of IMNV was amplified using the IMNV218F and IMNV682R primers (5′-GCT GGA CTG TAT TGG TTG AG-3′ and 5′-AAC CAA GTT CTT CTT CTC CAG TT-3′) based on the method described by Andrade et al. (2007). The primers were based on the published sequence for the ORF1 region of the IMNV genome (GenBank accession no. AY570982) (Poulos et al., 2006). Amplification was performed in 25-μl reaction volume with 1 μl of cDNA, 1 U of Taq polymerase, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 5 pmol of each primer and 1× PCR buffer. The amplification conditions were as follows: 39 cycles at 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s, with final extension at 72 °C for 10 min. The PCR product was analyzed by electrophoresis, purified using Illustra GFX PCR DNA and Gel Band Purification kit according to the manufacturer's protocol (Amersham/GE Healthcare, Buckinghamshire, UK) and cloned into the pGEM-T-Easy vector (Promega, Madison, WI, USA) following the manufacturer's protocol. The recombinant clones were determined by sequencing. The plasmid DNA of a recombinant clone was sequenced in an automated DNA sequencer (model ABI3100, Applied Biosystems, Foster City, CA, USA) using the BigDye® Terminator vs 3.0 kit (Applied Biosystems, Foster City, CA, USA) and the M13 universal primer (5′-TGTAAAACGACGGCCAGT-3′). The sequences were analyzed using the Basic Local Alignment Search Tool.

Recombinant plasmid DNA was quantified using a spectrophotometer (NanoVue Plus Spectrophotometer/GE Healthcare, Buckinghamshire, UK) and dilution series were made to generate the standard curves. Absolute quantitation of the standard curves was carried out based on the method described by Whelan et al. (2003), with the use of the known molecular weight of the plasmid and insert for the calculation of the number of copies. Thus, the dilution series of the plasmid standard for which the copy number was known was used as the positive control and standards for quantitation in all real-time assays.

Real-time PCR runs were conducted in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used for SYBR Green PCR are listed in Table 1. These primers were based on the published sequences for the ORF1 region of the IMNV genome (GenBank accession no. AY570982) (Poulos et al., 2006), as described by Andrade et al. (2007). Each PCR plate contained a dilution series of the plasmid standard (10^2–10^8 copies), two negative controls (water and Taura syndrome virus), an internal control gene of β-actin (Dhar et al., 2002) and samples of cDNA from each tissue. The reactions were carried out in a 96-well plate in a 25-μl reaction volume containing 1× SYBR Green Master Mix (Applied Biosystems, Warrington, UK), 0.2 μM of each primer and 1 μl of cDNA. The thermal profile was 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 1 min. All samples were carried out in duplicate in order to control intra-assay variation and all reactions were repeated at least twice independently to ensure reproducibility of the results. After the real-time PCR runs, data acquisition, analyses and the quantitation of the viral load of each sample were performed using the ABI 7500 program (version 2.0.1).

Mean and standard deviation (SD) values for each individual sample were determined and data were considered valid when varying less than 1 SD. The amplification efficiency (ε) of the target molecule was calculated from the slope of the standard curve (Too, 2003). Amplification specificity was confirmed by analyzing the dissociation curve of the target amplicons (Ririe et al., 1997) and testing the negative control (Taura syndrome virus).